CHARACTERIZATION OF THE ALPHAHERPESVIRUS TEGUMENT PROTEIN US2

by

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Abstract

Members of the Herpesviridae are large enveloped, double-stranded DNA viruses, whose virions are comprised of a viral genome-containing icosahedral capsid, a layer of tegument and a glycoprotein-embedded envelope. The tegument contains numerous viral proteins and cellular proteins. Most of the tegument proteins are poorly understood and require further investigation. This study focuses on one of the tegument proteins, Us2, and utilizes two model alphaherpesviruses: pseudorabies virus (PRV) and herpes simplex virus (HSV).

Us2 is conserved among all alphaherpesvirus with the exception of varicella-zoster virus (VZV). The amino acid sequence of all Us2 orthologs share three N-terminal conserved regions whereas the C-terminal sequences are highly variable. PRV Us2 contains a C-terminal prenylation motif that targets Us2 to the plasma membrane. Although it is indispensable for virus growth in cell culture, deletion of Us2 gene in PRV caused an accumulation of virions in the cytoplasm of infected primary cells. Furthermore, PRV Us2 spatially regulates MAPK ERK activity by sequestering it to the plasma membrane. Inhibition of ERK kinase activity caused a delay in the release of extracellular viruses and the defect was more profound in PRV Us2-null virus infected cells. Altogether, these data suggest a requirement for ERK activity and significance of PRV Us2-ERK interaction in virus egress. To understand the mechanism of Us2-ERK interaction, PRV Us2 determinants for ERK interaction were mapped. Our data revealed that the N-terminal 214 residues are the minimal sequence of Us2 required for interaction with ERK. In addition, PRV Us2 oligomerizes and forms complexes with ERK via the
ERK common docking (CD) domain that facilitates the interaction of ERK with many of its substrates.

Unlike PRV Us2, HSV-2 Us2 does not have any putative membrane targeting signals. However, our data revealed that HSV-2 Us2 localizes to the plasma membrane and is lipid raft associated. In addition, HSV-2 Us2 interacts directly with ubiquitin. As ubiquitination is responsible for proteasomal degradation and is involved in endocytosis and lysosomal degradation, these findings suggest that Us2 may be involved in proteasomal degradation pathways that counteract host defenses, or participate in final envelopment in endocytic compartments by facilitating the endocytosis of viral envelope proteins.
Co-Authorship

Chapter 4.1 is comprised of a scientific article that has been published. A former post-doc in our lab, Dr. Bibhuti Roy, performed membrane flotation assay (Figure 4-14A) in the Chapter 4.3.2, transferrin assay and Us2-Rab co-localization in Chapter 4.3.4, and the luciferase assay (Figure 5-3B) in Chapter 5.3.3. Dr. Roy also wrote Chapter 4.2.4, which is included in a manuscript prepared for journal submission. Dr. Bruce Banfield is the corresponding author for the manuscripts. HSV-2 Us2-null and repaired viruses were constructed by Dr. Valerie Le Sage.

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<th>Description</th>
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<tbody>
<tr>
<td>CCSC</td>
<td>C-capsid specific component</td>
</tr>
<tr>
<td>CD domain</td>
<td>Common docking domain</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>CR</td>
<td>Conserved region</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EE</td>
<td>Early endosome</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Egr-1</td>
<td>Early growth response gene 1</td>
</tr>
<tr>
<td>EHV-1</td>
<td>Equine herpesvirus type I</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-regulated kinase</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosoaminoglycan</td>
</tr>
<tr>
<td>GAL4-AD</td>
<td>GAL4 activation domain</td>
</tr>
<tr>
<td>GAL4-DBD</td>
<td>GAL4 DNA binding domain</td>
</tr>
<tr>
<td>HCF-1</td>
<td>Host cell factor-1</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human cytomegalovirus</td>
</tr>
<tr>
<td>HHV</td>
<td>Human herpesvirus</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpes simplex virus type I</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Herpes simplex virus type II</td>
</tr>
<tr>
<td>HVEM</td>
<td>Herpesvirus entry mediator</td>
</tr>
<tr>
<td>ICP</td>
<td>Infected cell protein</td>
</tr>
<tr>
<td>IE, E, L</td>
<td>Immediate early, early, late</td>
</tr>
<tr>
<td>IF</td>
<td>Intermediate filaments</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi’s sarcoma-associated herpesvirus</td>
</tr>
<tr>
<td>KSR</td>
<td>Kinase suppressor of Ras-1</td>
</tr>
<tr>
<td>LE</td>
<td>Late endosome</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>Major capsid protein</td>
</tr>
<tr>
<td>MNK</td>
<td>MAPK-interacting kinase</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubules</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular bodies</td>
</tr>
<tr>
<td>NEC</td>
<td>Nuclear envelopment complex</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Oct-1</td>
<td>Octamer binding transcription factor-1</td>
</tr>
<tr>
<td>PKD</td>
<td>Protein kinase D</td>
</tr>
<tr>
<td>PRV</td>
<td>Pseudorabies virus</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RE</td>
<td>Recycling endosome</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>RSK</td>
<td>Ribosomal S6 kinase</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SNAP</td>
<td>Soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein</td>
</tr>
<tr>
<td>SNAPE</td>
<td>SNAP receptor</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
<tr>
<td>Vhs</td>
<td>Virion host shutoff</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella-zoster virus</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

The Alphaherpesvirinae is a subfamily of the Herpesviridae family. They are an important group of human and animal pathogens, the majority of which establish lifelong latent infections in the peripheral nervous systems of their hosts. Herpesviruses are large enveloped viruses that contain linear double-stranded DNA genomes that encode between 70 to 200 viral proteins [1]. Between the icosahedral nucleocapsid, which contains the virus genome, and the virion envelope is a structure called the tegument. The tegument is the most complex subvirion compartment, in herpes simplex virus type I (HSV-1) and pseudorabies virus (PRV), housing 20 or so different virus-encoded proteins and about 49 host proteins [2, 3]. In recent years, significant insight has been gained into the complex series of protein-protein interactions required for tegument assembly [4]. Despite these advances, little is known about the functions of most tegument proteins. Because tegument proteins are delivered to the cytoplasm of cells upon virus infection, they have an opportunity to function prior to the expression of virus genes. A widely held view is that one role of the tegument is to establish a cellular environment conducive to virus infection [5]. In addition to these functions that take place early in infection, it is clear that a number of tegument proteins function later in infection, during virion maturation and assembly [6].
This study focuses on one of the tegument proteins called Us2. The Us2 gene encodes a tegument component that is conserved among most of the alphaherpesviruses, with one notable exception being varicella-zoster virus (VZV). While nonessential for the growth of virus in cell culture, the deletion of Us2 gene from an attenuated PRV vaccine strain, Bartha, as well as from the attenuated equine herpesvirus 1 (EHV-1) vaccine strain, RacH, strongly suggests that Us2 is an important virulence determinant during natural infections [7, 8]. Studies of the EHV-1 Us2 protein have shown that it associates with cellular and viral membranes and enhances virus entry and the cell-to-cell spread of infection [9].

The swine pathogen PRV is a well-studied alphaherpesvirus whose analysis has contributed greatly to our understanding of the molecular and cellular biology of virion assembly, as well as how viruses of this group spread and cause disease in the nervous system [10]. Our lab previously demonstrated that PRV Us2 binds to the extracellular-signal regulated kinase (ERK), an important effector serine/threonine kinase in the Raf/MEK/ERK signaling module [11]. The ERK mitogen-activated protein kinase (MAPK) pathway facilitates cellular responses to extracellular stimuli. It is a key signaling pathway that is important for the regulation of cell growth, division, apoptosis, and differentiation, and it is activated upon the binding of numerous growth factors, hormones, and cytokines to their cognate receptors [12-15]. Upwards of 70 different substrates have been identified for ERK [16, 17]. While the majority of ERK substrates are transcription factors that reside in the nucleus of the cell, others are found in the
cytosol, on the cytoskeleton, and on the cytoplasmic face of membranes. Importantly, after ERK activation by a given stimulus, only a subset of ERK substrates are phosphorylated. The restriction of ERK activity toward select substrates is thought to be mediated in part by ERK scaffolding molecules that spatially regulate ERK enzymatic activity and direct it toward the appropriate, stimulus-specific substrates [16, 18-20]. Our lab has shown that PRV Us2 contains a prenylation CAAX motif that targets it to the plasma membrane [21]. The PRV Us2 interaction with ERK re-localizes ERK to the plasma membrane as well as to a perinuclear vesicular compartment, thereby preventing the translocation of ERK into the nucleus [11]. However, Us2 does not prevent ERK activation, nor does it inhibit ERK enzymatic activity [11]. Furthermore, both ERK and Us2 activities are required for the efficient release of infectious virus from cells [11], and enveloped viruses accumulate in cytoplasmic vesicles in cells infected with Us2 null mutants [22]. Thus, it appears that Us2 acts as an ERK scaffold used by the virus to divert ERK activity to cellular membranes during a late stage of virus egress. In this study, we sought to define the mechanism by which Us2 and ERK interact.

Other than PRV, the human pathogen herpes simplex virus (HSV), another member of alphaherpesvirinae, has also been intensively studied. HSV-1 is widely used to study the molecular and cellular biology of alphaherpesvirus infection. Compared to PRV Us2, little is known about HSV-1 Us2 and herpes simplex virus type II (HSV-2) Us2. Despite three N-terminal conserved regions, the amino acid sequences located between conserved regions as well as the C-terminal sequences are highly variable
amongst Us2 orthologs [21]. PRV Us2 and HSV-2 Us2 share only 30% identity in their overall protein sequences. In addition, unlike PRV Us2, HSV-2 Us2 does not contain a CAAX motif for prenylation or any other putative post-translational modification motifs for membrane targeting. This raises a few questions: does HSV-2 Us2, like PRV Us2, localize to the plasma membrane and intracellular membranes? If it does, what is the mechanism of membrane localization? Also, does it share interacting partners with and share similar properties as PRV Us2? To investigate the function of HSV-2 Us2, its cellular localization and membrane association were studied using both microscopy and biochemical methods. To gain insight into Us2 function, an HSV-2 Us2 deleted mutant virus was constructed to monitor phenotypes, such as virus replication, virion egress and virus resistance to interferon. Cellular and viral interacting partners of HSV-2 Us2 were also identified using co-immunoprecipitation (co-IP) and LC-MS/MS analysis. By identifying HSV-2 Us2 interacting proteins, clues to the function of Us2 during viral infection were provided.
Chapter 2

Literature Review

2.1 The Herpesviridae Family

The Herpesviridae are large enveloped, double-stranded DNA viruses consisting of three main subfamilies: alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae. They are sub-classified on the basis of genome characteristics (e.g. DNA sequence homology and genomic sequence arrangement), pathogenesis and host range [23, 24]. A feature shared within the Herpesviridae is that the viruses establish a lifelong latency in their hosts. The three subfamilies can be distinguished by their biological properties, such as host range, duration of replicative cycle and mechanism by which they maintain the latent state. Members of the alphaherpesvirinae have a variable host range, a rapid reproductive cycle and establish latent infections in sensory ganglia. Members of the betaherpesvirinae have a restricted host range, a long reproductive cycle and are able to establish latency in lymphoreticular cells and cells within secretory glands [23, 24]. Members of gammaherpesvirinae infect lymphocytes and usually maintain their latent state in lymphoid tissues. [23, 24].

There are eight human herpesviruses (Table 2-1). The commonly known human alphaherpesviruses include HSV-1, HSV-2 and VZV. HSV infection will be described in Chapter 2.3. Primary VZV infection commonly occurs in childhood, which causes varicella (chickpox) [25]. Intimate contact with zoster lesions and respiratory droplets of
infected individuals are the major routes of VZV transmission. Initial infection sites are thought to be mucosal epithelial cells of the oropharynx [25, 26]. Spread of the viruses to the regional lymph nodes leads to primary viremia, which results in dissemination of the viruses to the liver or other mononuclear phagocyte system (MPS, which includes bone marrow progenitors, blood monocytes and tissue macrophages [27, 28]) and subsequently causes secondary viremia [25, 29]. Infected peripheral blood mononuclear cells (e.g. lymphocytes and monocytes) allow migration of the viruses to the skin where the viruses infect sensory neurons and are transported in a retrograde direction to the sensory ganglion where latency is established [25, 26]. Reactivation of VZV causes herpes zoster (shingles), which manifests as a localized, painful vesicular rash [25, 26]. The swine pathogen, PRV, also known as suid herpesvirus 1, belongs to this subfamily, too. Although PRV is not a human herpesvirus, it has been widely used as a model virus to study interactions of tegument proteins, virus assembly and transport of the virus in the nervous system [30].

Table 2-1. The human herpesviruses.

<table>
<thead>
<tr>
<th>Alpha</th>
<th>Beta</th>
<th>Gamma</th>
</tr>
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<tbody>
<tr>
<td>HSV-1 (HHV-1)</td>
<td>CMV (HHV-5)</td>
<td>EBV (HHV-4)</td>
</tr>
<tr>
<td>HSV-2 (HHV-2)</td>
<td>HHV-6</td>
<td>KSHV (HHV-8)</td>
</tr>
<tr>
<td>VZV (HHV-3)</td>
<td>HHV-7</td>
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</table>

Human cytomegalovirus (HCMV, a.k.a. HHV-5) is the prototypical member of the betaherpesvirinae and has been intensively studied. HCMV is highly prevalent, with approximately 70-100% of population in the world carrying the viruses. HCMV causes
both acute and chronic diseases [31, 32]. Infection can be prenatal by transplacental transmission. Postnatal transmission routes include ingestion of breast milk, contact with body fluids (e.g. saliva, urine, cervical and vaginal secretions), sexual activity, blood transfusion and organ transplantation [31]. Saliva is thought to be an important vehicle of HCMV transmission and children are considered as the major source of virus dissemination because infected infants excrete large amount of viruses for prolonged periods of time [31, 33]. Acute HCMV infection is usually asymptomatic in healthy individuals but may manifest in mononucleosis-like illness, such as fever, fatigue and sore throat, depending on the immunologic status of the host [33, 34]. Congenital infection can cause cognitive and perceptual disorders in children [33]. In immunocompromised individuals, HCMV infection is one of the major causes of morbidity and mortality [33]. Chronic infection results in persistent shedding of virus, termed persistence, and is associated with chronic vascular diseases [33]. Endothelial cells are important sites for HCMV persistence [35]. Lytic HCMV replication occurs in a variety of cell types, including fibroblasts, epithelial cells, endothelial cells and monocytes [36, 37], whereas latency is restricted to cells of monocyte/myeloid lineage [38].

In contrast to HCMV, the clinical significances of HHV-6 and HHV-7 are just beginning to emerge. There are two variants of HHV-6: HHV-6A and HHV-6B. HHV-6B has been identified as the major causative agent of exanthem subitum (roseola), which is characterized by a high fever lasting for a few days, followed by a rash on the trunk
There is some evidence that HHV-6A is associated with multiple sclerosis [41]. HHV-7 also causes exanthem subitum [42]. HHV-6 and HHV-7 are ubiquitous, with more than 90% of adults in the human population seropositive. Primary infection occurs in early childhood. Sites for HHV-6 latency include monocytes and bone marrow progenitor cells [43] whereas HHV-7 establishes latency in T lymphocytes [44, 45].

Epstein-Barr virus (EBV, HHV-4) and Kaposi’s sarcoma-associated herpesvirus (KSHV; HHV-8) are members of the gammaherpesvirinae. In normal hosts, primary EBV infection is usually asymptomatic in children but may manifest as infectious mononucleosis, which is characterized by fever, fatigue and sore throat, in adolescence and early adulthood [46]. After primary infection, virus is frequently detected in saliva, and therefore, usually transmitted through salivary exchange [47]. EBV infection occurs in epithelial cells of the oropharynx and latency is established in B lymphocytes [47]. In immunocompromised individuals, EBV infection is associated with a variety of malignancies, such as Burkitt’s lymphoma, Hodgkin’s disease and nasopharyngeal carcinoma [48, 49]. In contrast to other human herpesviruses, KSHV is rarely observed in normal adults [50]. Similar to EBV, virus is commonly detected in saliva after primary infection and therefore, transmission in childhood is considered to be saliva-mediated [51, 52]. Infection in immunocompetent children may appear as a febrile illness and maculopapular rash [51]. Transmission of KSHV can also be mediated via sexual activity and organ transplantation [52]. In AIDS patients, KSHV infection can result in Kaposi’s sarcoma [53] and is also found to associate with multicentric Castleman’s disease and
primary effusion lymphoma (PEL) [54, 55]. Similar to EBV, KSHV establishes latency in B lymphocytes and endothelial cells [56].

2.2 Structure of the Herpesvirus Virion

Viruses in the Herpesviridae family have a similar architecture [23, 24]. Herpesvirus virions are comprised of four major components: 1) an envelope with glycoprotein spikes, 2) a proteinaceous tegument lying between the envelope and capsid and 3) an icosahedral nucleocapsid, which contains 4) a large linear double-stranded DNA genome (Figure 2-1).

The herpesvirus envelope is derived from cellular membranes and contains phospholipids and several viral membrane proteins and glycoproteins [57]. The nucleocapsid is a highly ordered T=16 icosahedral structure with a size about 100-150nm in diameter [58, 59]. A herpesvirus capsid contains five conserved proteins: major capsid protein (MCP; VP5 in HSV-1), triple monomer protein (TRI-1; VP19c in HSV-1), triplex dimer protein (TRI-2; VP23 in HSV-1), smallest capsid protein (SCP; VP26 in HSV-1) [60] and the portal protein (UL6 in HSV-1). Between the envelope and the capsid is the tegument, which contains various cellular and viral proteins (Chapter 2.6) [61]. Inside the capsid is a large double stranded linear DNA genome, the size of which ranges from approximately 120kbp to 235kbp, encoding from 70 to 200 genes [58].
Figure 2-1. Structure of herpesvirus virion and genome. (A) Cartoon of herpesvirus structure (Los Alamos National Laboratory). (B) 3D cryo-electron structure of the virion. Light blue: capsid, dark blue: envelope, yellow: glycoprotein, orange: tegument [59]. (C) Structure of HSV genome. The HSV genome contains two unique regions, U_L and U_S (L for long and S for short). The unique regions are flanked by direct and inverted repeat regions (R_L and R_S). It also contains terminally redundant sequences, a few hundred base pairs (termed the a sequence, a’ for the inverted orientation) at the genome termini and between R_L and R_S. (Arrows indicate the orientations of the repeat regions).

Herpesvirus protein nomenclature:
2.3 Alphaherpesviruses: PRV and HSV

PRV is a swine pathogen that causes Aujeszky's disease. The disease was termed “mad-itch” when an outbreak in cattle was observed in 1930 and an extreme pruritus was the salient symptom of the infected cows [62]. PRV is not related to rabies virus, which is a member of the Rhabdoviridae family, but acquires its name because its clinical symptoms resemble rabies in infected dogs. It can cause disease in a broad range of domestic and wild animals [62, 63]. The infection is mild in adult swine but usually fatal in other species at any age [62, 64, 65]. Infected animals have pruritus after an incubation period, followed by neurological symptoms, such as trembling and convulsion, and usually die with rapid respiration soon after symptoms appear [62, 63]. Piglets under 5 weeks of age are most susceptible and the fatality rate is high for those under 2 weeks of age [63]. Infected adult pigs may develop fever and have transient illness. They usually survive from the infection and serve as virus reservoirs to infect other swine or species [63, 65-67].

HSV-1 and HSV-2 are human pathogens and are highly prevalent, affecting 60-95% of adults worldwide. Transmission of HSV requires intimate contact with mucosal surfaces or abraded skin of an infected individual who is shedding viruses. After primary infection, virus replication occurs in the mucosal epithelium. Viruses are then transported retrograde to the sensory neurons that innervate the mucosal surface where the viruses replicate and establish a life-long latency in the dorsal root ganglion of the peripheral nervous system [68]. Recurrence of infection can be stimulated by stress, fever or
exposure to UV (ultraviolet) light. There are two types of HSV: HSV-1 and HSV-2. Both types of HSV cause mucocutaneous infections. Primary HSV-1 infection causes intraoral lesions and recurrent infection manifests as herpes simplex labialis (cold sores) [69, 70]. HSV-2 infection is the leading cause of genital herpes [69, 70]. Keratoconjunctivitis, which may result in visual loss, is also one of the clinical manifestations of HSV infection and is mostly caused by HSV-1 [71]. In rare cases, HSV can invade the central nervous system and cause life-threatening encephalitis. HSV infection is usually mild and asymptomatic. However, it can be fatal for immunocompromised people, such as HIV patients, and neonates [69]. Once infected, there is no cure. Current medication can only lessen the severity of symptoms and reduce the frequency of recurrence.

Many studies have used PRV as a model virus to study the mechanism of alphaherpesvirus infection since most viral proteins are conserved amongst the alphaherpesviruses. Reasons for utilizing PRV are not only to seek strategies for prevention of the spread that can cause an impact on the swine industry, but also its ease of manipulation, compared to HSV. In addition, PRV grows faster and produces higher virus yield in cell culture than HSV. However, there are restrictions in utilizing wildtype PRV strains in Canada because PRV is not found naturally in Canada. While many experiments involve infection of wildtype strains to study the mechanism of infection, PRV may not be a good model virus for alphaherpesvirus research in Canada because of the regulatory restrictions. Since there is currently no cure for HSV infection, utilizing HSV as a model virus is beneficial for understanding the mechanisms by which these
viruses counteract cellular responses. Therefore, analysis of HSV infection has the potential to uncover new targets for antiviral interventions and curtail the dissemination of virus throughout the human population.

2.4 Alphaherpesvirus Replication

Entry of herpesviruses into cells involves two steps: 1) binding of the virion to the cell and 2) fusion of the virion envelope with the cell plasma membrane. They can also enter through a pH-dependent endocytic pathway, which depends on cell type [72, 73]. Three glycoproteins essential for virus entry are gB, gH and gL, which all are conserved throughout the three Herpesviridae subfamilies [58]. In HSV-1, five glycoproteins participate in virus entry: gB, gC, gD, gH and gL. The initial virus-cell contact is mediated by binding of gB and/or gC to ubiquitously expressed cell surface heparan sulfate, a glycosoaminoglycan (GAG) [74]. Fusion of the virion envelope to the cell surface is triggered by the binding of gD to one of these cell surface receptors: 1) HVEM (herpesvirus entry mediator), a member of the TNF-α (tumor necrosis factor-α) receptor family, 2) nectin-1 and nectin-2, members of the immunoglobulin superfamily, and 3) specific sites generated by specific 3-O-sulfotransferase (3-OST-3) on GAGs [75]. Glycoprotein D contains two distinct regions: N-terminal receptor binding site [76] and C-terminal profusion domain (PFD) [77]. Binding to the cell receptor leads to a conformational change of gD and recruits gB and the gH/gL heterodimer to the complex
**Figure 2-2.** Replication of the alphaherpesviruses [78]. Viruses can enter cell by fusion of viral envelope with the plasma membrane, or through a pH-dependent endocytic pathway, which depends on cell type [72, 73] (not shown in the figure). The outer tegument proteins are dissociated from the capsid in the cytoplasm while the inner tegument proteins remain attached to assist transporting the capsid to the nucleus along microtubules. The capsid docks at a nuclear pore complex (NPC) and ejects the viral genome into the nucleus. Transcription of viral immediate early (IE) genes is initiated by a VP16-induced complex. Products of IE genes initiate the transcription of early and late genes. The resulting mRNAs are transported to the cytoplasm for translation. Capsid proteins are imported into the nucleus for procapsid assembly and viral DNA packaging. The procapsid is converted into a mature capsid after packaging of the viral genome. The mature capsid buds into the perinuclear space and acquires its primary envelope from the inner nuclear membrane. Virions formed in the perinuclear space (perinuclear enveloped virions) enter the cytoplasm by fusion of the primary envelope with the outer nuclear membrane, a process termed de-envelopment, and lose their primary envelopes. Non-enveloped capsids assemble with the tegument proteins in the cytoplasm, acquire the final envelope from the TGN-derived membranes, and become mature virions. Mature virions are transported to the plasma membrane via intracellular vesicles. At the cell surface, vesicles fuse with the plasma membrane and release virions from the cell.
All four of gB, gD, gH and gL are required for virus entry and the assembly of gB, gH and gL executes the fusion at the cell surface [58, 81, 82].

Upon fusion of the viral envelope with the plasma membrane, nucleocapsid and tegument proteins are released into the cytoplasm (Figure 2-2). Most of the tegument proteins are dissociated from the nucleocapsid, however, UL36, UL37 and Us3, components of the inner tegument, remain bound to the nucleocapsid and may interact with cellular motor proteins for the transport of the capsid along the microtubules to the nucleus [83, 84]. The nucleocapsid docks at a nuclear pore complex (NPC) and releases the viral genome into the nucleoplasm through the nuclear pores [85]. Transcription of immediate early (IE), early (E) and late (L) genes occurs in three sequential phases. The IE genes are the first set of genes to be transcribed. Initiation of IE gene transcription requires the tegument protein VP16 (UL48 gene product), which acts as a transactivator, and two cellular proteins, Oct-1 (octamer binding transcription factor-1) and HCF-1 (host cell factor-1) [86]. Oct-1, a member of the POU family, recognizes and binds to the DNA octamer motif, ATGCAAAT, found in the promoters of cellular genes [87]. Upstream regions of IE genes, in the case of HSV-1, contain multiple copies of a consensus sequence similar to the octamer, TAATGARAT, termed the inducible enhancer core element [88]. After being released into the cytosol, VP16 interacts with HCF-1 whereas Oct-1 binds to the enhancer core for subsequent assembly with VP16-HCF-1 complexes [86]. VP16 contains a transcriptional activation domain and a central conserved core, which is responsible for interaction with Oct-1, HCF-1 and DNA [89, 90]. Assembly of
this VP16-induced complex initiates viral gene transcription, which utilizes the cellular RNA polymerase II machinery. Some IE gene products act as regulators for E and L gene transcription. ICP4 (infected cell protein 4), for example, one of the IE gene products, is required for E and L gene transcription. It acts as a transactivator and promotes formation of transcription preinitiation complexes [91]. Early gene products are required for viral DNA replication while L products are mostly structural proteins.

In HSV-1, the major capsid protein, VP5, interacts with the C-terminus of the scaffolding protein (pre-VP22a, gene product of UL26.5) while VP19c interacts with two VP23 molecules to form a triplex [92]. The VP5-pre-VP22a interaction is prompted by the triplex [93, 94]. Both VP5 and VP23 require pre-VP22a or VP19c to translocate into the nucleus to form a procapsid [92, 95]. The portal protein, UL6, interacts with the scaffolding protein and serves as an entry site for viral DNA into the procapsid [96-98]. The scaffolding protein is then released from the procapsid by a cleavage at its C-terminus that is catalyzed by the protease (gene product of UL26) [60, 93]. The cleavage of the scaffolding protein is followed by the recruitment of the smallest capsid protein, VP26 [99]. The procapsid is converted into a mature nucleocapsid after packaging of the viral genome by the terminase complex, encoded by UL15, UL28 and UL33 [100, 101]. The mature nucleocapsid leaves the nucleus for the cytoplasm, where assembly of virions occurs (Chapter 2.5). The assembly and egress of alphaherpesvirus are described in Chapter 2.5-6.
2.5 Egress of Alphaherpesviruses

Virus assembly and egress are important processes for virus spread between cells and among hosts. The envelopment of herpesviruses has been debated vigorously (Figure 2-3). Darlington and his colleagues proposed that the virion obtains its final envelope from the inner nuclear membrane through a budding process (Figure 2-3A) [102]. Single or multiple enveloped virions are enclosed in a transport vesicle derived either from the outer lamella of the perinuclear cisterna or from a lamella of the ER when leaving the nucleus or the ER. The virions at this stage carry immature oligosaccharides on their envelope glycoproteins. The immature glycoproteins are processed and mature while the virion-containing vesicles travel along and interact with membranes of the secretory or exocytic pathway [103, 104]. Processing of the viral glycoproteins mainly occurs within the Golgi [103, 104]. Once they arrive at the cell surface, vesicles fuse with the plasma membrane where the mature virions are released from the cells [102]. Wild and colleagues, using electron microscopy, suggested two pathways for this single envelopment model based on observations obtained during HSV-1 infection [105]. They suggested that capsids acquire their envelopes at the inner nuclear membrane and are transported via a continuous membrane system through the ER into Golgi cisternae. Enveloped virions would then be packaged into vesicles at the Golgi and transported to the plasma membrane to be released from the cell. Alternatively, Wild and colleagues proposed that capsids enter the cytoplasm through impaired, dilated nuclear pores and
Figure 2-3. Two model pathways of alphaherpesvirus egress. (A) The capsid forms in the nucleus and acquires its envelope from the nuclear membrane while budding into the perinuclear space. Single or multiple enveloped virions are enclosed in a transport vesicle. Virion-containing vesicles travel through the secretory or exocytic pathway and reach the Golgi, where the viral glycoproteins are processed and matured. Mature virions are transported to the plasma membrane to be released from the cell. (B) The capsid acquires a primary envelope while budding into the perinuclear space. The primary virion formed in the perinuclear space (primary enveloped perinuclear virion) is then de-enveloped by fusion of the envelope with the outer nuclear membrane and is released into the cytoplasm. Non-enveloped capsids migrate to the TGN and acquire final envelopes. Mature virions are transported to the plasma membrane via vesicles and are released from the cell. INM: inner nuclear membrane, ONM: outer nuclear membrane (Modified from [103])
migrate to the Golgi, where they would acquire envelopes by being wrapped into transport vesicles [106]. The capsids entering the cytoplasm through the impaired nuclear pores can also bud at the outer nuclear membrane and RER membranes [106].

Opposing Darlington and Wild’s speculations, another model suggested that the virion obtains its primary tegument and envelope at the inner nuclear membrane and then de-envelopes while fusing with the outer nuclear membrane (Figure 2-2 and 2-3B). Primary envelopment occurring at the inner nuclear membrane requires a nuclear envelopment complex (NEC), which contains UL31 and UL34 [107]. UL31 is recruited to the inner nuclear membrane through the interaction with UL34, a type II membrane protein [107, 108]. The NEC recruits protein kinase C (PKC) to the nuclear rim to phosphorylate the nuclear lamin protein, lamin B, which, along with the other two lamin proteins, lamin A and lamin C, forms the nuclear lamina lining the nucleoplasmic face of the inner nuclear membrane [109]. UL31 and UL34 also induce re-localization of lamin A/C [110]. In addition, the viral protein kinase, Us3, phosphorylates lamin A/C and disrupts the nuclear lamina [111, 112]. Disruption of the nuclear lamina allows the nucleocapsid to bud into the perinuclear space and the acquisition of the primary envelope from the inner nuclear membrane [113]. Primary virions formed in the perinuclear space then enter the cytoplasm through de-envelopment, which is achieved by fusion of the primary envelope with the outer nuclear membrane [113]. This fusion requires gB, gD and gH/L heterodimers, which are recruited to the inner nuclear membrane and incorporated into the primary envelope [114-116], and is regulated by the
kinase Us3 [117]. Non-enveloped capsids released into the cytoplasm gain their final tegument and envelope at TGN-derived membranes [118-121]. The latter model is currently viewed as the most plausible and accepted pathway [122]. Evidence suggests that virion assembly is a two-step envelopment process by the fact that, in the case of PRV, two tegument proteins conserved in the herpesviridae, UL31 and UL34, required for primary envelopment, cannot be found in mature virions [120, 123]. In addition, major tegument proteins, such as UL46, UL47 and UL48, can only be found in extracellular virions but not in primary enveloped perinuclear virions [118, 124, 125]. Furthermore, ultrastructural studies on HSV-1 and PRV demonstrate that the morphology of extracellular virions is different from that of primary virions in the infected cells. The spikes protruding from the envelope, demonstrated to be glycoproteins, of extracellular virions are absent from the primary virions [126, 127]. Although in HSV-1, gB and gH are shown to be required for the fusion of primary virions with the outer nuclear membrane during nuclear egress [114, 128], and gB and gD are detected in the perinuclear virions [115], these three glycoproteins are not essential in PRV nuclear egress and are absent in the primary perinuclear virions [129]. Furthermore, the phospholipid composition of the extracellular virion envelope is different from that of the nuclear membrane [130]. Thus, current evidence favors the two-step envelopment hypothesis.

There are two prominent tegument subassembly sites in the cytoplasm: the capsid and the trans-Golgi network (TGN). After primary envelopment, the capsid is released
from the outer nuclear membrane into the cytoplasm, where it assembles with the inner
tegment proteins. Outer tegument proteins and glycoproteins reside at the TGN, waiting
for the inner tegument accompanied capsid to migrate to this subassembly site for final
envelopment. Here, the mature virion forms by budding of the capsid into the TGN
membrane and acquires its final envelope [122]. Although many studies have suggested
that the TGN is the major site for herpesvirus assembly [131], late endosomes (LE) [132]
and multivesicular bodies (MVB) [133, 134] also have been proposed as alternative
envelopment sites.

2.6 Tegument Assembly and Envelopment

There are twenty or so viral proteins in the tegument (Table 2-2) [2, 3], which
includes both structural proteins and a variety of enzymes. The functions of most
tegment proteins are poorly understood. In recent years, many studies have gained
insight into the protein-protein interactions within this complex and have revealed the
significance of the tegument during virus assembly (review in [6, 135]). Tegument
proteins can be divided into two major groups: inner tegument proteins that interact with
the capsid and outer tegument proteins that interact directly with membranes or
glycoproteins embedded in the cellular membranes (Figure 2-4). Protein-protein
interactions in the tegument are complicated. There are three types of tegument protein
interactions involved in virus assembly: 1) capsid interaction with inner tegument
proteins, 2) interactions between inner and outer tegument proteins, and 3) outer
tegument protein interactions with the envelope. Each type of interaction is important in facilitating virus assembly.

**Table 2-2.** Viral proteins in alphaherpesvirus virions.

<table>
<thead>
<tr>
<th>Capsid</th>
<th>Tegment Primary&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Secondary</th>
<th>Envelope</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inner</td>
<td>Outer</td>
<td>Un-grouped</td>
</tr>
<tr>
<td>UL6</td>
<td>UL31</td>
<td>UL14</td>
<td>UL4</td>
</tr>
<tr>
<td>UL18 (VP23)</td>
<td>UL34</td>
<td>UL11&lt;sup&gt;*&lt;/sup&gt;</td>
<td>UL10 (gM)</td>
</tr>
<tr>
<td>UL19 (VP5)</td>
<td>US3 (PK&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>UL13 (PK)</td>
<td>UL20</td>
</tr>
<tr>
<td>UL35 (VP26)</td>
<td>UL16</td>
<td>UL41 (vhs)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>UL22 (gH)</td>
</tr>
<tr>
<td>UL38 (VP19c)</td>
<td>UL21</td>
<td>UL46 (VP11/12)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>UL27 (gB)&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>UL36 (VP1/2)</td>
<td>UL47 (VP13/14)</td>
<td>UL43</td>
</tr>
<tr>
<td>UL17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>UL37</td>
<td>UL48 (VP16)</td>
<td>UL44 (gC)&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>UL25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>US3 (PK&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>UL49 (VP22)&lt;sup&gt;**&lt;/sup&gt;</td>
<td>UL49.5 (gN)</td>
</tr>
<tr>
<td></td>
<td>ICP0</td>
<td>UL51&lt;sup&gt;**&lt;/sup&gt;</td>
<td>UL53 (gK)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US2</td>
<td>UL56&lt;sup&gt;*&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>US11</td>
<td>US5 (gJ)</td>
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<tr>
<td></td>
<td></td>
<td>ICP4</td>
<td>US6 (gD)&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ICP34.5</td>
<td>US7 (gI)</td>
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<td></td>
<td>US8 (gE)&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>US9&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*</sup> Proteins reported to be associated with membrane rafts.
<sup>**</sup> Tegument proteins that interact with membrane but not lipid raft-associated.
<sup>a</sup>: “Primary” indicates proteins that are involved in the primary envelopment but may not in the secondary envelopment, as UL31 and UL34 participate only in the primary envelopment and are not appeared in the extracellular virions.
<sup>b</sup>: minor capsid proteins
<sup>c</sup>: PK stands for protein kinase
Figure 2-4. Alphaherpesvirus tegument proteins involved in final envelopment. This figure reflects known protein-protein interactions but not subvirion localization. Colour code: yellow - inner tegument proteins; orange - outer tegument proteins; pink - tegument proteins interacting with the TGN membrane (envelope); blue and purple - kinases (transient interactions with tegument proteins); olive - cellular proteins. (Modified from [136])
2.6.1 Capsid-Tegument protein interactions

UL36, UL37 and Us3, are the first three proteins identified that remain associated with the nucleocapsid after virus entry and are considered as inner tegument proteins [83]. The capsid-proximal tegument is likely involved in cytoplasmic transportation of capsids to a docking site at the TGN during egress. However, it is still unclear whether all these proteins participate in primary envelopment and remain associated after nuclear egress, or if they are recruited in the cytoplasm after de-envelopment. Experiments that treated purified HSV-1 virions with different concentrations of salt to determine the strength of capsid-tegument protein interactions also suggest the interactions of Us3, UL36 and UL37 with the capsid [137]. Other tegument proteins identified to associate with the capsids include ICP0, UL14, UL16 and UL21, and of which, the interactions of UL14, UL16 and UL21, with capsid proteins were also determined in yeast two-hybrid assays [138]. UL14 interacts with the capsid protein, UL35 (VP26). Both UL16 and UL21 interact with UL35 and the major capsid protein, UL19 (VP5) [138]. Association between UL16 and capsids has been demonstrated in HSV-1 infected cells although it is thought to be a transient interaction [139]. UL36 is the largest tegument protein with its N-terminal tail interacting with UL37 and C-terminal tail interacting with UL25 [140, 141]. In PRV-infected cells, the absence of UL37 causes nucleocapsid aggregates to form in the cytoplasm that lack most of the tegument proteins of mature virions except UL36 [142]. Deletion of UL37 in HSV-1 also blocks virus maturation [143, 144]. These data suggest that UL37 is required to recruit and direct other tegument constituents to where
they need to be [78, 143] and is not required for UL36 to interact with the capsid, which is consistent with the fact that UL36 interacts with capsid-associated UL25 directly. UL36 in PRV is required for transporting capsids via microtubules in the cytoplasm [145]. Defects in UL36 in HSV-1 and PRV resulted in failure of virion formation [119, 145, 146]. Recent studies on HSV-1 UL36 reported that it is also involved in virus entry [144]. Together, the data suggest an essential function of UL36 in an early stage of infection and also in transporting capsids to the TGN for final envelopment.

Another two inner tegument proteins, UL17 and UL25, together termed the CCSC (C-capsid specific component; C-capsid is also known as mature capsid) or CVSC (capsid-vertex specific component) are also considered as minor capsid proteins [147] and are required for viral genome packaging into the capsids [148]. The two proteins interact with each other and directly contact with the capsid [148-150]. Deletion of UL17 in HSV-1 reduces the amount of UL25 present in the capsids, suggesting that it is required for efficient binding of UL25 to the capsids [151]. UL25 interacts directly with the capsid and UL36 [141, 152]. Deletion of UL25 results in failure of recruiting UL36 to the capsid, indicating its essential role in linking tegument proteins and capsid [141]. Cryo-EM analysis also suggests an interaction between UL36 and CCSC [153].

2.6.2 Tegument protein-Tegument protein interactions

Interaction between inner and outer tegument proteins is significant for virus assembly. In HSV-1, the abundant tegument protein UL48 interacts with several other
proteins: UL41, which requires UL48 to be incorporated into the virion [154], UL46, UL47 [155], UL49 [156] and the inner tegument protein UL36 [155]. Deletion of UL48 causes non-enveloped nucleocapsids to accumulate in the cytoplasm and secondary envelopment in HSV-1 and PRV infected cells is blocked [118, 157]. This suggests a significant role for UL48 in linking capsid and envelope-associated tegument during virion formation (See Chapter 2.6.3).

Another series of protein-protein interactions includes UL11, UL16 and UL21. UL16 is found to physically interact with UL11 [155, 158] and UL21 [159, 160]. At least one of UL11 and UL21 is required for incorporation of UL16 into virions [161]. UL11 interacts with membranes directly and will be discussed further in the protein-envelope interaction section (Chapter 2.6.3). Besides its interaction with UL16, UL21 is found to be essential for incorporation of UL46, UL49 and Us3 into virions inasmuch as deletion of UL21 results in drastic decreases in the incorporation of the UL46, UL49 and Us3 into mature PRV virions [162].

2.6.3 Tegument protein-Envelope interactions

Interaction between outer tegument proteins and membranes is important for assembly and final envelopment since herpesvirus virion acquires its envelope from the TGN. Outer tegument proteins interacting with membranes are listed in Table 2-2. They can interact with cellular membranes either directly by post-translational modification, such as acylation, or indirectly, through interaction with glycoproteins.
Tegument proteins interacting with the TGN membrane via glycoproteins include UL11, UL13, UL48 and UL49. They are proposed to interact with the cytoplasmic tails of glycoproteins. Recent studies on HSV-1 UL11 discovered its interaction with gE and each is required for efficient incorporation of the other into virions [163]. In HSV-1, UL13 interacts with gE [164]. In PRV-infected cells, UL48 interacts with gB, gD and gH [165] while UL49 interacts with gD [166], gE and gM [167, 168]. Glycoprotein M has been implicated in localizing other glycoproteins to the membranes inasmuch as the complex of gM/UL49A in HSV-1 and gM in PRV localize to the TGN and are able to re-localize other membrane proteins to TGN-like compartments [168]. Furthermore, deletion of gE/I and gM leads to a failure to incorporate UL49 into virions [167]. HSV-1 UL49 also interacts with membranes directly through a 100 amino acid sequence enriched in basic residues [169]. All of the above examples demonstrate that the interaction between tegument proteins and membranes destined to serve as the virion envelop are critical for final virion assembly [170].

Despite interacting through glycoproteins, some tegument proteins interact with cellular membranes directly via acylation (myristoylation and palmitoylation). UL51, a phosphoprotein, is palmitoylated and this post-translational modification is required for Golgi targeting in transfected cells [171]. Although UL51 is dispensable for viral replication in cell culture, viruses deleted for UL51 in PRV and HSV-1 produce substantially smaller plaques compared to the wildtype virus, suggesting a role in virus egress [172, 173]. Besides its interaction with gE, HSV UL11 has been shown to interact
with the cytoplasmic face of TGN-derived membranes through myristoylation and palmitoylation [170, 174]. Lack of UL11 in both HSV-1 and PRV caused a reduction in virus production and accumulation of non-enveloped capsids in the cytoplasm [175, 176]. A delay in the release of extracellular viruses was also observed in an HSV-1 UL11-null infection [175]. Together, these data suggest a role for UL11 in final assembly and virus egress. In addition, both PRV and EHV-1 UL11-null mutant viruses produce smaller plaques compared to the wildtype strains, revealing the involvement of UL11 in cell-to-cell spread [176, 177]. Despite its membrane association via dual acylation, UL11 is also found to interact with gE [178]. As mentioned previously, UL11 also binds to another conserved tegument protein, UL16, in HSV-1 infected cells [158] and UL16 interacts with UL21 in PRV infected cells [159]. Combined with the fact that UL21 is required for efficient incorporation of UL46, UL49 and US3 [162], the cumulative data support the roles for UL11 in cellular trafficking and directing other tegument components to the site of secondary envelopment by interacting with UL16 or other tegument constituents.

UL41 (Vhs) is also suggested to interact with the membranes although it does not contain any putative post-translational modification signals for membrane targeting nor interact with glycoproteins [179, 180]. Recent studies show that the N-terminal 42 amino acids are responsible for membrane association and incorporation into virions of HSV-1 UL41 [180]. Interestingly, membrane association is infection dependent, suggesting that other viral proteins are involved [180]. Previous studies have shown that UL41 interacts with UL48 (VP16) [154] and UL48 interacts with UL49 [156]. Both UL48 and UL49
associate with the membranes via interaction with glycoproteins, which in turn may imply that the membrane association of UL41 is facilitated by multiple interactions.

Another tegument protein, UL46, is also shown to associate with cellular membranes and HSV-1 capsids [181]. The interaction with the membranes is probably through multiple domains in UL46 and occurs independently of other viral proteins [181].

Us9 and UL56 are not glycoproteins but are considered as envelope proteins inasmuch as they have transmembrane domains. HSV-2 UL56, for example, proposed to be a tail-anchored type II membrane protein, associates with membranes through its C-terminal hydrophobic region [182]. PRV Us9 is demonstrated to be a type II tail-anchored membrane protein [183].

2.7 Alphaherpesvirus Us2

The genome of the attenuated PRV vaccine strain, Bartha, lacks gI, gE, Us9 and Us2 genes [184, 185]. Although the deletion of Us2 coding sequences did not show apparent defects, deletion of Us2 in the genome of Bartha that is repaired with gE/gI/Us2/Us9 caused a drastic decrease in virulence [186]. Previous studies have shown that a PRV mutant harbouring a deletion of Us2 resulted in an accumulation of virions in the cytoplasm of primary cultures [22]. Our lab has previously reported that PRV Us2 interacts with MAPK ERK and spatially regulates its activity [11]. Inhibition of ERK kinase activity causes a delay in the release of extracellular viruses and this phenotype is more profound during PRV Us2-null virus infection [11]. Moreover, an EHV-1 Us2-null
virus has been reported to produce slightly, but significantly, smaller plaques than those of wildtype and repaired viruses, although no effect on growth kinetics was observed [9]. Collectively, these data suggest a role for Us2 in the release of virions from infected cells. However, the mechanism of how Us2 acts in virus assembly and egress remains undefined. PRV Us2 contains a C-terminal CAAX motif for prenylation and localizes to the plasma membrane and intracellular vesicles [21]. In contrast, HSV Us2 and EHV-1 Us2 do not contain any conventional membrane targeting signals. EHV-1 Us2 has been demonstrated to associate with both cellular membranes and the viral envelope [9]. Together, these data implicate that Us2 promotes virus assembly and egress by bridging interaction between the tegument and envelope as well as promoting the fusion of virion transport vesicles to the plasma membrane.

2.8 The MAPK ERK Signaling Pathway

ERK is one of the MAPKs that regulates diverse cellular programs. ERK1 and ERK2 (ERK1/2) are closely related, sharing 83% identity, and are the most characterized members within the ERK family [187, 188]. MAPK pathways consist of a series of phosphorylation events and each of them is comprised of at least three enzymes: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. There are four well-characterized MAP kinase pathways in mammals: ERK1/2 [188, 189], p38 (α/β/γ/δ), SAPK/JNK and ERK5/BMK1 [190, 191]. (MAPK mentioned in the following text refers to all the MAPKs, if not specifically indicated.)
The MAPK signaling pathway can be triggered by different extracellular stimuli through the binding of ligands to specific receptors expressed on the cell surface. Diverse extracellular signals converge into a MAPK signaling pathway to produce distinct cellular responses. For example, activation of ERK signaling promotes cell growth, differentiation and development.

Ligands for the MAPK ERK signaling cascade include growth factors, such as epidermal growth factor (EGF) and nerve growth factor (NGF), that bind to receptor tyrosine kinases (RTKs) (Figure 2-5). Binding of the growth factors stimulates autophosphorylation and activation of the receptors (i.e. RTKs). Active receptors subsequently phosphorylate adaptor proteins, such as Grb2 and Shc [192]. Adaptors interact with the GTP-exchange factor (GEF) SOS (son of sevenless) [192, 193]. SOS exchanges GDP that is bound to Ras for GTP, which leads to a conformational change of Ras [194, 195]. Active GTP-bound Ras interacts with Raf family kinases and recruits them to the membrane for phosphorylation and activation by kinases, such as PAK (p21 activated kinase) and Src [196]. Raf then activates MAPKKs, MEK1 and MEK2 (“MEKs” will be used in the following context) [197, 198], and recruits them to the plasma membrane. In resting cells, MEKs bind to ERK1/2 in the cytoplasm. Upon activation, Raf recruits and activates MEKs by phosphorylation [199]. Active MEKs then
Figure 2-5. MAPK ERK signaling pathway. Extracellular stimuli (e.g. growth factors) activate receptors at the cell surface. Activated receptor phosphorylates adaptor proteins (e.g. Grb2 and Shc), which then interact with GTP-exchange factor (GEF) SOS. SOS exchanges GDP from Ras for GTP, which leads to a Ras conformational change and recruitment of Raf. Raf is activated by kinases, such as PAK and Src. Active Raf phosphorylates MEK, which subsequently phosphorylates ERK. Active ERK can activate its cytosolic substrates or translocate into the nucleus and activate its nuclear substrates. (Modified from [200])
phosphorylate and release ERK1/2. Active ERK1/2 can activate their cytosolic substrates or translocate into the nucleus and activate their nuclear substrates, such as Elk-1.

ERK1/2 are serine/threonine protein kinases that recognize consensus sequences with serine or the threonine residues located in the proximity of proline residues, usually Pro-X-(Ser/Thr)-Pro [201]. Although ERK1 and ERK2 phosphorylate the same sites with different efficiency in vitro [201], their high degree of similarity is usually considered to be functionally redundant. Substrates of ERKs are distributed both in the cytoplasm and in the nucleus. Active ERK1/2 homodimerizes and phosphorylates its substrates in the cytoplasm, such as ribosomal S6 kinases (RSKs) and MNK (MAPK interacting kinase). To activate nuclear substrates, such as Elk-1 and other transcription factors, ERK1/2 translocates into the nucleus. Transcription factors activated by ERK1/2 subsequently promote transcription of various genes. The genes expressed can be responsible for cell proliferation and differentiation, such as c-fos and early growth response gene 1 (egr-1).

The structure of ERK2 has three notable features: an activation/phosphorylation site, a common docking domain for substrate docking site, termed the CD domain (residues 312-320), and a MEK insertion site, termed the MEK insert (residues 241-272) (Figure 2-6). The activation loop contains a threonine (T183) and a tyrosine (Y185) residue (TXY motif), which are both required to be phosphorylated by MEK1/2 to fully activate ERK. MEK1 and MEK2 are highly similar, in the case of Homo sapiens, sharing 80% identity and 87% similarity, but show differential ability in activating ERK.
Figure 2-6. Structure of ERK2. MAPK ERK contains a phosphorylation/activation site, a common docking domain for substrate docking, termed CD domain (residues 312-320) and a MEK insertion site, termed MEK insert (residues 241-272). The activation site contains T183 and Y185 residues that are phosphorylated by its upstream kinase, MEK. [202]
MEKs are dual specificity kinases (i.e. phosphorylate both serine/threonine and tyrosine residues) and recognize only the native forms of ERK1/2 [204]. ERK1/2 is, so far, the only known substrate of MEKs and is exclusively activated by MEKs [204, 205].

The substrate docking site is required for efficient phosphorylation for both ERK substrates and ERK, and also considered to confer signal specificity through specific binding to its substrates. ERK1/2 interacts with its substrates via the CD domain and phosphorylates their serine or threonine residues. The CD domain contains two aspartic acids (D316 and D319 in the murine ERK2 ortholog) that are important for electrostatic interactions. The D domain, also known as the LXL motif or the DEJL (Docking site for ERK and JNK, LXL) motif, is a cluster of basic residues followed by a consensus sequence, Leu-X-Leu, that can be recognized by MAPKs including ERK1/2, JNK and p38. Proteins containing the LXL motif include the transcription factors, Elk-1 and c-Jun, substrates for ERK and JNK, respectively, and are usually located N-terminal to the transcription-activation domain. MAPKKs, including MEKs, also contain sequences very similar to the D domain, which is comprised of a cluster of basic residues followed by hydrophobic residues, at their N-termini. Mutations on either aspartic acids in the ERK CD domain or basic and hydrophobic residues in MEK D domain reduce MEK-ERK docking activity, suggesting that both hydrophobic and electrostatic interactions are involved in mediating high affinity interactions [206-208].

Another motif recognized by ERK1/2 is the FXF motif, also known as the DEF motif (Docking site for ERK, FXF), located downstream of the substrate phosphorylation
site. It is found on a number of proteins, including MAPK substrates (e.g. Elk-1) and scaffolding proteins (e.g. kinase suppressor of Ras-1 (KSR-1)). ERK1/2 interacts with FXF-containing proteins through the MAPK insert, which locates between residues 243 and 273, close to the phosphorylation lip. The FXF motif is required for efficient phosphorylation by ERK [209]. MAPK substrates can possess either the LXL or FXF motif, or both, which can be recognized specifically by their MAPKs. For example, FXF motifs mediate high affinity interaction between ERK, but not JNK, and their substrates [209]. Previous studies using Elk-1, which contains both motifs, as a model substrate show that these two motifs can function either independently or synergistically [209].

Between MEKs and ERK, multiple interacting sites provide specific phosphorylation toward ERK by MEK. While the CD domain is important for efficient ERK phosphorylation, the MAPK insert has been shown to be an interaction determinant for MEK docking to ERK [210] and is required for specific phosphorylation by MEKs [205]. An additional sequence that can be recognized by ERK is Leu-X-X-Arg-Arg (LxxRR) followed by several basic residues. The LxxRR motif can be found in MAPK-activated protein kinases (MAPKAPKs), such as MNK (MAPK-interacting kinase) and MSK (mitogen- and stress-activated protein kinase), for efficient phosphorylation, and protein tyrosine phosphatase (PTPs), such as STEP (striatum-enriched phosphatase) and PTP-SL for ERK binding and regulation [211, 212]. Although these motifs are shared among MAPKs, their substrates and upstream MAPKKs, the variability in the number and position of the basic and hydrophobic residues within the docking sites as well as the
number of docking sites contained in the substrates may contribute to the specificity of their interactions and subsequent signaling and cellular responses.

Other than substrate docking determinants, signaling specificity can also be regulated spatially by scaffolding proteins. Scaffolding proteins have been suggested to participate in kinase activation. As positive regulators, they can recruit a complex of consecutive kinases to an activated receptor and sequester them from binding to other proteins to facilitate signal transduction. Several scaffolding proteins have been reported to negatively regulate the ERK MAPK signaling by controlling subcellular localization of key components in the pathway [18, 19]. ERK bound to a scaffold is unable to translocate into the nucleus to activate its nuclear substrates and subsequent gene transcription. The purpose of such negative regulation is still unclear. Hypothesized functions include facilitating activation of cytosolic substrates for specific cellular responses.

There are several scaffolding proteins known to tether and confine ERK to the cytoplasm, such as KSR (kinase suppressor of Ras). Previous studies have revealed that co-operation between Ras and KSR accelerates activation of MEK1 and ERK. KSR act as a positive regulator by facilitating MEK activation by Raf [213, 214]. In resting cells, MEK is associated with KSR in the cytoplasm. Upon growth factor stimulation, MEK is recruited to the plasma membrane by KSR for phosphorylation [215]. Further investigation showed that the biological effects of KSR depended on the level of KSR expressed. In KSR knockout mice, growth factor-induced ERK activation is reduced and the reintroduction of KSR into the mice shows ERK activation in a KSR dose-dependent
manner [216]. Optimal level of KSR induces prolonged activation and an increased capability of cell proliferation [216, 217]. Those results demonstrate the role of KSR as a scaffolding protein, which positively regulates MAPK pathway [218].

Previous studies have shown that PRV Us2 interacts with ERK and spatially regulates ERK activity by sequestering it to the plasma membrane (Figure 2-5). In this study, various approaches were employed to understand the mechanism of interaction between PRV Us2 and ERK.

2.9 Protein regulation through ubiquitination

Proteins can be covalently attached with a monoubiquitin, multi-mono-ubiquitin (monoubiquitin on several lysine residues) or a polyubiquitin chain. Ubiquitin, a small 76 amino acid protein, contains seven lysine residues: K6, K11, K27, K29, K33, K48 and K63 (Figure 2-7). Proteins are modified through the linkage between the C-terminal glycine of an ubiquitin to their lysine residues. Conjugation of ubiquitin to a protein substrate involves three enzymes, E1, E2 and E3 ligase, and three successive steps: 1) the ubiquitin activating enzyme (E1) forms a thiol-ester bond with an ubiquitin in an ATP-dependent manner, 2) the activated ubiquitin is transferred to the ubiquitin conjugating enzyme (E2), generating an E2-ubiquitin thiol-ester intermediate, 3) the ubiquitin is transferred to a substrate by forming an isopeptide bond between the C-terminal glycine of the ubiquitin and a lysine residue in the substrate protein. The last step is catalyzed by an E3 ligase, which confers substrate specificity [219]. Attachment of an ubiquitin to a
Figure 2-7. Structure of ubiquitin. There are seven lysine residues in the amino acid sequence of ubiquitin. The lysine residue used to form a linkage between ubiquitin molecules leads to different types of polyubiquitin chains. Each linkage associates with distinct cellular functions as indicated in the figure. [220]
protein acts as a priming event for subsequent addition of ubiquitin molecules to synthesize a polyubiquitin chain. A polyubiquitin chain forms when the C-terminal glycine of an ubiquitin is covalently linked to a lysine residue of another ubiquitin. Linkages can form on all seven lysine residues and individual linkage could lead to distinct topology of a polyubiquitin chain and distinct cellular functions [220].

Ubiquitination is well-known for its role in proteasomal degradation. After several rounds of addition of an ubiquitin molecule to K48 of the previous conjugated ubiquitin, a polyubiquitin chain is formed. The substrate protein tagged with a K48-linked polyubiquitin chain is recognized by the 26S proteasome for degradation. Substrate proteins for proteasome include not only misfolded, damaged and excess proteins, but also a variety of critical regulatory proteins, such as transcription factors and membrane receptors (e.g. growth factor receptors), that control diverse cellular programs, including cell cycle and signal transduction, to maintain cell homeostasis [221]. In recent years, the non-proteasomal functions of ubiquitination, including DNA damage response, endocytosis and protein sorting signal for lysosomal degradation, were discovered [222]. The outcome of ubiquitination is determined by the linkage type, for example, proteins modified by a K48-linked polyubiquitin chain are targeted to the proteasome whereas proteins modified with a K63-linked polyubiquitin chain are sorted to lysosomes. Quantitative proteomic studies reveal that all non-K63-linkages can mediate proteasomal degradation [223]. K11-linked and K48-linked polyubiquitin chains are the most abundant types in vivo (Figure 2-7) [223]. Other than proteasomal degradation, K11-
linked polyubiquitin chains are also involved in the ER-associated degradation (ERAD) pathway [223].

Monoubiquitination can serve as an endocytic signal at the plasma membrane and as an endosomal sorting signal for lysosomal degradation, which is important for down-regulation of signal transduction by degradation of cell surface receptors (Figure 2-8). Ubiquitin itself can function as an internalization signal. Previous studies have shown that a ubiquitin moiety fused in-frame to a receptor lacking internalization signals or ubiquitination sites is able to stimulate internalization of the receptor [224]. In addition, a mutant ubiquitin that lacks all the lysine residues and is therefore unable to form polyubiquitin chains can mediate internalization of the receptor that it is fused to, suggesting that the ubiquitin itself is recognized as an internalization signal [224, 225]. Although monoubiquitination is sufficient to trigger internalization, the extended conformation of K63-linked polyubiquitin chains could enhance the endocytic process [226].

Proteins at the plasma membrane, such as receptors, can be endocytosed through either a clathrin-dependent or a clathrin-independent pathway. Previous studies have shown that when the epidermal growth factor receptor (EGFR) is not ubiquitinated, it is internalized through the clathrin-dependent pathway. However, when it is ubiquitinated, a substantial fraction of EGFR is internalized through the clathrin-independent pathway [227]. After entering early endosomes, membrane proteins can be either recycled back to the plasma membrane or directed to lysosomes for degradation (Figure 2-8). At the
Figure 2-8. Roles of ubiquitin in the endocytic pathway. Ubiquitin functions as an endocytic signal at the plasma membrane. Monoubiquitination triggers internalization of the ubiquitinated membrane proteins. Ubiquitin also acts as a sorting signal at the early endosomes, targeting ubiquitinated proteins into the LE/MVB pathway for lysosomal degradation. [228]
endosomes, ubiquitin serves as a signal for sorting proteins to mature late endosomes, also known as multivesicular bodies (MVB), and lysosomes for degradation [229].

Ubiquitin conjugated proteins are recognized by Vps27 (a.k.a. Hrs) through its ubiquitin-interaction motif (UIM). Vps27 recruits the endosomal complex required for transport (ESCRT) complexes and drives the ubiquitinated proteins into the MVB [229]. Previous studies on EGFR have shown that ubiquitination is essential for targeting it to lysosomes. Ubiquitinated EGFRs enter the LE/MVB pathway and are degraded in the lysosomes while non-ubiquitinated EGFRs are recycled back to the plasma membrane [226, 230].

Furthermore, LC-MS/MS analysis of the ubiquitinated EGFR shows that most of the EGFR-associated ubiquitin is in the form of a polyubiquitin chain and that the K63-linked polyubiquitin chain is more abundant than other linkages. Together, these findings suggest the role of ubiquitin in protein sorting signaling [226].
Chapter 3

Materials and Methods

3.1 Cell lines

Vero cells, HaCaT cells, Caco-2 cells, T12 cells, PK15 cells, and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) in a 5% CO2 environment. NIH 3T3 cells, obtained from the ATCC, were maintained in DMEM containing 10% newborn calf serum (NCS). Caco-2 cells were a kind gift from Dr. E. Petrof (Queen’s University, ON, Canada). HaCaT cells were kindly provided by Dr. Szewczuk (Queen’s University, ON, Canada). T12 cells were a gift from Dr. W. Bresnahan (University of Minnesota, MN, USA).

3.2 Expression plasmids

The PRV Us2 expression plasmids pCC34 (wildtype Us2), pCC35 (Us2GAAX), pJR73 (3XFLAG-Us2), and pJR47 (GAL4DBD-Us2) have been described previously (3, 18). The ERK2-enhanced green fluorescent protein (EGFP) expression construct was kindly provided by N. W. Bunnet, UCSF (5). The pCMV-myc vector was a kind gift from P. Sadowski, University of Toronto. Plasmids encoding FLAG-tagged PRV Us2 C-terminal truncation mutants were constructed by amplifying Us2 from pCC34 using Pfx polymerase (Invitrogen, Burlington, Ontario, Canada)-mediated PCR using forward
primer PRVUs2 F EcoRV and reverse primers PRVUs2N37, PRVUs2N71, PRVUs2N84, PRVUs2N146, PRVUs2N157, PRVUs2N214, N214CAAX R, N233CAAX R, N246CAAX R (see Appendix E). The PCR products were digested with EcoRV and SalI and cloned into pFLAG-CMV-2 (Sigma, St. Louis, MO) and pGBKTK7 (Clontech, Palo Alto, CA) that had been digested with EcoRV-SalI and SmaI-SalI, respectively. Plasmids encoding PRV Us2 LxxRR mutants were constructed by site-directed mutagenesis using primers PRVUs2 F EcoRI, R183K/R184K F/R, R183A/R184A F/R, R183-184A F/R (LAAAA), and PRVUs2 R SalI to amplify Us2 from pCC34. The PCR products were digested with EcoRI and SalI and cloned in frame into pCIneo and pGBKTK7 that had been digested with EcoRI and SalI. The PRV Us2 AAAAA (LTRRR/5A) mutant was constructed by amplifying Us2 from R183-185A-pCIneo plasmid using primers PRVUs2 F EcoRI, PRVUs2LxxRR5A F/R, and PRVUs2 R SalI. The PCR product was digested with EcoRI and SalI and cloned in frame into pCIneo and pGBKTK7. Myc-tagged PRV Us2 was constructed by amplifying Us2 from pCC34 using primers PRVUs2 F EcoRI and PRVUs2 R BglII. The PCR product was digested with EcoRI and BglII and cloned in frame into pCMV-myc that had been digested with EcoRI and BamHI. To subclone murine ERK2 into pACT2 (Clontech, Palo Alto, CA), pML32 (18) was digested with BamHI and the purified insert treated with the Klenow fragment to produce a blunt-ended product. The insert was ligated to pACT2 that was digested with NcoI and treated with the Klenow fragment. Plasmids encoding ERK2 mutants were constructed by site-directed mutagenesis using primers pACT2 MUT F2, H229R F/R, N235K F/R, Y260N
F/R, D315A/D318A F/R, and ERK2 R XhoI to amplify the genes from ERK2-pACT2. The PCR products were cloned into pCR-BluntII-TOPO vector using a Zero Blunt TOPO PCR cloning kit (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer’s instructions. Positive clones were digested with NcoI and XhoI, and the inserts were cloned in-frame into pACT2 that had been similarly digested. ERK2 D315A/D318A mutant was cloned into pEGFP-C1 (Clontech, Palo Alto, CA) by amplifying the gene from ERK2 D315A/D318A-pACT2 using forward primer ERK2 F HindIII and reverse primer ERK2 R SalI. The PCR product was cloned into pCR-BluntII-TOPO vector using a Zero Blunt TOPO PCR cloning kit. A clone containing the PCR product was digested with HindIII and SalI, and the insert was cloned in-frame into pEGFP-C1 that had been similarly digested. Plasmids encoding Flag-tagged HSV-2 Us2 and TAP-tagged HSV-2 Us2 were constructed by amplifying HSV-2 Us2 gene using primers HSV-2 Us2 F EcoRV and HSV-2 Us2 SalI. The PCR product was subcloned into pFLAG-CMV-2 and pNTAP-A (Stratagene, La Jolla, CA) using Zero Blunt TOPO PCR cloning kit as described previously. Yeast plasmid encoding HSV-2 Us2 fused to GAL4-DBD was constructed by amplifying HSV-2 Us2 gene using primers HSV-2 Us2 F EcoRI and HSV-2 Us2 SalI. The PCR product was subcloned into pGBKKT7 using Zero Blunt TOPO PCR cloning kit as described previously. The HSV-2 Us2 expression vector, HSV-2 Us2-pCIneo, was constructed by former post-doc Dr. B. B. Roy.
3.3 Antibodies

Antibodies against HSV-2 Us2 were prepared by our former lab technician, Ms. Susan Johnston. The HSV-2 Us2 gene was fused to the C-terminus of the glutathione S-transferase (GST) gene in plasmid pGEX-3X (Amersham Biosciences, Piscataway, NJ). An insoluble GST-Us2 fusion protein was isolated from IPTG induced Rosetta (DE3) E. coli using the B-Per Bacterial Protein Extraction Reagent (Pierce, Rockford, IL). The partially purified protein was subjected to 10% SDS-PAGE. A band containing the GST-Us2 fusion protein was excised and sent to Cedarlane Laboratories (Burlington, ON) for the production of polyclonal antiserum in Wistar rats. The resulting antiserum was used for Western blotting at a dilution of 1:500 and for indirect immunofluorescence microscopy at a dilution of 1:200. The production of PRV Us2 antiserum (used at a 1:10,000 dilution for immunoblotting and a 1:500 dilution for immunostaining) was described previously (3). Monoclonal antisera against total ERK1/2 (1:1,000 dilution for immunoblotting) and phospho-ERK1/2 (1:1,000 dilution for immunoblotting) were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antiserum against GFP (1:1,000 dilution for immunoblotting) was purchased from Clontech (Palo Alto, CA). Mouse monoclonal antiserum against c-myc (1:2,000 dilution for immunoblotting) was purchased from Roche Diagnostics (Laval, Canada). Anti-FLAG polyclonal antibody was purchased from Sigma (St. Louis, MO). Mono- and polyubiquitin antibody (FK2) was purchased from Enzo Life Sciences (Burlington, ON).
HSV ICP5, ICP8, ICP27 and gD were purchased from Virusys Corporation (Taneytown, MD).

3.4 Viruses

All virus strains were propagated in HaCaT cells. The HSV-2 recombinant virus encoding Flag-Us2 was constructed using en passant mutagenesis as described previously [231]. In brief, the sequence used for recombination was amplified using primers HSV2 Us2_Flag_BAC_F and HSV2 Us2_Flag_BAC_R (see Appendix E) from pEP-kan-S2 that contains a kanamycin (Kan) cassette as the marker and an I-SceI site. The PCR product was purified using a PCR Purification kit (QIAgen, Toronto, ON) and digested with DpnI overnight at 37°C. For the first recombinant, the *E. coli* GS1783 strain containing a HSV-2 BAC pYEbac373 containing a chloramphenicol (Cm) resistance gene was grown at 32°C until OD600 was between 0.6 and 0.8. Bacteria were then transferred to 42°C for 15 minutes to induce the expression of Red recombination components. Cells were then incubated on ice for 20 minutes and pelleted at 3,000 rpm for 15 minutes at 4°C. Pelleted cells were resuspended in cold sterile water. The DpnI digested PCR product was electroporated into GS1783 at 1.8kV, 200Ω and 2.5µF, and cells were plated on LB containing Cm and Kan. The success of recombination was confirmed by the presence of Kan cassette using colony PCR. For the second recombination, clones with BAC containing Kan cassette were grown at 32°C until OD600 was between 0.4 and 0.6 and then incubated in LB containing 2% arabinose for 1
hour to induce expression of I-SecI and restriction digestion. Cells were transferred to 42°C for 15 minutes to induce Red recombination. Cells were the incubated at 32°C for 1 hour and then plated on selection medium containing Cm and 1% arabinose. To screen for the success of second recombination, selected clones were plated in duplicate on LB containing either Kan or Cm. Clones that were resistant to Cm but sensitive to Kan were confirmed for the absence of Kan using colony PCR. BAC DNA of the derived recombinants was purified using Plasmid Mini Kit (QIAGen, Toronto, ON) and electroporated into Vero cells with Cre-expression plasmid, pOG231. Cells were plated on a 100mm culture dish containing 10ml DMEM and incubated overnight at 37°C. Medium was removed and replaced with 10ml methocel. Cells were incubated until plaques formed. Plaques were collected by pipetting the methocel surrounding the plaque area and transferred into a fresh microcentrifuge tube. Collected plaques were diluted into 1:10, 1:100 and 1:1000 in 500ul DMEM. Cell grown in 6-well plates were infection with diluted viruses and incubated until plaque formed. Single plaque was collected and amplified for future use. The HSV-1 recombinant virus encoding Flag-Us2 gene and HSV-2 Us2-deleted and repair recombinant virus were constructed using the same protocol. Primers used for constructing recombinant viruses are listed in Appendix E. The HSV-1 BAC pYEbac102 encoding HSV-1 wildtype strain F was kindly provided by Dr. Y. Kawaguchi (Tokyo Medical and Dental University, Tokyo, Japan) and Dr. G. Smith (Northwestern University, MI, USA). The HSV-2 BAC pYEbac373 encoding HSV-2 wildtype strain 186 was a kindly given by Dr. Y. Kawaguchi. The E. coli strain GS1783
and pOG132 were gifts from Dr. G. Smith. Dr. V Le Sage transferred pYEbac373 into GS1783 to facilitate the construction of HSV-2 recombinants.

3.5 Yeast two-hybrid analysis

The PRV Us2 C-terminal and N-terminal truncations and HSV-2 Us2 were cloned into pGBK7 (Trp) (Clontech, Palo Alto, CA). Wildtype ERK1, ERK2, and four ERK2 mutants, H229R, N235K, Y260N, and D315A/D318A, were cloned into pACT2 (Leu) (Clontech, Palo Alto, CA), as described in “Expression plasmids”. Plasmids were cotransformed into Saccharomyces cerevisiae strain yCH1. The absence of tryptophan from the growth medium selected for the Us2 plasmids, and the absence of leucine from the medium selected for the ERK2 plasmids. In these assays, interaction between Us2 and ERK brings the GAL4 DNA binding domain (DBD) and activation domain (AD) in proximity, leading to the transcription of the reporter genes HIS3 and ADE2, which allows yeast to grow on medium lacking histidine and adenine, respectively. Selected clones were grown on -Trp/-Leu/-His dropout medium (medium stringency) or -Trp/-Leu/-Ade/-His (high stringency) to select for two-hybrid interactions.

To construct PRV Us2 mutant library, primers PRVUs2 MUT F and PRVUs2 MUT R (see Appendix E) and the error-prone Mutazyme II DNA polymerase from Gene MorphII EZClone Domain Mutagenesis kit (Stratagene, La Jolla, CA) were used to amplify the Us2 gene that has already been cloned into a yeast plasmid (pJR47). The PCR products were used as “mega primers” to amplify the whole plasmid. The resulting
plasmids, which contain mutated Us2 genes, were treated with DpnI for 4 hours to digest the template plasmids and transformed into XL-10 Gold Ultracompetent cells (Stratagene, La Jolla, CA) for amplification. Transformed cells were spread onto plates to determine the numbers of clones, which represent the numbers of mutated Us2 genes. Clones were collected, inoculated into LB containing kanamycin and incubated at 30ºC for 4 hours, for amplification. Plasmids were extracted and co-transformed into yCH1 with ERK2 D315A/D318A-pACT2. Transformed cells were plated on –Leu/-Trp dropout medium and replica plated onto –Leu/-Trp/-His and –Leu/-Trp/-His/-Ade dropout medium. Plasmids from clones capable to grow on –Leu/-Trp/-His/-Ade dropout medium were extracted and sequenced. Mutants with single point mutation were co-transformed with ERK2-pACT2 and grown on selection plates to determine interactions.

3.6 Membrane flotation assay

HEK293T cells were grown to 80% confluence in 150-mm dishes and transfected with HSV-2 Us2 expression plasmid. Cells were liberated from the dish using trypsin and centrifuged at 200 x g for 7 minutes at 4ºC. Cells were washed with cold PBS once and then washed with homogenization buffer (0.25M sucrose, 10mM KCl, 10mM Tris, pH7.4, 1.5mM MgCl2). Cells were re-suspended in 500ul of homogenization buffer supplemented with protease inhibitors and incubated on ice for 30 minutes. Cells were homogenized by passing through a 26 gauge syringe whilst leaving nuclei intact. Cells were centrifuged at 600 x g for 10 minutes at 4ºC to collect post-nuclear supernatant
(PNS). Three hundred microlitres of PNS was mixed with 2.7ml of 85% sucrose (w/v) in TNE buffer (100mM NaCl, 10mM Tris, pH7.4, 1mM EDTA) and placed at the bottom of a SW41 centrifuge tube. Six millilitres of 65% of sucrose (w/v in TNE) were laid on the top of PNS/sucrose mixture, followed by 3ml of 10% sucrose (w/v in TNE) at the top. The sucrose gradient was centrifuged at 27,000 rpm for 18 hours at 4°C in a Backman SW41 rotor. Twelve 1ml fractions were collected from the top of the gradient. Fractions 3 and 4 from the top were diluted with 500ul of TNE buffer and centrifuged at 100,000 rpm for 15 minutes at 4°C with Beckman MLA130 rotor. Pellets were re-suspended with 60ul of PBS and 60ul of SDS loading buffer for Western blot analysis or with 1ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) for immunoprecipitation.

For digitonin assays, 500ul from the interface between the 10% and 65% sucrose was diluted with 500ul of TNE buffer and centrifuged at 100,000 rpm for 15 minutes at 4°C in a Beckman MLA130 rotor. Pellets were re-suspended in 1ml TNE and 500ul was incubated with 0.01% digitonin for 1 hour at 4°C, and then centrifuged at 100,000 rpm for 15 minutes, as described above, to pellet membranes. The pellet was re-suspended in 60ul of 1X SDS loading buffer. Five hundred microlitres of TNE buffer was added to the supernatant, followed by the addition of 250ul of 100% TCA (trichloroacetic acid), and incubated overnight at 4°C. The supernatant was centrifuged at 100,000 rpm for 15 minutes at 4°C in Beckman MLA130 rotor. Pellets was washed with acetone and re-
suspended in 60ul of 1X SDS loading buffer. Proteins were then subjected to Western blot analysis.

### 3.7 Immunoprecipitation (IP)

HEK293T cells were grown to 80% confluence in 150-mm dishes, then transfected with FLAG-tagged wildtype Us2 (pJR73) or C-terminal Us2 truncation expression plasmids and ERK2-EGFP, EGFP-ERK2 D315A/D318A, or pEGFP-C1 (Clontech, Palo Alto, CA) expression constructs. For Us2 oligomerization experiments, cells were co-transfected with pJR73 or pFLAG-CMV-2 and Myc-Us2 expression constructs. Twenty-four hours post-transfection, cells were washed with phosphate-buffered saline (PBS) and scraped into 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing protease inhibitors (Roche Diagnostics, Laval, Canada) and phosphatase inhibitors (50 mM NaF, 10 mM Na₃VO₄) and centrifuged at 13,000 rpm for 10 min. Supernatants were transferred to a fresh microcentrifuge tube containing 40 ul of anti-FLAG M2 affinity gel (Sigma, St. Louis, MO) and rocked overnight at 4°C. Anti-FLAG beads were pelleted by brief centrifugation at 9,000 rpm. Supernatants were discarded, and the beads were washed three times successively with 1 ml Tris-buffered saline (TBS). Immune complexes were re-suspended in 50 ul of SDS-PAGE sample buffer and boiled for 5 min. Samples were analyzed by SDS-PAGE on 12% gels, followed by Western blotting.
To determine if Us2 oligomers were capable of interacting with ERK, cells were transfected with pJR73 or pFLAG-CMV-2, Myc-Us2 or pCMV-myc, and ERK2-EGFP expression constructs. Co-IPs were performed as described above to isolate FLAG-Us2. The immune complexes were washed three times with TBS and then subjected to competitive elution by using 3XFLAG peptide (Sigma, St. Louis, MO) according to the manufacturer’s instructions. Eluates then were incubated with anti-myc antibody (Roche Diagnostics, Laval, Canada) overnight at 4°C. The antigen-antibody complexes were recovered using protein G agarose (Pierce Biotechnology, Rockford, IL). Protein G agarose was washed three times successively with 1 ml TBS. Protein complexes were re-suspended in SDS-PAGE sample buffer and boiled for 5 min prior to Western blot analysis.

To identify interacting partners and PRV-2 Us2, plasmid expressing Flag-Us2 was transfected into 293T cells grown in 150mm dishes. At 24 hours post-transfection, cells were collected in lysis buffer. Lysates were incubated with anti-FLAG M2 affinity gel overnight at 4°C. Anti-FLAG beads were washed three times with TBS. Precipitated protein complexes were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. Bands were excised and sent for LC-MS/MS at Queen’s University Mass Spectrometry and Proteomics Unit.

To identify interacting partners and HSV-2 Us2, plasmid expressing Flag-Us2 was transfected into 293T cells grown in 150mm dishes. At 24 hours post-transfection, cells were collected to purify membrane fractions using membrane flotation assay as
described previously. Membranes were incubated with anti-FLAG M2 affinity gel overnight at 4°C. Anti-FLAG beads were washed three times with TBS. Precipitated protein complexes were subjected to SDS-PAGE and stained using SimplyBlue™SafeStain according to manufacturer’s instruction (Invitrogen, Burlington, Ontario, Canada). Bands were excised and sent for LC-MS/MS at UVic Genome BC Protein Centre.

To determine if HSV-2 Us2 could interact with ubiquitin-conjugated proteins in infected cells, Vero cells grown in 150mm dishes were infected with HSV-2 wildtype strain 186, HSV-2 Us2-null and HSV-2 Us2 repair viruses at an MOI of 5. At 6 hour post-infection, cells were collected with lysis buffer and centrifuged at 14,000 x g for 20 minutes at 4°C. Lysates were incubated with anti-FLAG M2 affinity gel overnight at 4°C. Anti-FLAG beads were washed three times with TBS. Precipitated protein complexes were subjected to Western blotting. To determine if HSV-2 Us2 could be pulled down by ubiquitin-conjugated proteins, lysate were incubated with antibody against mono- and polyubiquitin (FK2) (Enzo Life Sciences, Burlington, ON) overnight at 4°C. Protein G agarose was added to antigen/antibody complexes and the mixtures were nutated at 4°C for 2 hours. Protein G agarose was washed three times with TBS, re-suspended in 1X SDS loading buffer, and analyzed by Western blotting.
3.8 Ubiquitin binding assay

To determine if HSV-2 Us2 interacted directly with ubiquitin, Vero cells grown in 150mm dishes were infected with wildtype HSV-2 at an MOI of 5 for 6 hours. Cells were collected in lysis buffer and centrifuged at 14,000 x g for 20 minutes at 4°C. Ubiquitin-conjugated beads were washed twice with 25mM HEPES, pH7.5, before use. Lysates were incubated with monoubiquitin-conjugated beads, K48-linked and K63-linked tetra ubiquitin-conjugated beads (Boston Biochem, Cambridge, MA) overnight at 4°C. Beads were washed three times with wash buffer (10mM Tris, pH 7.4, 100mM NaCl, 0.1% NP-40), re-suspended with 1X SDS loading buffer, and analyzed by Western blotting.

To determine the ubiquitin binding of Us2 in transfected cells, a N-terminal Flag-tagged HSV-2 Us2 expression plasmid was transfected into 293T cells growing on two 150mm dishes. At 24 hours post-transfected, cells were collected in 800ul of lysis buffer per dish and centrifuged at 14,000 rpm for 15 minutes. Supernatants were incubated with anti-FLAG M2 affinity gel (Sigma, St. Louis, MO) overnight at 4°C. The immune complexes were washed three times with TBS and then subjected to competitive elution by using 3XFLAG peptide (Sigma, St. Louis, MO) according to the manufacturer’s instructions. Eluates then from two dishes were pooled and incubated with monoubiquitin-conjugated beads (Boston Biochem, Cambridge, MA) overnight at 4°C. Beads were washed three times with wash buffer, re-suspended with 1X SDS loading buffer, and analyzed by Western blotting.
3.9 Tandem affinity purification (TAP)

Plasmid expressing TAP-Us2 was transfected into 293T cells growing in 150mm dishes. At 48 hours post-transfection, cells were collected in lysis buffer and freeze-thawed three times at -80ºC/37ºC. Lysates were centrifuged at 15,000 rpm for 10 minutes three times to pellet cell debris. Supernatants were collected and proteins purified using the InterPlay Mammalian TAP system kit (Stratagene, La Jolla, CA), according to manufacturer’s instruction. Purified proteins were subjected to SDS-PAGE electrophoresis and stained using SimplyBlue™ SafeStain according to manufacturer’s instruction (Invitrogen, Burlington, Ontario, Canada). Bands were excised and sent for LC-MS/MS at UVic Genome BC Protein Centre for identification.

3.10 Immunofluorescence microscopy

Vero cells were seeded onto glass coverslips in 6-well plates and grown to 30 to 40% confluence. Cells were transfected with 1 ug of plasmid DNA using FuGENE 6 (Roche, Laval, Canada) according to the manufacturer’s instructions. At 24 h post-transfection, cells were rinsed with PBS and then fixed in 4% paraformaldehyde-PBS for 10 min at room temperature. Cells were rinsed with PBS and permeabilized in 1% bovine serum albumin (BSA)-PBS containing 0.1% Triton X-100 at room temperature for 5 min. Cells were rinsed three times with PBS and incubated with Us2 goat polyclonal antiserum diluted in 1% BSA-PBS (1:500) for 1 h. Cells were rinsed three times with 1% BSA in TBS and incubated with Alexa 568-conjugated secondary antibodies (Invitrogen,
Burlington, Ontario, Canada) and diluted in 1% BSA-PBS (1:500) for 1 h. To visualize nuclei, cells were stained with Hoechst 33342 (Sigma, St. Louis, MO) diluted to 0.5 ug/ml in PBS for 7 min at room temperature, followed by three rinses with PBS. Coverslips were mounted onto glass slides, and digital images were captured using an Olympus FV1000 laser-scanning confocal microscope.

3.11 Fluorescence recovery after photobleaching (FRAP) analyses

PK15 cells seeded onto 35-mm glass-bottom dishes (MatTek, Ashland, MA) were co-transfected with the indicated plasmids using the FuGENE 6 transfection reagent. Twenty-four hours after transfection, the medium was replaced with warm DMEM (lacking phenol red)-10% FCS, and the cells were mounted onto an Olympus FV1000 confocal microscope and maintained at 37°C in a humidified 5% CO2 environment. All image acquisition parameters were controlled using Olympus Fluoview software version 1.7.3.0. EGFP was excited using a 488-nm laser line set at 5% power. Images (512 by 512 pixels) were obtained using a 60X (1.42-numerical aperture) oil immersion objective and a digital zoom factor of 4. Images were collected at a rate of 0.2 frames per second. Three frames were collected before the regions bounded by the red rectangles were photobleached by repeated scanning with a 488-nm laser set at 80% power for a total of 200 ms. Before, during, and after photobleaching, fluorescence intensity in bleached and unbleached control regions was measured using Fluoview 1.7.3.0, and the data were exported into Microsoft Excel for graphical presentation.
3.12 Elk-1 dual-receptor assay

Elk-1 activation was measured using the dual-luciferase reporter system (Promega, Madison, WI). NIH 3T3 cells were grown to 40 to 60% confluence in 24-well plates, and each well was co-transfected with 30 ng of GAL4-fused Elk-1 (pFA2-Elk-1) (Stratagene, La Jolla, CA), 200 ng of luciferase reporter linked to a GAL4 binding element (pFR-Luc) (Stratagene, La Jolla, CA), 50 ng Renilla-luciferase (pRL-SV40) (Promega, Madison WI), 30 ng of constitutively active MEK (pFC-MEK1) (Stratagene, La Jolla, CA), and 200 ng of wildtype Us2, mutant Us2, or pCIneo (empty vector control) expression plasmid using the TransIT-3T3 transfection kit (Mirus, Madison, WI) according to the manufacturer’s instructions. Transfections with each plasmid combination were performed in triplicate. Cells were incubated in medium containing 10% NCS for the first 24 h and in medium containing 0.5% NCS for the final 24 h to minimize background ERK activity. Elk-1-dependent transcription (i.e., luciferase activity) was measured using a Promega dual-luciferase reporter system. In some experiments, the MEK inhibitor U0126 (Promega, Madison WI) was included at a concentration of 30 μM.

3.13 Virion purification

HaCaT cells were infected with wildtype HSV-2, Us2-null and repair recombinant viruses at an MOI of 5 for 24 hours. Cells were scrapped into medium and
centrifuged at 3,500 rpm for 15 minutes at 4°C to pellet cells. Supernatant was collected and centrifuged twice more to completely remove cells and cell debris. Supernatants were laid over onto 3ml of 30% sucrose (v/w in PBS) in a SW41 tube and centrifuged at 24,000 rpm for 3 hours at 4°C in a Beckman SW41 rotor. Virion pellets were re-suspended in 1X SDS loading buffer and analyzed by Western blotting.
Chapter 4

Results

4.1 Interaction between PRV Us2 and the MAPK ERK

All alphaherpesvirus Us2 orthologs share three N-terminal conserved regions, however, their C-terminal sequences are highly variable. The difference in C-terminal amino acid sequences may therefore differentiate the mechanism they utilize to promote viral replication.

Previous studies have shown that PRV Us2 binds to ERK and inhibits the activation of ERK nuclear substrates by sequestering ERK at the plasma membrane [11, 232]. ERK is a serine/threonine kinase in a MAPK signaling cascade, responsible for cell growth, differentiation and development. Substrates of ERK reside both in the nucleus, such as Elk-1, and in the cytoplasm, such as MAPK-interacting kinase (MNK). After being phosphorylated by its upstream kinase, MEK, ERK dimerizes and translocalizes into the nucleus. Our lab had previously shown that PRV Us2 interacts with ERK and spatially regulates its activity [11]. However, the mechanism of interaction between Us2 and ERK is undefined. By utilizing a series of Us2 truncations, we mapped the determinants for Us2-ERK interaction.
4.1.1 PRV Us2 confers the properties of a membrane protein upon ERK

To examine the dynamics of ERK localization in Us2-expressing cells, we performed FRAP (Fluorescence recovery after photobleaching) experiments (Figure 4-1). Cells were co-transfected with either the empty vector pCIneo (Figure 4-1A, row i) or a Us2 expression plasmid (Figure 4-1A, row ii) along with an ERK2-EGFP expression construct. In the absence of Us2, ERK was localized diffusely throughout the nucleus and cytoplasm of transfected cells. In contrast, the majority of ERK localized to the plasma membrane, to the nuclear envelope, and to punctate vesicular structures in cells that were co-transfected with a Us2 expression plasmid. The photobleaching of ERK2-EGFP in pCIneo-co-transfected cells resulted in the rapid recovery of the fluorescent signal into the photobleached region within 4 s (Figure 4-1B, graph i). These data are consistent with ERK2-EGFP behaving as a soluble cytoplasmic protein. The photobleaching of ERK2-EGFP that was localized to the plasma membrane in Us2-expressing cells resulted in a much lower rate of recovery of fluorescence into the bleached region, with the maximal recovery observed by 35 s after bleaching (Figure 4-1B, graph ii). These findings suggest that the interaction between Us2 and ERK changes the diffusion properties of ERK such that, in the presence of Us2, ERK behaves like a membrane protein. PRV Us2 associates with membranes through the posttranslational addition of a farnesyl group to its C-terminal CAAX motif [21]. The ability of ERK to bind to membrane-tethered Us2 likely explains these findings.
Figure 4-1. FRAP analysis of ERK2-EGFP in the absence and presence of PRV Us2. (A) Images of live PK15 cells co-transfected with pCIneo and ERK2-EGFP (i), Us2 and ERK2-EGFP (ii), or Us2GAAX and ERK2-EGFP expression plasmids (iii) immediately before, immediately after, or 28.8 s (i), 40 s (ii), and 40.1 s (iii) after the area outlined in the red box were photobleached using an Olympus FV1000 laser-scanning confocal microscope. The boxed blue area is proximal to the photobleached region, and the boxed orange area is distal to the photobleached region. The fluorescence signal is ERK2-EGFP. Scale bars are 10 µm. (B) Quantitation of fluorescence intensity of the photobleached area, as well as control proximal and distal areas, over time. Shown are data for PK15 cells co-transfected with pCIneo and ERK2-EGFP (i), cells co-transfected with Us2 and ERK2-EGFP expression plasmids (ii), and cells co-transfected with Us2GAAX and ERK2-EGFP expression plasmids (iii).
To determine the role of Us2 prenylation in ERK membrane localization, we mutated the cysteine of the Us2 CAAX motif to a glycine, thereby abrogating the ability of Us2 to be prenylated inside cells [21, 233]. This mutant Us2 protein was designated Us2GAAX. When Us2GAAX and ERK2-EGFP were co-expressed, ERK2-EGFP localized to large cytoplasmic vesicles but was not present at the plasma membrane, indicating that the prenylation of Us2 is necessary for plasma membrane localization (Figure 4-1A, row iii). When the ERK2-EGFP in one half of a large cytoplasmic vesicle was photobleached, the migration of ERK2-EGFP from the non-bleached portion of the vesicle into the bleached region occurred with maximal recovery observed by 40 s after bleaching (Figure 4-1A, row iii, and B, graph iii). These findings suggest that, like wildtype Us2, Us2GAAX imparts the properties of a membrane protein upon ERK2-EGFP.

The finding that nonprenylated Us2 maintained the ability to associate with membranes was unexpected, because the loss of this lipid modification was predicted to result in the loss of Us2-membrane association. The majority of alphaherpesvirus Us2 proteins do not contain signals for prenylation or other lipid modifications, nor do they contain putative membrane-spanning domains. Curiously, Meindl and Osterrieder have demonstrated that EHV-1 Us2, a nonprenylated protein, is indeed membrane associated [9]. It may be that nonprenylated Us2 orthologs associate with membranes through interaction with other membrane components.
4.1.2 Mapping PRV Us2 determinants required for interaction with ERK

To identify the minimal portion of Us2 required for interaction with ERK, we began by constructing a series of C-terminal truncation mutants (Figure 4-2A). Each truncation was cloned into a yeast expression vector, pGBK7, for yeast two-hybrid assays and a mammalian expression vector, pFLAG-CMV-2, for co-immunoprecipitation (co-IP) studies and Elk-1 trans-reporter assays. We first examined protein-protein interactions in yeast by co-expressing Us2 truncation mutants fused to the GAL4 DNA binding domain (DBD) and ERK2 fused to the GAL4 activation domain (AD). Only yeast strains co-transformed with ERK2 and wildtype Us2 or Us2N214 expression plasmids were able to grow on medium lacking histidine, indicating a two-hybrid interaction (Figure 4-2, A and B). We also found that the deletion of the first 14 N-terminal residues (ΔCR1) of Us2 disrupted the ERK interaction (Figure 4-2, A and B). We then performed IP experiments to determine if N214 was able to interact with ERK in mammalian cells. Immunoprecipitates and total cell extracts were analyzed by Western blotting (Figure 4-2C). The results show that while both FLAG-tagged Us2 and EGFP-fused ERK2 were expressed efficiently, only full-length Us2 could pull down ERK2-EGFP.

Elk-1 trans-reporter assays that utilize an ERK-dependent Elk-1-responsive luciferase expression construct were performed to determine the effects of Us2 truncations on the ERK signaling pathway. Upon the activation of ERK by MEK, ERK dimerizes and enters the nucleus, where it phosphorylates many transcription factors,
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Figure 4-2. Mapping Us2 determinants required for interaction with ERK. (A) Cartoon of Us2 truncated mutants. The top line shows full-length Us2 protein and highlights the conserved regions CR1, CR2, and CR3, the C-terminal CAAX motif, which serves as a signal for Us2 prenylation, and an LTRRR motif located at residues 181 to 185. The structure of six C-terminal truncation mutants and one N-terminal truncation mutant analyzed in this study are shown. (B) Truncation mutant N214 interacts with ERK in yeast. Expression plasmids for the Us2 truncations shown in panel A fused to the Gal4 DBD were co-transformed with a Gal4 AD ERK2 expression plasmid into yeast. Transformants were plated on medium lacking leucine, tryptophan, and histidine (medium stringency). (C) Co-IP of Us2 and ERK. FLAG-tagged Us2 and ERK2-EGFP fusion proteins were co-expressed in HEK293T cells and immunoprecipitated with anti-FLAG agarose. Total cell extracts (left panels) and immunoprecipitates (right panels) were analyzed by Western blotting using antibodies reactive with Us2 (α-Us2; bottom blots) and EGFP (α-EGFP; top blots). Asterisks shown in the top right blot indicate the position of background levels of EGFP and ERK2-EGFP that bind nonspecifically to FLAG-Us2 fusion proteins. (D) Elk1 luciferase reporter assays of Us2 truncation mutants. NIH 3T3 cells were transfected with constitutively active MEK, empty vector (EV) or Us2, GAL4-fused Elk-1, and luciferase reporter linked to GAL4 binding sequences. Luciferase activity is expressed as relative light units. Combinations of plasmids are indicated under the x axis. Each assay was performed in triplicate, and the error bars represent the standard deviations between replicate assays.
including Elk-1. Previous studies indicated that wildtype Us2 sequestered ERK in the cytoplasm, preventing it from entering the nucleus to activate Elk-1 and resulting in a dramatic decrease in luciferase activity from the reporter construct [11]. In the absence of constitutively active MEK, a basal level of luciferase activity was observed (Figure 4-2D). As expected, the inclusion of a constitutively active MEK expression construct in the transfection cocktail led to a 240-fold activation of luciferase activity. The incubation of these cells with the MEK inhibitor, UO126, dramatically decreased luciferase activity to below basal levels. These control experiments confirmed that the assay was dependent upon ERK activity for the production of luciferase. Similarly to what has been reported previously, the inclusion of wildtype Us2 led to a 67-fold reduction in luciferase activity [11]. Consistent with the yeast two-hybrid assays (Figure 4-2B), the N157 and N146 truncation mutants did not inhibit luciferase activity, whereas the N214 truncation showed a 2-fold inhibition of ERK-dependent Elk-1 activity. In summary, the N214 mutant demonstrated a strong interaction with ERK in yeast cells and demonstrated the modest inhibition of ERK signaling in NIH 3T3 cells. However, FLAG-N214 was unable to co-precipitate ERK2-EGFP from HEK293T cell lysates (Figure 4-2C), which may indicate that the N214-ERK interaction is not strong enough to withstand the presence of detergents in the co-IP assays. The co-expression of the N214 truncation with ERK2-EGFP and subsequent analysis by immunofluorescence microscopy revealed the substantial co-localization of the two proteins; however, as both proteins localized
throughout the cytoplasm and to cytoplasmic aggregates, these data could not reliably report on the interaction between these molecules (data not shown).

### 4.1.3 LxxRR is not a functional motif for interaction with ERK

Between residues 157 and 214 of Us2, there is a putative LxxRR motif that we hypothesized interacted with ERK. The LxxRR motif, found in the ERK substrates MNK1 and MNK2 [234] and all Rsk isoforms [235], is required for binding to the ERK CD domain. MNK1 contains an LARRR motif, whereas MNK2 and the Rsk isoforms contain an LAQRR motif. The LTRRR motif of PRV Us2 is located from residues 181 to 185 (Figure 4-2A). To determine whether the LTRRR motif is required for Us2 binding to ERK, we first constructed LTRKK (R184K/R185K) and LTRAA (R184A/R185A) mutants that replaced the last two arginine residues with either lysine or alanine [236]. Both mutants maintained their ability to interact with ERK in yeast (Figure 4-3). In addition to the R184K/R185K and R184A/R185A mutants, we also constructed an LAAAA (R183 to 185A) mutant to abolish positive charges in this motif and an AAAAA (LTRRR/5A) mutant to additionally replace the leucine, which might be critical in ERK docking [234], with alanine. Both the R183 to 185A and LTRRR/5A mutants were able to interact with ERK (Figure 4-3), indicating that LTRRR is not a functional ERK binding motif. Taken together, these data suggest that the N-terminal 214 amino acids of Us2 are necessary and sufficient for an interaction with ERK in yeast cells but are not sufficient for detectable interactions in co-immunoprecipitation experiments. These
Figure 4-3. PRV Us2 LTRRR mutants interact with ERK in yeast. Expression plasmids for the indicated Us2 mutants and ERK2 were cotransformed into yeast and plated onto medium lacking leucine, tryptophan, histidine, and adenine (high stringency). Growth is indicative of a two-hybrid interaction. EV: empty vector.
findings suggest that Us2 secondary or tertiary structure, rather than a linear amino acid sequence, is required for the ERK interaction. To analyze this further, we examined the ability of Us2 to form higher-order structures.

4.1.4 PRV Us2 forms oligomeric structures

In the course of our studies, we noted an additional band that reacted with the anti-FLAG antiserum on Western blots of Us2 immunoprecipitates (Figure 4-4A, asterisk). This species had a relative mobility of roughly twice that of Us2, which is consistent with the idea that Us2 can form dimers. To test this idea, we constructed a Myc-tagged Us2 expression plasmid and co-expressed it with FLAG-Us2 and FLAG-Us2N214 expression constructs. If Us2 forms oligomers, then one would predict that Myc-Us2 would be immunoprecipitated by FLAG-Us2. FLAG-tagged Us2s were immunoprecipitated, and the precipitated material was analyzed for the presence of Myc-Us2 (Figure 4-4B). Whereas FLAG-Us2 and FLAG-Us2N214 were efficiently expressed and recovered in the immunoprecipitates (Figure 4-4A), only FLAG-Us2 pulled down Myc-Us2 (Figure 4-4B). The data show that Myc-Us2 formed oligomers with full-length Us2 but not with the Us2N214 truncation mutant. To investigate whether oligomeric Us2 was capable of interacting with ERK, we performed sequential co-immunoprecipitation experiments. Protein complexes from cell lysates containing FLAG-Us2, Myc-Us2, and ERK2-EGFP first were immunoprecipitated by using anti-FLAG affinity gel, and the protein complexes were competitively eluted using a FLAG peptide. The eluted
**Figure 4-4.** PRV Us2 forms oligomers. FLAG-tagged and Myc-tagged Us2 expression constructs were co-transfected into HEK293T cells. Proteins were immunoprecipitated using anti-FLAG agarose and analyzed by Western blotting. Total cell extracts and IPs were probed with anti-FLAG (A) and anti-myc (B) antibodies to detect the presence of Us2. An asterisk indicates a protein species with a relative mobility roughly twice that of Us2. (C) FLAG-tagged Us2, Myc-Us2, and ERK2-EGFP expression constructs were co-transfected into 293T cells, and protein complexes were immunoprecipitated using anti-FLAG agarose and subsequently eluted using a FLAG peptide. The top three blots show the analysis of FLAG-Us2, Myc-Us2, and ERK2-EGFP present in the eluted fraction. Protein complexes were immunoprecipitated from the FLAG elutes using anti-myc antibody and detected for the presence of ERK2-EGFP (asterisk) by Western blotting (bottom blot).
complexes then were immunoprecipitated by using anti-myc antibody, and these immune complexes were analyzed for the presence of ERK2-EGFP by Western blotting. The data demonstrate that ERK2-EGFP was immunoprecipitated specifically in the complexes containing oligomeric Us2 (Figure 4-4C). Taken together, these findings raise the possibility that Us2 oligomerization is required for robust interaction with ERK, and that the inability of Us2N214 to oligomerize might explain why it fails to interact with ERK efficiently.

4.1.5 Mapping ERK determinants required for interaction with PRV Us2

The ERK structure has three notable features: an activation loop, a substrate docking site, called the CD domain, and a MEK binding site, termed the MAPK insert. To identify the Us2 binding site on ERK, we constructed four ERK mutants, H229R, N235K, Y260N, and D315A/D318A (Figure 4-5A). Mutants H229R and N235K locate close to the MAPK insert, whereas Y260N locates in the MAPK insert. Based on previous studies, the H229R and Y260N mutations prevent MEK binding and the N235K mutation inhibits MEK binding [210]. The D315A/D318A mutations are in the CD domain and have been shown to prevent the binding of the ERK substrate, MNK, to ERK [210]. A yeast two-hybrid assay was used to determine the ability of these mutants to interact with Us2, MNK, and MEK K97M, a kinase-dead mutant of MEK that shows stronger interaction with ERK than wildtype MEK [210]. As expected, the H229R and Y260N mutants lost their interaction with MEK K97M, whereas N235K maintained a...
Figure 4-5. PRV Us2 interacts with ERK at its common docking domain. (A) Structure of ERK2. Four mutations, H229R, N235K, Y260N, and D315A/D318A, were introduced into ERK2, and the positions of these key residues are indicated by the arrows. (B) Yeast two-hybrid analysis. Expression plasmids for the ERK2 mutants were co-transformed into yeast strain YCH1 along with empty vector (EV), wild-type Us2, the ERK substrate MNK, or the MEK kinase-dead mutant K97M to determine protein-protein interactions as described for Figure 4-2. The top image indicates the position of the ERK-interacting partners on the plates shown underneath. Growth is indicative of a two-hybrid interaction. (C) Co-immunoprecipitation of Us2 and ERK. FLAG-tagged Us2 was co-transfected with either wild-type ERK2-EGFP fusion or EGFP-D315A/D318A for 24 h. Proteins were immunoprecipitated using anti-FLAG agarose and subjected to Western blot analysis. Cell lysates (left panel) and immunoprecipitates (right panel) were probed using Us2 (α-Us2) antiserum, anti-EGFP (α-EGFP), anti-ERK1/2 (α-ERK1/2), and anti-phospho-ERK1/2 (α-P-ERK1/2) antibodies.
weak interaction with MEK K97M (Figure 4-5B). All three of these mutants interacted with MNK, which was expected because the CD domain remained intact. Furthermore, these mutants maintained the ability to bind to Us2. Whereas the D315A/D318A mutant interacted with MEK K97M, it failed to bind MNK, as shown previously (Figure 4-5B) [210]. Significantly, the D315A/D318A mutant also failed to interact with Us2, indicating that Us2 binds to ERK via its CD domain.

Co-IP experiments were performed to confirm the interaction of Us2 with the ERK CD domain in mammalian cells. FLAG-tagged Us2 and ERK2 or D315A/D318A-EGFP expression plasmids were co-transfected into HEK293T cells for 24 h. Us2 was immunoprecipitated with anti-FLAG affinity gel. Immunoprecipitates and total cell extracts were analyzed by Western blotting using antiserum against Us2, EGFP, ERK1/2, and phospho-ERK1/2. Both Us2 and wildtype ERK2-EGFP or EGFP-D315A/D318A, or EGFP alone, were efficiently co-expressed in cells (Figure 4-5C, left). Moreover, the ERK and D315A/D318A-EGFP fusion proteins were readily detected with anti-phospho-ERK1/2 antibody, indicating that these proteins maintained their ability to interact with, and be phosphorylated by, MEK (Figure 4-5C, left). Consistent with the yeast two-hybrid results (Figure 4-7B), only wildtype ERK2-EGFP and endogenous ERK could be pulled down by Us2. These findings indicate that ERK D315 and D318 residues are critical for Us2 interaction in animal cells (Figure 4-5C, right).

Us2 and the ERK EGFP expression plasmids were co-transfected into Vero cells to determine their subcellular localization (Figure 4-6). In the absence of Us2, EGFP,
Figure 4-6. PRV Us2 does not alter subcellular localization of EGFP-D315A/D318A. Vero cells were co-transfected with Us2 and EGFP-ERK2expression plasmids. Twenty-four hours after transfection, cells were stained for Us2 using Us2 antiserum. Arrowheads indicate points of interest. Scale bars are 10 µm. (A) Localization of Us2 (red) and wildtype ERK2-EGFP (green). (B) Localization of Us2 and EGFP-D315A/D318A. (C) Localization of EGFP-ERK2 in the absence of Us2. (D) Localization of EGFP-D315A/D318A in the absence of Us2. (E) Localization of EGFP in the absence of Us2.
ERK2-EGFP, and EGFP-D315A/D318A, all showed a diffuse nuclear and cytoplasmic distribution (Figure 4-6, C to E). When co-expressed with PRV Us2, wildtype ERK2-EGFP was re-localized to the plasma membrane, where it co-localized with Us2 (Figure 4-5A, arrowhead). In sharp contrast, the localization of the EGFP-D315A/D318A mutant was unaffected in the presence of Us2 (Figure 4-6B, arrowhead).

FRAP experiments were performed to determine the influence of wildtype Us2 expression on the diffusion properties of EGFP-D315A/D318A (Figure 4-7). In the absence of Us2, EGFP-D315A/D318A (Figure 4-7A, row i, and B, graph i) behaved very similarly to ERK2-EGFP (Figure 4-1A, row i, and B, graph i), with the rapid recovery of the fluorescent signal into the photobleached region occurring within 5 s. The diffusion of EGFP-D315A/D318A in the presence of Us2 (Figure 4-7A, row ii, and B, graph ii) was indistinguishable from its behavior in the absence of Us2. Collectively, these data indicate that Us2 interacts with ERK via the CD domain, and that this interaction facilitates the tethering of ERK to the plasma membrane.
Figure 4-7. FRAP analysis of EGFP-D315A/D318A in the absence and presence of PRV Us2. (A) Images of live PK15 cells co-transfected with pCIneo and EGFP-D315A/D318A (i) or Us2 and EGFP-D315A/D318A expression plasmids (ii) immediately before, immediately after, or 24 (i) and 20.1(ii) s after the area outlined in the red box was photobleached using an Olympus FV1000 laser-scanning confocal microscope. The boxed blue area is proximal to the photobleached region, and the boxed orange area is distal to the photobleached region. The fluorescence signal is ERK2-EGFP. Scale bars, 10 µm. (B) Quantitation of the fluorescence intensity of the photobleached area, as well as control proximal and distal areas, over time. Data for PK15 cells co-transfected with pCIneo and EGFP-D315A/D318A (i) and cells co-transfected with Us2 and EGFP-D315A/D318A expression plasmids (ii) are shown.
4.1.6 Yeast two-hybrid screen for PRV Us2 mutants interacting with ERK D315A/D318A

To determine which portion of PRV Us2 interacts with ERK, yeast two-hybrid assays were used to screen for PRV Us2 mutants that gain the ability to interact with ERK D315A/D318A. The isolation of a single point mutation that allow Us2 to interact with ERK D315A/D318A but disrupts interaction between Us2 and wildtype ERK would suggest that the corresponding substituted amino acid is responsible for the interaction. A PRV Us2 mutant library was constructed using error-prone DNA polymerases to amplify the Us2 gene that had been cloned into the pGBKT7 vector and fused to a GAL4 DNA binding domain (GAL4-DBD). The PCR products that contained the mutated Us2 gene were then used as “mega primers” to amplify and reconstitute the whole yeast plasmids. The template plasmid that contained the wildtype Us2 gene was digested with the restriction enzyme, DpnI, to remove wildtype Us2 gene. The resulting library plasmids carrying various mutated Us2 genes were amplified in E. coli and purified. Plasmids expressing Us2 mutants were then co-transformed with the plasmid expressing ERK D315A/D318A fused to a GAL4 activation domain (GAL4-AD), into the yeast strain, yCH1, which carries two reporter genes, adenine and histidine. The GAL4-AD-ERK D315A/D318A expression plasmid was constructed in pACT2, which carried a leucine biosynthesis gene whereas pGBKT7 carried a tryptophan biosynthesis gene. Interaction between Us2 mutants and ERK D315A/D318A brought GAL4-AD and GAL4-DBD in close proximity and activated the transcription of the reporter genes, which in turn allowed yeast to grow on the medium lacking adenine and histidine. Transformed cells
were plated on the selection medium lacking both leucine and tryptophan (-Leu/-Trp) to allow yeast receiving both plasmids to form colonies, which were then replica plated onto higher stringency medium (-Leu/-Trp/-His and -Leu/-Trp/-His/-Ade) to determine if there was an interaction between Us2 and ERK D315A/D318A. Plasmids containing PRV Us2 mutants from the yeast that could grow on the -Leu/-Trp/-Ade/-His medium were purified.

Table 4-1. PRV Us2 mutants identified from the yeast two-hybrid screen.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4</td>
<td>truncated: G246STOP (245 a.a.)</td>
</tr>
<tr>
<td>P6</td>
<td>same as P4</td>
</tr>
<tr>
<td>P12</td>
<td>1 base pair deletion at the n.t. 698</td>
</tr>
<tr>
<td>P16</td>
<td>same as P12</td>
</tr>
<tr>
<td>P23</td>
<td>A134T, missing 34 base pairs from n.t. 693-747</td>
</tr>
<tr>
<td>P24</td>
<td>truncated: E248STOP (247 a.a.)</td>
</tr>
<tr>
<td>P25</td>
<td>same as P12</td>
</tr>
<tr>
<td>P37</td>
<td>A88V, F90S, 1 base pair deletion at n.t. 698</td>
</tr>
<tr>
<td>P40</td>
<td>STOP257Y</td>
</tr>
<tr>
<td>P48</td>
<td>I255N</td>
</tr>
<tr>
<td>P53</td>
<td>same as P37</td>
</tr>
<tr>
<td>P56</td>
<td>1 base pair deletion at n.t. 749</td>
</tr>
<tr>
<td>P69</td>
<td>1 base pair deletion at n.t. 724</td>
</tr>
<tr>
<td>P77</td>
<td>Same as P12</td>
</tr>
<tr>
<td>P91</td>
<td>R210Q</td>
</tr>
<tr>
<td>P94</td>
<td>Same as P94</td>
</tr>
</tbody>
</table>

Note: Most of them have 1 base pair deletion and results in a frameshift. These mutants have an N-terminal sequence different from the wildtype Us2 and the differences start at or downstream of the residue 233. (Bold: clones contain a single point mutation, a.a.: amino acid, n.t.: nucleotide)
Out of 17,000 clones screened, only 96 clones were able to grow on the -Leu/-Trp/-Ade/-His medium. Plasmids containing PRV Us2 mutants purified from the 96 clones were co-transformed with the plasmid expressing wildtype ERK to determine interactions between PRV Us2 mutants and wildtype ERK. Transformed yeast was first plated on the -Leu/-Trp medium and then replica plated on the -Leu/-Trp/-His medium to determine the interactions between PRV Us2 mutants and ERK. Plasmids from the yeast that exhibited no growth or little growth were purified and sequenced (Table 4-1). Seven of them contained a single base pair deletion, which resulted in a frameshift and consequently have different N-terminal sequences beyond amino acid residue 233. Only six isolates contain a single point mutation as listed in Table 4-1. P4 and P6 carry the same mutation, G246STOP, while both P91 and P94 carry a R210Q mutation. Plasmids purified from the clones listed in Table 4-1 were re-cotransformed with the plasmids expressing either wildtype ERK or ERK D315A/D318A mutant to confirm their interactions (Table 4-2). In the repeated transformation, all of Us2 mutants still maintained interaction with ERK D315A/D318A. Except P48, all other mutants also showed interaction with wildtype ERK. The gain of the ability of PRV Us2 mutants to interact with ERK D315A/D318A may suggest that the mutated amino acid is responsible for Us2-ERK interaction.
Clones | WT ERK | D315A/D318A
<table>
<thead>
<tr>
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<td></td>
<td>M</td>
<td>H</td>
<td>M</td>
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<td></td>
</tr>
<tr>
<td>EV</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>P4, P6</td>
<td>+</td>
<td>W</td>
<td>+</td>
<td>+</td>
<td>G246STOP (N245)</td>
</tr>
<tr>
<td>P24</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>E248STOP (N247)</td>
</tr>
<tr>
<td>P48</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>I255N (CTNS)</td>
</tr>
<tr>
<td>P91, P94</td>
<td>+</td>
<td>-</td>
<td>W</td>
<td>-</td>
<td>R210Q</td>
</tr>
<tr>
<td>N214</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tbody>
</table>

Table 4-2. PRV Us2 mutants identified from yeast two-hybrid screen using ERK D315A/D318A as the bait. M: medium stringency, H: high stringency, EV: empty vector, WT: wildtype Us2, W: weak interaction.

Three of the clones (P4, P6 and P24) are truncated mutants while one (P48) contains a mutation in the prenylation motif. P48 lost its interaction with both wildtype ERK and the D315A/D318A mutant, which may suggest that the CAAX motif is involved in the Us2-ERK interaction. However, the truncated mutants, P4, P6 and P24, which lacked a CAAX motif showed strong interaction with wildtype ERK. To understand the mechanism of interaction, four Us2 C-terminal truncated mutants were constructed, based on the yeast two-hybrid results, in mammalian expression plasmids: N233, N246 and those with a CAAX motif attached to the C-terminus (N233CAAX and N246CAAX). These mutants, along with N214 and full length Us2, also represent sequences containing different numbers of the arginine residues (R) at the C-terminus (Figure 4-8). Previous studies on p21\textsuperscript{Ras} have shown that the CAAX motif requires a palmitoylation site or a polybasic domain for plasma membrane localization [237, 238]. In the case of PRV Us2, it contains 9 arginine residues upstream of the CAAX motif. The
Figure 4-8. Schematic structure of the PRV Us2 C-terminal truncated mutants that contain different numbers of arginine residues. Wildtype HSV-2 Us2 contains 9 arginine residues, N246CAAX contains 6 arginine residues and N233 contains only 1 arginine residue (Bold: arginine residues).
truncated mutant N246 (Chapter 4.1.2) and the three mutants, P4, P6 and P24, identified in the yeast two-hybrid screen have 6 arginine residues. PRV Us2 N246 was constructed to represent those mutants that contain 6 arginine residues. Plasmids expressing N233, N233CAAX, N246 and N246CAAX were transfected into Vero cells to determine their subcellular localization and the ability to re-localize ERK.

The immunofluorescence results showed that the CAAX motif could only restore plasma membrane localization of N246 but not N233 (Figure 4-9), consistent with the fact that the CAAX motif requires a polybasic domain for the plasma membrane localization [237, 238]. ERK localizes diffusely in the cytoplasm in the absence of Us2 and is re-localized to the plasma membrane when Us2 is present (see Figure 4-6). Although N246CAAX was able to alter ERK localization, it could not sequester it to the plasma membrane (Figure 4-9B). Instead, it re-localized ERK to punctate structures in the cytoplasm. N233, N233CAAX and N246 also re-localized ERK to cytoplasmic punctate structures, however, they did not show co-localization (Figure 4-9). Altered ERK localization may be either due to transient interaction between PRV Us2 truncated mutants and ERK or mediated through other proteins, for example, the Us2-ERK complex contains another ERK interacting protein, which sequesters ERK at those punctuate structures after Us2 dissociates from the complex.

Co-IP experiments were also performed to determine physical interactions between the Us2 truncated mutants and ERK as well as the capability of the Us2 truncated mutants to form oligomers. Plasmids expressing N-terminal Flag-tagged N233,
<table>
<thead>
<tr>
<th></th>
<th>Us2</th>
<th>EGFP</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>EGFP</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>N233</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
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</tr>
<tr>
<td>ERK2-EGFP</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>N233CAAX</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
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<tr>
<td>EGFP</td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
</tr>
<tr>
<td>N23CAAX</td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
<td><img src="image21.png" alt="Image" /></td>
</tr>
<tr>
<td>ERK2-EGFP</td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 4-9. The CAAX motif restores the plasma membrane localization of N246 but not N233. Plasmids expressing PRV Us2 N233, N233CAAX (A), N246 and N246CAAX (B) were co-transfected with the plasmid expressing either EGFP or ERK-EGFP into Vero cells for 24 hours. Cells were stained using antibodies against PRV Us2.
N233CAAX, N246 and N246CAAX were individually co-transfected with the plasmid expressing EGFP-ERK2 into 293T cells. At 24 hours post-transfection, cells were collected and lysates were incubated with anti-Flag affinity gel to immunoprecipitate protein complexes. Precipitated proteins were analyzed by Western blotting and the results show that all of the truncated mutants, except N214, were able to pull down ERK (Figure 4-10A). The correlation between Us2-ERK interaction and oligomerization was further investigated. Plasmids expressing Flag-Us2, myc-Us2 and EGFP-ERK2 were co-transfected into 293T cells. At 24 hours post-transfection, cells were collected and protein complexes were immunoprecipitated using anti-Flag affinity gel. The results showed that the addition of a CAAX motif to N214 did not rescue its interaction with ERK in the co-IP experiment (Figure 4-10B). Furthermore, Flag-N233CAAX could not co-precipitate myc-Us2, suggesting that N233CAAX could not oligomerize, nonetheless, it maintained the ability to interact with ERK. Together, it may suggest that Us2 can interact with ERK both as a monomer or a dimer and that the sequence contained within residues 233 to 246 (N233-246) is significant for oligomerization (Figure 4-11). In addition, the data suggested that the sequence contained within residues 214 and 233 (N214-233), rather than the CAAX motif, contributes to the interaction between Us2 and ERK. An alternative explanation for the results (Figure 4-10B) could be that N214-N233 is the determinant, regardless of the presence of a CAAX motif, for proper Us2 folding, which is required for the Us2-ERK interaction. In summary, proposed determinants of Us2 mediating interaction with ERK and oligomerization are illustrated in Figure 4-11.
Figure 4-10. Mapping PRV Us2 determinants for interaction with ERK and oligomerization. (A) PRV Us2 does not require a CAAX motif to interact with ERK. Plasmids expressing N-terminal Flag-tagged Us2 truncated mutants were co-transfected with the plasmid expressing EGFP-ERK2 into 293T cells. At 24 hours post-transfection, cells were collected with lysis buffer and lysates were incubated overnight with anti-Flag agarose. Immunoprecipitated proteins were analyzed by Western blotting. Proteins were probed with antibodies against Flag epitope and GFP. (B) N233-246 is required for Us2 oligomerization. Plasmids expressing N-terminal Flag-tagged Us2 truncated mutants, N-terminal myc-tagged Us2 and ERK2-EGFP were co-transfected into 293T cells. At 24 hpt, cells were collected and co-IP was performed as described in (A). Immunoprecipitated protein complexes were analyzed by Western blotting. Proteins were detected using antibodies against Flag, myc and GFP. WT: wildtype, arrow: EGFP-ERK2, asterisk: heavy chain.
Figure 4-11. Proposed PRV Us2 determinants for ERK binding, oligomerization and plasma membrane (PM) localization. The N-terminal 214 amino acids are required for Us2 proper folding and the interaction with ERK. Determinant for Us2 oligomerization is contained within the residues 214 and 233. The 6 arginine residues contained within the residues 233 and 246 are required for plasma membrane localization of Us2.
4.2 Other PRV Us2 interacting partners

Previous studies have shown that ERK kinase activity is required for efficient virus egress [11], which might suggest the involvement of ERK substrates. To determine if any ERK substrates are present in the Us2-ERK complex, plasmid expressing N-terminal Flag-tagged Us2 was transfected into 293T cells. At 24 hours post-transfection, cells were collected in lysis buffer. Lysates were incubated with anti-Flag affinity gel to immunoprecipitate protein complexes. Precipitated proteins were applied to SDS-PAGE. Bands were excised and sent for LC-MS/MS analysis for identification. Although precipitated proteins did not seem to contain ERK substrates, two Us2 interacting proteins were found: vimentin and beta-tubulin. Co-IP and Western blotting were performed to confirm the interaction. Plasmids expressing N-terminal Flag-tagged PRV Us2 N157 truncated mutant, Flag-PRV Us2, Flag-HSV-2 Us2 and Flag-PRV Us3 were transfected into 293T cells. The Flag-PRV Us2 N157 mutants and Flag-PRV Us3 were included as negative controls. At 24 hours post-transfection, cell lysates were prepared and incubated with anti-Flag affinity gel to immunoprecipitate proteins. Precipitated proteins were analyzed by Western blotting and the results suggest that both vimentin and beta-tubulin physically interact with PRV Us2 (Figure 4-11).
Figure 4-12. PRV Us2 interacts with beta-tubulin and vimentin. Plasmids expressing Flag-PRV Us2 N157, Flag-PRV Us2, Flag-HSV-2 Us2 and Flag-PRV Us3 were transfected into 293T cells. At 24 hours post-transfection, cells were collected and lysates were incubated overnight with anti-Flag agarose to immunoprecipitate protein complexes. Precipitated proteins were probed with antibodies against Flag, vimentin and beta-tubulin. Arrowhead (in IB: b-tubulin): beta-tubulin. Asterisk: double bands are immunoglobulin heavy chains.
4.3 Characterization of HSV-2 Us2

The tegument protein, Us2, is conserved throughout the alphaherpesvirinae with the exception of VZV. All Us2 orthologs have three conserved regions at their N-termini whereas the sequences at the C-termini are highly variable. The differences in the C-terminal sequences may differentiate the mechanisms by which Us2 influences viral infection. Previous studies have shown that the deletion of the Us2 gene in PRV resulted in an accumulation of virions within primary cells [22]. Our lab has previously shown that PRV Us2 contains a prenylation motif for membrane targeting and that prenylated PRV Us2 recruits the MAP kinase, ERK, to the plasma membrane via interactions with the ERK CD domain (Chapter 4.1) [11, 232]. Inhibition of ERK activity also causes virions to accumulate in infected cells and this phenotype is more profound in PRV Us2-null virus infection [11]. In addition, EHV-1 Us2-deleted viruses have smaller plaques compared to the wildtype strain [9]. Altogether, these findings suggest that Us2 functions in virus egress. Unlike PRV Us2, EHV-1 Us2 and HSV-2 Us2 do not possess a C-terminal prenylation motif or any other predicted membrane targeting signals. However, EHV-1 Us2 is capable of interacting with membranes. These data may suggest that Us2 orthologs utilize different mechanisms to interact with membranes and facilitate viral egress. In this study, we sought to understand how Us2 promotes viral replication by determining whether HSV-2 Us2 shares properties with PRV Us2.

In this section, the subcellular localization of HSV-2 Us2 was examined using indirect immunofluorescence microscopy. Physical properties, including membrane
association and the ability to oligomerize, were also determined using biochemical methods. To further investigate the roles of Us2 in HSV-2 infection, HSV-2 recombinant virus encoding an N-terminal Flag-tagged Us2 was constructed to identify its cellular and viral interacting partners. By identifying the interacting partners and their functions, we hoped to gain insight into the roles of Us2 in viral replication.

4.3.1 HSV-2 Us2 localizes to the plasma membrane and vesicles

Subcellular localization of HSV-2 Us2 was studied in both transfected and infected cells. Plasmids expressing either untagged HSV-2 Us2 or N-terminal Flag-tagged Us2 were transfected into Vero cells for 24 hours. Cells were stained with antibodies against HSV-2 Us2. The immunofluorescence data show that, like PRV Us2, both untagged HSV-2 Us2 and Flag-HSV-2 Us2 localized to the plasma membrane (Figure 4-13, A and B). Localization of HSV-2 Us2 was also observed in HSV-2 infected cells at 5 hours post-infection. Consistent with the localization in transfected cells, HSV-2 Us2 localizes to the plasma membrane and vesicles in infected cells (Figure 4-13C).

4.3.2 HSV-2 Us2 is tightly associated with cellular membranes at lipid rafts

Although HSV-2 Us2 does not contain any putative membrane targeting signals, it localizes to the plasma membrane like PRV Us2. To determine if HSV-2 Us2 physically associates with the membranes, membrane flotation experiments were performed as described in Materials and Methods. As shown in Figure 4-14A, a portion
Figure 4-13. HSV-2 Us2 localizes to the plasma membrane and cytoplasmic vesicles in both transfected and infected cells. For localization in transfected cells, plasmids expressing N-terminal Flag-tagged Us2 (A) or untagged Us2 (B) were transfected into Vero cells for 24 hours. For localization in infected cells, Vero cells were infected with wildtype HSV-2 for 5 hours (C). Cells were stained using HSV-2 Us2 polyclonal antiserum.
Figure 4-14. Physical properties of HSV-2 Us2. (A) HSV-2 Us2 is associated with the membranes. Plasmid expressing either HSV-2 Us2 or EGFP was transfected into 293T cells for 24 hours. Cells were collected, homogenized and centrifuged. The supernatant (PNS) was subjected to centrifugation on sucrose gradients to purify membrane fractions. Twelve 1 ml fractions were collected from the top of the centrifuge tube. Top to bottom: Fraction #1-12. Fraction 3 and 4: cellular membranes, Fraction 10-12: cytosolic proteins, Fraction 13: pellet at the bottom of the tube. (B) Membranes (Fraction 3 and 4) were either untreated or treated with 0.01% digitonin at 4°C for 1 hour and then centrifuged at 100,000 rpm for 15 minutes. P: pellet, S: supernatant. (C) HSV-2 Us2 forms oligomers. Plasmids expressing Flag-Us2 and TAP-Us2 were co-transfected into 293T cells for 24 hours. Cells were collected and lysates were incubated overnight with anti-Flag agarose at 4°C. Immunoprecipitated proteins were analyzed by Western blotting.
of HSV-2 Us2 was in the membrane-enriched fractions (Fraction 3 and 4), consistent with the idea that HSV-2 Us2 is membrane associated. EGFP, as a control, did not float to membrane fractions but only in the cytosolic fractions (Fraction 10-12) (Figure 4-14A).

There are several ways for proteins to associate with the membranes, one of which is via electrostatic interactions. Proteins interact with negatively charged lipid bilayers through their positively charged residues. Our data showed that the Us2-membrane association could not be disrupted by high salt (1M NaCl) and high pH (0.2M Na₂CO₃, pH 11) treatments, suggesting that the association was not due to electrostatic interaction. Furthermore, treatment of membranes with non-ionic detergent (1% Tx-100) at low temperature released only a portion of Us2 from the membranes, suggesting that HSV-2 Us2 is associated with lipid rafts.

Lipid rafts are dynamic microdomains enriched in sphingolipids, cholesterol and phospholipids with saturated fatty acid side chains. They are biochemically defined as detergent insoluble at low temperature and float to low density in sucrose gradients, and are therefore also termed detergent-resistant membranes (DRM) [250-252]. Lipid rafts are tightly packed liquid-ordered domains embedded in surrounding disordered membranes. DRM exist predominantly in the plasma membrane and some are found in endocytic membranes. During lipid raft formation, cholesterol synthesized in the ER travels to and assembles with sphingolipid in the Golgi to form cholesterol-sphingolipid raft domains [253, 254]. These microdomains move toward the plasma membrane and cycle between cell surface, endosomes and the Golgi, and thereby play an important role
in vesicle sorting and trafficking [250]. Previous studies have shown that crosslinking of raft proteins led to coalescence of multiple rafts and selective recruitment of raft proteins while non-raft proteins like the transferrin receptor were excluded [255]. The properties of clustering proteins, which include receptors, kinases and scaffolds, enable rafts to serve as platforms for signal transduction [256]. Proteins can associate with rafts temporarily or permanently through dual acylation (i.e. myristoylation and palmitoylation), glycosylphosphatidylinositol(GPI)-anchor, transmembrane domain and cholesterol-binding [250, 251]. HSV-2 Us2 does not seem to have any post-translational motifs for acylation or GPI-anchors. Nonetheless, HSV-2 Us2 contains a putative cholesterol binding motif, CRAC (cholesterol recognition/interaction amino acid consensus) (L/V-X_{1,5}-Y-X_{1,5}-R/K) [257], which is contained within sequence residues 134-144 (VPFFEYAQKTR). To determine whether this is a functional CRAC motif, membrane fractions collected in membrane flotation experiments were treated with a cholesterol sequestering agent, digitonin, to release cholesterol binding proteins from the membranes. The association between the membranes and actinin, a cytoskeleton element that has previously been shown to be a cholesterol-dependent membrane binding protein [258], was disrupted after digitonin treatment (data not shown). However, HSV-2 Us2 could not be liberated from the membranes by digitonin, suggesting that it is not a cholesterol binding protein (Figure 4-14B). From these results, we concluded that the membrane association might be due to the interaction between HSV-2 Us2 and other membrane proteins.
4.3.3 HSV-2 Us2 forms oligomers

PRV Us2 has previously been shown to form oligomers (Chapter 4.5). To determine if HSV-2 Us2 also possesses this property, either a TAP (containing both a calmodulin binding peptide and a streptavidin binding peptide) or a Flag epitope was fused to the N-terminus of HSV-2 Us2. Plasmids expressing TAP-Us2 and Flag-Us2 were co-transfected into 293T cells. At 24 hours post-transfection, cells were collected and lysates were incubated with anti-Flag affinity gel. Precipitated protein complexes were subjected to Western blotting to probe for the presence of Flag-Us2 and TAP-Us2. The data show that TAP-Us2 was pulled down with Flag-Us2, indicating that, like PRV Us2, HSV-Us2 form oligomers (Figure 4-14C).

4.3.4 HSV-2 containing vesicles share the properties of recycling endosomes

The data presented so far suggest that Us2 is associated with detergent insoluble membranes and localizes predominantly to the plasma membrane and cytoplasmic vesicles. To determine if Us2 containing cytoplasmic vesicles represent components of the biosynthetic secretory pathway, the dynamic movement of HSV-2 Us2 inside the cell was monitored using live cell imaging. Imaging of Us2 dynamics in live Vero cells transiently transfected with mCherry-Us2 expression constructs revealed that Us2 containing cytoplasmic vesicles are highly dynamic and were frequently observed to form long filamentous tubules that are a hallmark of recycling endosomal compartments [259]. These results prompted us to examine the association of Us2 with endosomes more closely. Upon binding to its cell surface receptor, diferric transferrin is internalized into
clathrin coated vesicles and delivered to an early endosomal compartment [260]. Two Fe\textsuperscript{3+} atoms are released from transferrin in the acidic environment of the early endosome and the resultant apotransferrin, still bound to its receptor, traffics to an endocytic recycling compartment where it is sorted back to the plasma membrane [260]. Alternatively, the apotransferrin/receptor complex can bypass the recycling compartment and is transferred directly to the plasma membrane from early endosomes [261, 262]. Once at the cell surface, the apotransferrin dissociates from its receptor and is again free to scavenge iron. This movement through the endocytic pathway can be monitored using fluorescently conjugated diferric transferrin. Vero cells were transfected with a mCherry-Us2 expression plasmid. At 24h post-transfection cells were incubated with Alexa Fluor 647-conjugated transferrin on ice for 30 minutes. Unbound transferrin was washed away and the cells were transferred to 37°C for the indicated times before cell fixation and analysis by confocal microscopy (Figure 4-15). After 5 minutes at 37°C the majority of the transferrin localized to the cell surface and did not co-localize with HSV-2 Us2 (Figure 4-15A). By 12 minutes after the shift to 37°C transferrin was found in vesicles and a small fraction of which co-localized with Us2 (Figure 4-15B). At 30 minutes after incubation at 37°C, extensive co-localization was observed between transferrin and Us2 (Figure 4-15C). The kinetics of transferrin/Us2 co-localization is consistent with Us2 localizing to an endocytic recycling compartment [263, 264].

To more precisely define the identity of Us2 containing endosomal membranes cells were transfected with plasmids encoding either the early endosomal (EE) marker,
Figure 4-15. HSV-2 Us2 co-localizes with endosomal vesicles. Vero cells transfected with mCherry-Us2 expression plasmids were incubated with Alexa Fluor 647-conjugated transferrin for 30 minutes at 4°C, washed and shifted to 37°C for 5 minutes (A), 12 minutes (B), and 30 minutes (C) prior to fixation and staining the cells for Us2. Transferrin signal is shown in green. Us2 signal is red. Nuclei were detected using the DNA stain Hoechst 33342 (blue). Representative images are shown.
Rab5-GFP, the late endosomal (LE) marker, Rab7-GFP, or the recycling endosomal (RE) marker, Rab11-GFP, along with a Us2 expression plasmid, and the cells examined by confocal microscopy (Figure 4-16). Consistent with the transferrin trafficking data shown above, minimal co-localization was observed between Us2 and Rab5-GFP (Figure 4-16A) or Rab7-GFP (Figure 4-16B), however, Us2 showed significant co-localization with Rab11-GFP (Figure 4-16C). Taken together, these findings strongly suggest that Us2 localizes to recycling endosomes.

**4.3.5 Identification of cellular interacting partners of HSV-2 Us2**

Our previous data revealed that HSV-2 Us2 associated with membranes and that this association was not facilitated by membrane targeting motifs, electrostatic interaction, cholesterol binding or transmembrane domains (Chapter 4.3.2). We therefore hypothesized that Us2-membrane association might be mediated by a cellular membrane protein. To identify membrane-associated interacting partners of HSV-2 Us2, a plasmid expressing Flag-Us2 was transfected into 293T cells. At 24 hours post-transfection, cells were collected and homogenized. Membrane fractions were purified as described in Materials and Methods. Protein complexes were immunoprecipitated by incubating the membrane fractions with anti-Flag affinity gel. Empty vector was used as a negative control. Precipitated proteins were identified by LC-MS/MS analysis. Proteins interacting with Us2 were also pulled down using tandem affinity purification (TAP). A plasmid expressing N-terminal TAP (calmodulin peptide and streptavidin peptide)-tagged HSV-2
Figure 4-16. HSV-2 Us2 co-localizes with Rab11, a marker of recycling endosomes. Vero cells were co-transfected with plasmids encoding HSV-2 Us2 and either Rab5-GFP (A), Rab7-GFP (B), or Rab11-GFP (C). Cells were fixed at 24 hours post-transfection and stained for Us2. Images of stained cells were captured by confocal microscopy. Rab signals are EGFP fluorescence and are displayed in green. Us2 signal is red. Nuclei were detected using the DNA stain Hoechst 33342 (blue). Arrowheads in panel (C) highlight co-localization of Us2 and Rab11 signals. Representative images are shown.
Us2 was transfected into mammalian cells. At 24 hours post-transfection, cells were collected with lysis buffer and total cell lysates were used for TAP purification, as described in Materials and Methods. Protein complexes purified were identified by LC-MS/MS analysis. Identified proteins are listed in Table 4-3 (MS#1 and MS#2 indicate two independent co-IPs).

**Table 4-3. Proteins identified from Flag and TAP purifications.**

<table>
<thead>
<tr>
<th>Size (kDa)</th>
<th>MS#1</th>
<th>MS#2</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>mito pyruvate carboxylase</td>
<td>\textit{N.D.}</td>
</tr>
<tr>
<td></td>
<td>kinesin-1 heavy chain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RasGTPase-like IQGAP1</td>
<td></td>
</tr>
<tr>
<td>70-80</td>
<td>calnexin</td>
<td>calnexin</td>
</tr>
<tr>
<td></td>
<td>glucosidase 2 subunit beta</td>
<td>glucosidase 2 subunit beta</td>
</tr>
<tr>
<td></td>
<td>GRP78/BiP</td>
<td>GRP78/BiP</td>
</tr>
<tr>
<td></td>
<td>delta-1-pyrroline-5-carboxylate synthetase</td>
<td>Endoplasmic (GRP94)</td>
</tr>
<tr>
<td></td>
<td>AFG-3 like (paraplegin-like protein)</td>
<td>Galectin-3-binding protein</td>
</tr>
<tr>
<td></td>
<td>HSP70, HSP90</td>
<td>Na/K transporting ATPase A1</td>
</tr>
<tr>
<td>30-35</td>
<td>Sec61a</td>
<td>Sec61a</td>
</tr>
<tr>
<td></td>
<td>Erlin-1</td>
<td>Erlin-1/2</td>
</tr>
<tr>
<td></td>
<td>TIM50</td>
<td>poly(rC) binding protein</td>
</tr>
<tr>
<td></td>
<td>poly(rC) binding protein</td>
<td>nuclear ribonucleoprotein C</td>
</tr>
<tr>
<td></td>
<td>calponin-3</td>
<td>reticulocalbin-1</td>
</tr>
<tr>
<td></td>
<td>Ser/Thr protein phosphatase 1 (PP1a)</td>
<td>60S ribosomal protein L40</td>
</tr>
<tr>
<td></td>
<td>lysozyme C</td>
<td>ubiquitin</td>
</tr>
<tr>
<td></td>
<td>ribosomal protein S27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ubiquitin</td>
<td></td>
</tr>
</tbody>
</table>

Note: Identified proteins listed in 30-35kDa and 70-80kDa are from two independent Flag IPs. The 150kDa ones are from TAP purification.) N.D.: not determined.
Of the proteins identified, Erlin and calnexin are membrane proteins that might be predicted to contribute to the membrane association of HSV-2 Us2. Reciprocal co-IPs were performed in both transfected and infected cells to confirm these interactions. However, neither calnexin nor Erlin could pull down HSV-2 Us2 in either transfected or infected cells (data not shown) and were not considered further.

TIM50 localizes to the inner membrane of mitochondria. It complexes with another mitochondrial protein, TIM23, to form a translocase and provides integrity of mitochondrial membranes [265, 266]. Interestingly, it was also identified as one of the PRV Us2 interacting partners identified in yeast two-hybrid screen [267]. The interaction between HSV-2 Us2 and TIM50 was confirmed by yeast two-hybrid assay and co-immunoprecipitation. The results showed that TIM50 interacted with HSV-2 Us2 in yeast two-hybrid assay and could be pulled down by HSV-2 Us2 in transfected cells (data not shown). However, TIM50 did not seem to be pulled down in infected cells (data not shown). Also, HSV-2 Us2 does not localize to mitochondria. We, therefore, concluded that HSV-2 Us2 does not interact with TIM50 during infection or that this interaction is transient and specific to a certain stage of infection.

Other than TIM50, ubiquitin was also present in the protein complexes co-precipitated with Flag-Us2. The interaction was further confirmed by co-IP, reciprocal IP and ubiquitin binding assay, and the results are discussed in the next few sections (Chapter 4.3.6 to 4.3.9)
4.3.6 HSV Us2 interacts with ubiquitin-conjugated proteins

Of the proteins identified in LC-MS/MS analysis (Chapter 4.3.5), we noted that approximately 3% of the proteins co-precipitated with HSV-2 Flag-Us2 were ubiquitin, which might suggest an interaction between them. Ubiquitin is a small 76 amino acid protein that contains 7 lysine residues (K6, K11, K27, K29, K33, K48 and K63). Ubiquitination is a post-translational modification, in which an ubiquitin molecule is covalently attached to a lysine residue of a substrate protein. A polyubiquitin chain forms when the C-terminus of another ubiquitin molecule is covalently linked to a lysine residue of an already conjugated ubiquitin. Depending on the lysine used to link the ubiquitin in the chain, there could be different fates for the ubiquitinated proteins [220, 268, 269]. Monoubiquitin and K63-linked polyubiquitin chains, usually found in membrane proteins, are involved in endocytosis and targeting of proteins for lysosomal degradation [222]. K48-linked polyubiquitin chains are well known for targeting proteins for proteasomal degradation. In this study, HSV recombinant viruses encoding an N-terminal Flag-tagged Us2 gene were constructed to determine whether HSV Us2 interacts with ubiquitin during viral infection.

The HSV-1 and HSV-2 Flag-Us2 recombinant viruses were constructed using en passant mutagenesis as described in Materials and Methods. To determine whether HSV Us2 interacts with ubiquitin or ubiquitin-conjugated proteins during infection, Vero cells were infected with wildtype HSV-1 or HSV-2, HSV-2 Us2-null virus and HSV-1 and HSV-2 Flag-Us2 recombinant viruses. Cell lysates were prepared at 6 hpi and incubated
with anti-Flag affinity gel. Precipitated protein complexes were subjected to Western blotting and probed with antibodies against mono- and polyubiquitin (FK2). The FK2 monoclonal antibody detects only protein-associated ubiquitin but not free ubiquitin molecules. The results showed that both HSV-1 and HSV-2 Us2 were able to pull down ubiquitin-conjugated proteins (Figure 4-17).

The fact that both HSV-1 Us2 and HSV-2 Us2 were capable of pulling down ubiquitin-conjugated proteins may suggest that: 1) they interacted either with the same proteins or directly with ubiquitin, and 2) the portion of amino acid sequence they used to interact with either ubiquitin or ubiquitin-conjugated proteins share high homology. HSV-1 Us2 and HSV-2 Us2 share 76% identity and 82% similarity (Figure 4-18). Other than the three conserved regions, the sequences between the conserved regions are also highly similar. In contrast, the C-terminal sequences downstream of the third conserved region are more variable, especially the C-terminal 50 amino acids. If HSV-1 Us2 and HSV-2 Us2 have common interacting partners, the three conserved regions and the sequences in between might be the sites for interactions.

4.3.7 HSV-2 Us2 is co-precipitated by ubiquitin-conjugated proteins

Reciprocal co-IP experiments were performed to confirm the interaction between HSV-2 Us2 and ubiquitinated proteins. Vero cells were infected and lysates were prepared at 6 hpi. Cell lysates were incubated overnight with antibodies against mono- and polyubiquitin (FK2). The antigen/antibody complexes were precipitated using Protein G agarose. The Western blot results show that Us2 could be pulled down using
**Figure 4-17.** HSV-1 Us2 and HSV-2 Us2 interact with ubiquitin or ubiquitin-conjugated proteins. Vero cells were infected with wildtype HSV-2, HSV-2 Us2-null virus and HSV-2 Flag-Us2 recombinant viruses (A) or wildtype HSV-1 and HSV-1 Flag-Us2 recombinant viruses (B). At 6 hpi, cell lysates were prepared and proteins were immunoprecipitated using anti-Flag affinity gel. Immunoprecipitated proteins were detected using antibodies against HSV-1 Us2, HSV-2 Us2 and mono- and polyubiquitin (FK2). W: wildtype, Δ: Us2-deleted mutant virus, M: mock infected, B: HSV-1 or HSV-2 Flag-Us2 recombinant viruses. (arrow: Flag-Us2 expressed in HSV-1 recombinant virus infected cells, asterisk: heavy chain)
Figure 4-18. Pairwise alignment of HSV-1 Us2 and HSV-2 Us2. HSV-1 Us2 and HSV-2 Us2 share 76% identity and 82% similarity. The amino acid sequences between three conserved regions are highly similar whereas the C-terminal sequences downstream of the third conserved region are more variable, especially the C-terminal 50 amino acids. Box: conserved regions in all Us2 orthologs.
the FK2 antibody, suggesting an interaction between HSV-2 Us2 and ubiquitin-conjugated proteins (Figure 4-19). As a negative control, other viral proteins, including the major capsid protein, ICP5, tegument protein, UL21, regulatory protein, ICP27 and glycoprotein gD, were detected to determine if this interaction is specific with Us2. The results indicate that ubiquitin or ubiquitin-conjugated proteins interacted specifically to Us2 (Figure 4-19).

### 4.3.8 HSV-2 Us2 interacts directly with ubiquitin

The FK2 antibody can only detect the ubiquitin that is conjugated to a protein. Above, we demonstrated that Us2 could be pulled down using FK2 antibodies, suggesting that it interacts either with ubiquitin directly, or with numerous ubiquitin-conjugated proteins. To determine whether HSV-2 Us2 interacts directly with ubiquitin, Vero cells were infected with wildtype HSV-2. At 6 hpi, cell lysates were prepared and incubated with either Protein G agarose, as a negative control, or monoubiquitin-conjugated agarose. Precipitated complexes were washed and subjected to Western blotting for analysis. The results showed that Us2 could be pulled down by monoubiquitin, suggesting that it interacts directly with ubiquitin (Figure 4-20, first 3 lanes).

To determine if the interaction between HSV-2 Us2 and ubiquitin requires other viral proteins, N-terminal Flag-tagged Us2 was transfected into 293T cells. In previous co-IP experiments, no ubiquitin-conjugated proteins were detected by the FK2 antibody
Figure 4-19. HSV-2 Us2 co-immunoprecipitates with ubiquitin-conjugated proteins from infected cells. Vero cells were infected with wildtype HSV-2 or Us2-null virus for 6 hours. Cells were collected and lysates were incubated overnight with antibodies against mono- and polyubiquitin (FK2). Proteins were pulled down using Protein G agarose and subjected to Western blotting to detect for the presence of Us2, ICP5, ICP27, UL21 and gD. M: mock, Δ: Us2-null virus, W: wildtype HSV-2. (Asterisk: Ig heavy chain)
Figure 4-20. HSV-2 Us2 interacts directly with ubiquitin. Vero cells were infected with wildtype HSV-2 for 6 hours. Cell lysates were prepared and incubated overnight with ubiquitin-conjugated beads. Proteins pulled down were subjected to Western blotting and probed with antibody against Us2. G: Protein G agarose (negative control), mono: monoubiquitin-conjugated agarose, K48: K48-linked tetraubiquitin-conjugated agarose, K63: K63-linked tetraubiquitin-conjugated agarose, L: lysate, M: mock infected, W: wildtype HSV-2 infected.

Figure 4-21. HSV-2 Us2 interacts with ubiquitin in the absence of other viral proteins. N-terminal Flag-tagged Us2 was transfected into 293T cells. At 24 hours post-transfection, cells were collected with lysis buffer and lysates were incubated overnight with anti-Flag beads at 4°C. Transfection and IP were preformed in duplicate. Immunoprecipitated proteins were eluted using 3XFLAG peptides. Eluates from two IPs were combined and incubated overnight with either Protein G agarose or monoubiquitin-conjugated beads at 4°C. Beads were washed three times with wash buffer (see Materials and Methods). Proteins pulled down were subjected for Western blotting and probed with anti-Flag antibodies. G: Protein G agarose, Ubi: monoubiquitin-conjugated beads, EV: empty vector.
in the protein complexes pulled down by Flag-Us2 from transfected cells (data not shown), which might be due to low expression levels of Flag-Us2. To overcome this problem, transfection and IP were performed in duplicate. Protein complexes were first immunoprecipitated using anti-Flag affinity gel, then eluted using 3X Flag peptides to compete for binding with Flag antibodies. The eluates from the two IPs were pooled to increase Flag-Us2 protein level and then incubated with monoubiquitin-conjugated beads. Proteins pulled down were subjected to Western blotting to detect the presence of Us2. The results showed that HSV-2 Us2 interacted with ubiquitin from transfected cells, suggesting that the interaction does not require other viral proteins (Figure 4-21).

To study the Us2-ubiquitin interaction further, HSV-2 infected cell lysates were incubated with either K48-linked or K63-linked tetraubiquitin-conjugated agarose. Ubiquitin binding proteins can interact with either ubiquitin molecules or the linkages between conjugated ubiquitin molecules. The preference of binding toward a specific linkage is associated with the function of ubiquitin binding proteins. We hypothesized that if HSV-2 Us2 was involved in the proteasomal degradation pathway, it would interact with K48-linked polyubiquitin chains with higher affinity. Alternatively, if Us2 participated in endocytosis or lysosomal degradation, it would be predicted to preferentially interact with K63-linked polyubiquitin chains. The pull-down results showed that HSV-2 Us2 interacted with K48-linked tetraubiquitin and K63-linked tetraubiquitin with similar affinity (Figure 4-20), which may suggest that it interacts with
the ubiquitin molecules, instead of with the linkages per se, or it interacts with both K48- and K63-linkages but with equal affinity.

4.3.9 Incorporation of ubiquitin-conjugated proteins into virions occurs in an Us2-independent manner

Previous studies on identification of components in HSV-1 virions show that ubiquitin is packaged into extracellular virions [2]. To determine if this packaging is Us2 dependent, HaCaT cells were infected with wildtype HSV-2, HSV-2 Us2-null or Us2 repair recombinant viruses at an MOI of 5 for 24 hours. The HSV-2 Us2-null and repair recombinant viruses were constructed. Virions were purified by centrifugation through a 30% sucrose cushion, as described in Materials and Methods. Purified virions were analyzed by Western blotting for the presence of ubiquitin-conjugated proteins.

The Western blot results show that the profile of ubiquitin-conjugated proteins incorporated into virions was indistinguishable in the presence or absence of Us2, indicating that this process is Us2 independent (Figure 4-22). However, we cannot exclude the possibility that certain species of ubiquitinated proteins requires Us2 for virion incorporation since numerous proteins were detected on the Western blot. The disappearance of a specific species (i.e. a single band) in the Us2-null sample but not in
Incorporation of ubiquitin-conjugated proteins into virions is Us2-independent. HaCaT cells were infected with wildtype HSV-2, Us2-deleted and repair recombinant viruses for 24 hours. Cells were scraped into the medium and centrifuged at low speed to pellet cells and cells debris. Supernatants were collected and laid onto 30% sucrose (v/w in PBS) in centrifuge tubes for centrifugation at 24,000 rpm for 3 hours. Pelleted virions and cell lysates were subjected to Western blotting for analysis. Proteins were detected with antibodies against Us2, ICP5 and mono- and polyubiquitin (FK2). M: mock, W: wildtype HSV-2, ∆: Us2-null mutant virus, R: Us2 repair virus. (Asterisk: protein species that were specifically incorporated into extracellular virions) A band, same molecular weight as the bands representing Us2 in wildtype and Us2 repair viruses, appeared in the cell lysates of HSV-2 Us2-null infected cells may be due to spill-over from the neighbouring lanes inasmuch as it was not incorporated into extracellular virions produced by HSV-2 Us2-null viruses.
the wildtype and repair samples could not be discounted. Identification of packaged ubiquitinated proteins, either viral or cellular, that interact with Us2 could provide direction for further studies.
Chapter 5
Discussion

5.1 Interaction between PRV Us2 and ERK

By utilizing a series of PRV Us2 truncated mutants, we determined that the minimal portion of Us2 required for interaction with ERK is contained within its amino-terminal 214 amino acids. The loss of the ability of Us2 to bind to ERK in co-immunoprecipitation (co-IP) experiments was accompanied by a failure of Us2 to form oligomers, raising the possibility that higher-order Us2 structures are required for ERK interaction. To map the Us2 interaction site on ERK, we introduced mutations into the region of ERK that interacts with the ERK kinase, MEK, or into the common docking (CD) domain that mediates interactions with many ERK substrates. Our data showed that ERK carrying mutations within the MEK binding region maintained the ability to bind Us2, whereas ERK carrying mutations within the CD domain did not (Figure 4-5). Furthermore, the ERK CD domain was required for the Us2-mediated recruitment of ERK to membranes (Figure 4-6).

Previous studies showed that ERK isolated in the complex with PRV Us2 maintained the ability to phosphorylate Elk-1 in vitro, suggesting that Us2 did not inhibit ERK enzymatic activity [11]. Interestingly, the ERK-mediated activation of Elk-1 was inhibited by Us2 in transfected cells (Figure 4-2D) [11]. Because Elk-1 normally resides
in the nucleus and Us2 restricts ERK localization to the cytoplasm, these observations suggest that Us2 spatially regulates ERK activity. The data presented in this study indicate that, like many ERK substrates, Us2 interacts with ERK via the CD domain. However, our experiments suggest that Us2 is not modified by phosphorylation (data not shown). It may be that Us2 also regulates ERK activity by competing for ERK substrate binding. How might ERK bound to Us2 interact with its substrates? While many ERK substrates bind to the CD domain, ERK substrates that contain an FXFP motif bind to the ERK MAP kinase insert, which is distal to the CD domain [209]. It may be that ERK bound to Us2 has access only to substrates that bind the MAP kinase insert. Experiments to identify proteins phosphorylated by ERK in the presence of Us2 should help sort this out. It is also possible that ERK bound to Us2 is capable of activating all of its potential substrates, and that Us2 spatially regulates ERK activity simply by restricting its cellular localization. Previous studies have shown that the cytoplasmic ERK scaffold, KSR, binds to the ERK MAPK insert via its FXFP motif [209]. However, KSR binding to ERK does not interfere with its interaction with other substrates that also use the MAPK insert to bind ERK [270]. The dimerization of ERK allows it to form simultaneous interactions with both KSR and cytoplasmic substrates [270]. Our data suggest that Us2 can interact with ERK both as a monomer or a dimer (Figure 4-10B) but we do not know which is more preferential. Determining the stoichiometry of the Us2-ERK interaction will provide important clues to further our understanding of the regulation of ERK activity by Us2 and help define the role of this complex in alphaherpesvirus egress.
5.2 Other PRV Us2 interacting partners

Other than the MAPK ERK, PRV Us2 also interacts with an intermediate filament (IF) protein, vimentin, and a building block of the microtubules (MT), beta-tubulin (Figure 4-12). Intermediate filaments, microfilaments (MF; also called actin filaments) and microtubules, are the three main components of the cytoskeleton.

The role of microtubules in intracellular trafficking is well-characterized. Microtubules are comprised of alpha- and beta-tubulin. The microtubule network involves two general classes of motors: kinesin and dynein, which move toward the plus (+) ends and the minus (-) ends of microtubules, respectively. Microtubules emanate toward the cell periphery from the microtubule-organizing centre (MTOC), where the minus ends are located. Cellular cargos can be transported to the plasma membrane or destined organelles by utilizing these motors. Other than the roles in transportation of cellular cargoes, such as endocytic/exocytic vesicles and organelles, viruses also use this machinery to facilitate their replication. In HSV-1 infected cells, retrograde transport of incoming capsids from cell surface to the nucleus is mediated by microtubules [84, 271] and intact MT are required for nuclear targeting of the capsids [272]. Previous studies have shown that HSV inner tegument proteins are required for capsid binding to the motors and promote the movement along microtubules in vitro [273]. Live cell imaging experiments tracking the motion of the mRFP-fused capsid protein, VP26, upon PRV infection demonstrate that inner tegument protein, VP1/2 and UL37 moved along with the mRFP-VP26 to the nucleus [274]. Microtubules are utilized not only for capsid
trafficking to the nucleus after entry of viruses, but also for antegrade transport of mature virions to the plasma membrane for the release from the infected cells [275]. Tegument proteins, HSV-1 Us11[276] and HSV-2 UL56 [277], were reported to interact with a kinesin. Also, disruption of microtubules reduces yield of virus production [278]. The interaction with beta-tubulin may suggest the involvement of Us2 in the trafficking of viruses.

Intermediate filaments are comprised of a variety of proteins, with about 70 IF proteins identified, and are classified into five groups, which are expressed in different cell types [239, 240]. Type I and II IF are acid and basic/neutral keratin, respectively, expressed in epithelial cells. Members of Type III IF include vimentin and desmin. Vimentin can be found in various cell types, including fibroblasts and smooth muscle cells, whereas desmin is expressed specifically in muscle cells. Type IV IF proteins are neurofilaments (NF), which are expressed in neurons, supporting the long, thin processes. Type V IF proteins are the nuclear lamina, components of the inner nuclear envelope.

Unlike MT and MF, IF do not have polarity and have only been known for their roles in structural and mechanical support of cells. Only recently, their roles in membrane trafficking have been discovered [279]. Intracellular trafficking generally involves two steps: 1) budding of a transport vesicle from an organelle or the plasma membrane and 2) fusion of the transport vesicle with the destination organelle. Previous studies have shown that vimentin interacts with the AP-3 adaptor complex [241], which is involved in sorting membrane proteins to lysosomes [242, 243] and is essential in cargo transport from the Golgi to lysosomes [244, 245]. In addition, vimentin has been shown to serve as
a reservoir for SNAP-23 [246], a component of SNARE (soluble NSF attachment protein receptor) complexes. SNAP-23 is a ubiquitously expressed homolog of SNAP-25, which is only found in neural cells [247]. SNAP-25, together with synaptobrevin/VAMP and syntaxin, serve as a docking site for vesicle fusion [248, 249]. Together, these findings may implicate the involvement of vimentin in vesicular trafficking. Involvement of vimentin in viral replication is less well understood. Some studies report that disruption of vimentin reduced virus yield of blue tongue virus (BTV) [280], Junin virus (JUNV) [281] and Dengue virus (DENV) [282], suggesting an importance of vimentin in viral egress. In HCMV, a member of the betaherpesvirinae, disruption of vimentin filaments greatly reduces viral entry, suggesting that an intact vimentin skeleton is important for the onset of virus replication [283]. In addition, the absence of vimentin causes prolonged retention of HCMV capsids in the cytoplasm before entering the nucleus for DNA replication, indicating that vimentin is required for efficient trafficking of the capsids into the nucleus [283].

Our lab has previously shown that a nonprenylated form of Us2 localizes to uniform punctuate structures associated with microtubules in both transfected and infected cells [21]. In addition, PRV Us2 interacts with and re-localizes MAPK ERK, without inhibiting its kinase activity, which is significant for virus egress [11]. Interestingly, vimentin has recently been identified in a retrograde transport protein complex that contains dynein, importin-β and phosphorylated ERK (pERK) in injured nerves [284, 285]. Vimentin interacts with pERK in a calcium-dependent manner in vitro.
Elevation in calcium concentration, which also occurs in virally infected cells, facilitates vimentin binding to pERK and the binding protects pERK from being dephosphorylated by a phosphatase [284]. These studies also showed that pERK-dynein interaction was lost and that phosphorylation of ERK and Elk were not observed downstream of the lesion site in the injured nerve from vim\(^{-}\) mice whereas both pERK-dynein interaction and phosphorylation of ERK and Elk were exhibited in the vim\(^{+/}\) injured nerves [284]. These data suggest a role of vimentin in the transport of active ERK. Together, it may implicate that PRV Us2 acts with both ERK and vimentin to facilitate the migration of virions from the assembly site (TGN/LE) to the cell surface. However, further investigation (i.e. determining whether the Us2-ERK complexes also contain vimentin) is required to support this hypothesis.

5.3 Characterization of HSV-2 Us2

Although the Us2 gene is conserved among all alphaherpesviruses, with the exception of VZV, their amino acid sequences do not share considerable homology. Despite the three conserved regions at the N-terminus, the sequences between the conserved regions and at the C-terminus are quite different, which may differentiate the mechanisms they use to facilitate virus replication. Compared to PRV Us2, HSV-2 Us2 does not contain any classical post-translational modification signals for membrane localization. Interestingly, our immunofluorescence microscopy results show that HSV-2 Us2 localizes to the plasma membrane and vesicles like PRV Us2 (Figure 4-13). HSV-2
Us2 association with membranes was also confirmed biochemically using membrane flotation assays (Figure 4-14A). Proteins can associate with the membranes via various ways, such as electrostatic interactions and post-translational modifications. To determine the mechanism of Us2-membrane association, membrane fractions were treated with high salt and high pH to disrupt membrane association that is contributed by electrostatic interactions. However, the results showed that HSV-2 Us2 remained associated with the membranes. One possibility for the tight membrane association may be that HSV-2 Us2 localizes to lipid rafts. Lipid rafts are dynamic microdomains, enriched in sphingolipids, cholesterol and phospholipids with saturated fatty acid sidechains. They are found predominately in the plasma membrane and some in the endocytic membranes. They are insoluble in non-ionic detergents at low temperature. The treatment of membranes with TX-100 at 4°C could not liberate HSV-2 Us2 from the membranes, suggesting that HSV-2 Us2 is lipid raft-associated.

Proteins can associate with lipid rafts via dual acylation, a GPI-anchor, cholesterol binding or via a transmembrane domains. Previous studies have shown that many viral proteins are raft-associated, and enveloped viruses, such as HIV and influenza, use lipid rafts in their assembly and egress. The envelope of influenza virus has been shown to contain lipid ordered domains insofar as that it contains a higher amount of cholesterol and saturated lipids compared to other enveloped viruses, such as vesicular stomatitis virus (VSV) and Semliki Forest virus (SFV) [286]. Glycoproteins, HA and NA, of influenza were demonstrated to be raft associated through their cytoplasmic tails

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and via palmitoylation. A mutant virus with HA and NA lacking their cytoplasmic tails contains reduced amounts of cholesterol and sphingolipids in purified virions, suggesting that influenza virus acquires its envelope from lipid rafts [287]. The Gag protein and glycoprotein Env of the retrovirus HIV are also raft-associated [288]. The envelope of HIV particles was found to contain raft-associated molecules, such as ganglioside GM1 and GPI-linked proteins [288]. Furthermore, cholesterol depletion, which causes disruption of the rafts, leads to reduction in release of HIV particles and reduced infectivity [289]. These data suggest that HIV utilizes lipid rafts for assembly and budding.

In contrast to HIV and influenza, there is no direct evidence indicating that herpesviruses utilize lipid rafts for egress. However, a subset of alphaherpesvirus tegument proteins, including Us2, exhibit associations with lipid rafts. Tegument proteins that have been demonstrated to be raft-associated include UL11, UL41 and UL46. UL11 associates with lipid rafts by dual acylation [170]. UL41, which encodes a virion host shutoff (vhs) protein, interacts with membrane rafts via its N-terminal sequence [180]. UL46 seems to interact with membranes through multiple sites within its amino acid sequence [181]. Deletion of UL46 in PRV does not affect virus replication but causes a reduction in plaque size [290], which may suggest its involvement in virus egress. Envelope proteins, including gB, gC, gD, gE, UL56 and Us9, which all contain transmembrane domains, are also reported to be raft-associated proteins [182, 291, 292].
Although many glycoproteins and tegument proteins are raft associated, whether herpesviruses utilize lipid rafts for egress requires further investigation.

How HSV-2 Us2 associates with lipid rafts is not yet understood. Despite lacking a conventional post-translational motif and transmembrane domains, it contains a putative cholesterol binding motif, CRAC [257]. Purified membranes were treated with cholesterol sequestering agent, digitonin, to release cholesterol binding proteins. However, our data show that HSV-2 Us2 remained associated with the membranes after digitonin treatment (Figure 4-14B), strongly suggesting that it does not bind to cholesterol. Altogether, our data suggest that Us2-membrane association may be mediated by other membrane proteins. Identification of Us2 interacting partners could provide clues to not only the mechanism of membrane association, but also the function of Us2. To identify membrane-associated interacting partners of HSV-2 Us2, Flag-Us2 was transfected into mammalian cells and protein complexes were immunoprecipitated and identified by LC-MS/MS analysis. Although the two membrane proteins, calnexin and Erlin, identified appeared to be false positives, large amounts of ubiquitin were present in the precipitated protein complexes, suggesting that HSV-2 Us2 might interact with ubiquitin. The interaction between HSV-2 Us2 and ubiquitin was further confirmed by IP, reciprocal IP and ubiquitin binding assays. Altogether, these results indicate that Us2 interacts directly with ubiquitin and that this interaction is specific for Us2 while other viral proteins, such as ICP5, ICP27, UL21 and gD, do not exhibit an interaction with ubiquitin (Figure 4-17, 4-19, 4-20 and 4-21).
The best characterized function of ubiquitin is to serve as a signal for proteasomal degradation, which regulates diverse cellular programs, by controlling protein turnover, to keep cells in a homeostatic state. Proteins that are misfolded, damaged or considered unnecessary are modified with a polyubiquitin chain and targeted to proteasome for degradation. To maintain the balance inside cells, regulatory proteins, such as transcription factors and membrane receptors, are continuously degraded to modulate the cell cycle, and signal transduction, etc [221, 293]. The ubiquitin-proteasome system is also involved in host responses against viral infection. Under normal conditions, MHC class I (MHC-I) molecules continuously present peptides derived from cellular proteins, generated by the proteasomal degradation, at the cell surface for surveillance by cytotoxic T cells (CTLs) [294, 295]. Upon viral infection, viral proteins synthesized in the cells may be degraded and presented by MHC-I at the cell surface, and subsequently recognized by CTLs [294, 295]. The infected cells are then eliminated by CTLs. However, viruses can hijack the proteasome machinery to counteract host responses in various ways. Some viruses, such as HPV, encode proteins to co-opt cellular E3 ligase complexes to alter substrate specificity. HPV E6 protein binds to the cellular E3 ligase E6AP and directs its ubiquitination activity toward p53, which subsequently results in p53 degradation and thereby prevents initiation of apoptosis and maximizes viral replication [296, 297]. Viruses can also encode their own ubiquitin ligases. An example is HSV-1 ICP0, an E3 ligase that induces polyubiquitination and degradation of various cellular proteins [298], including tumor suppressors, PML (promyelocytic leukemia
protein) [299] and p53 [300]. ICP0 also interacts with cellular deubiquitinase, USP7, to protect itself from being ubiquitinated and degraded [301, 302]. In addition, viruses can manipulate the ubiquitin-proteasome system to promote viral immune evasion. Another member of the Herpesviridae, MHV-68 (murine gamma-herpesvirus 68), encodes a PHD/LAP finger E3 ligase, MK3, that down-regulates the presentation of MHC-I at the cell surface through the proteasomal degradation pathway [303-305]. Inhibition of the proteasome reduced MHC-I degradation and induced accumulation of ubiquitinated MHC-I molecules whereas inhibition of lysosomes had no effect on MHC-I degradation [303].

Besides proteasomal degradation, ubiquitin has recently been discovered to participate in other cellular functions that are associated with specific linkages of polyubiquitin chains [220, 268]. While K48-linked polyubiquitination is responsible for proteasomal degradation, monoubiquitination and K63-linked polyubiquitination are involved in endocytosis and lysosomal degradation pathways [222, 229, 306]. Monoubiquitin, usually found on membrane proteins, serves as an endocytic signal at the plasma membrane and as a protein sorting signal at early endosomes. Proteins attached with monoubiquitin or K63-linked polyubiquitin chains are endocytosed and transported to the early endosomes where they are subsequently sorted to LE/MVB for lysosomal degradation. Viruses can also promote viral invasion through lysosomal degradation pathway. Examples of viral proteins include KSHV E3 ligases, K3 and K5 [304]. They
downregulate MHC-I presentation at the cell surface by enhancing endocytosis through ubiquitination and subsequent lysosomal degradation [307-309]

Ubiquitin binding proteins participating in proteasomal pathways interact preferentially with K48-linkage while the ones in the LE/MVB pathway prefer K63-linkages [310]. To determine which pathway HSV-2 Us2 is involved with, interactions with both K48-linkage and K63-linkage were determined and the results showed that HSV-2 Us2 has similar affinity to monoubiquitin, K48-linkages and K63-linkages (Figure 4-20), which may suggest that HSV-2 Us2 recognizes ubiquitin itself, not the linkage connecting ubiquitin molecules. An alternative explanation is that Us2 interacts with both the ubiquitin molecules and the linkages, but with equal affinity, suggesting that it is involved in both proteasomal and lysosomal pathways.

Our data showed that HSV-2 Us2 localized to cellular vesicles that share the properties with recycling endosomes, forming long filamentous tubules, and that HSV-2 Us2 co-localized with a marker of recycling endosomes, Rab11 (Figure 4-15 and 4-16). Recent ultrastructural studies showed that the HSV-1 capsid is enveloped in endocytic tubules and that Rab5 and Rab11, which regulate endocytic tubules, are required for efficient virus production [311]. Rab5 is involved in endocytosis and trafficking from the plasma membrane to early endosomes, while Rab11 is involved in trafficking from the TGN or recycling endosomes to the plasma membrane [312, 313]. Furthermore, Hollinshead and colleagues found that gD and gE localized to the cell surface, were then endocytosed, and that gD that once was on the plasma membrane was present in the
envelope of released virions [311]. Some glycoproteins have endocytosis signals in their cytoplasmic tails and gB was found to be ubiquitinated with a K63-linked polyubiquitin chain [133]. An envelope protein, Us9, is also ubiquitinated and localizes to the plasma membrane [314]. Hollinshead and colleagues therefore proposed that virus envelope proteins are first transported to the plasma membrane and then endocytosed for envelopment at the plasma membrane derived tubules (Figure 5-1) [311].

From the data presented, we can only conclude that HSV-2 Us2 interacts with ubiquitin, how and why it interacts remains undefined. Proteins interact directly with ubiquitin via ubiquitin binding domains (UBDs), which can be found in ubiquitin ligase, deubiquitinase and ubiquitin receptors. There are a variety of ubiquitin binding motifs in the UBD family, including UIM (ubiquitin interacting motif), UBA (ubiquitin associated domain), UEV (ubiquitin E2 variant domain), CUE (coupling of ubiquitin conjugation to ER degradation domain), etc [219, 315]. Viral proteins known to interact directly with ubiquitin include viral E3 ligase (e.g. HSV-1 ICP0, KSHV K3 and K5) and deubiquitinase (e.g. HSV-1 UL36). Intriguingly, HSV-2 Us2 does not resemble any of these. Ubiquitin receptors function to recognize and interpret signals from ubiquitin-conjugated substrates. Examples of cellular ubiquitin receptors include Tsg101 (tumor susceptibility gene 101), which recognizes ubiquitinated proteins targeted to MVB for lysosomal degradation [316]. HSV-2 Us2 appears to interact directly with ubiquitin via a domain that is not classified into any of the known UBDs. We do not know whether it
Figure 5-1. Model for virus envelopment via an endocytic pathway. (1) Newly synthesized glycoproteins are transported to the plasma membrane. (2) Glycoproteins are endocytosed through the early endosomes to produce tubules for envelopment. (3) Cytoplasmic capsids acquire envelope from the plasma membrane-derived, glycoprotein-containing tubules. [311]
interacts with specific linkages, but it is more likely that it interacts with the ubiquitin itself rather than the linkages, and is involved in endocytosis mediated by ubiquitination. Together with the evidence that it localizes to recycling endosomes, we may hypothesize that HSV-2 Us2 is involved in endocytosis of glycoproteins mediated by ubiquitination and trafficking of virions from the endosomes to the plasma membranes (Figure 5-1, route 2 and 3). HSV-2 Us2 may facilitate the endocytosis through its interaction with ubiquitin, however, the mechanism of interaction and how does the interaction influence the endocytosis require further investigation. Furthermore, the interaction of HSV-2 Us2 with ubiquitin conjugated to membrane proteins may mediate the membrane association of HSV-2 Us2. More experiments are required to prove this speculation.

5.4 Comparison between PRV Us2 and HSV-2 Us2

5.4.1 Membrane targeting signals

Orthologs of the alphaherpesvirus tegument protein Us2 share three conserved regions at their N-terminus. In contrast, the C-terminal sequences are highly variable. As shown in Figure 5-2, the C-terminus of PRV Us2 contains a CAAX motif and an upstream polybasic domain. The polybasic domain of PRV Us2 is comprised of 9 arginine residues. Both the CAAX and the polybasic domains are required for the plasma membrane localization [237, 238]. Previous studies have shown that the PRV Us2 GAAX mutant, in which the cysteine residue of the CAAX motif is replaced by a glycine residue,
**Figure 5-2.** Comparison of PRV Us2 and HSV-2 Us2 amino acid sequences. (A) Structure scheme of PRV Us2 and HSV-2 Us2. (B) Alignment of PRV Us2 and HSV-2 Us2 amino acid sequences. PRV Us2 and HSV-2 Us2 share three conserved regions. Sequences between the three conserved regions and the C-terminal sequences are very variable between PRV Us2 and HSV-2 Us2. CR (A) and boxes (B): conserved regions, arrows: the arginine residues upstream of the CAAX motif (polybasic domain).
lost its plasma membrane localization but maintained the ability to localize to vesicular membranes [21]. The vesicular localization could be facilitated by its polybasic domain, insofar as the positive charges of the arginine residues can associate with the negatively charged membranes through electrostatic interaction. EHV-1 has been shown to associate with the membranes, regardless of the lack of the conventional membrane targeting signals. Nonetheless, the EHV-1 Us2 C-terminus contains a cluster of arginine residues, which may contribute to its membrane association. In contrast, HSV-2 Us2 does not contain any putative membrane targeting signals or polybasic domains for membrane targeting, but intriguingly, it localizes to the plasma membrane. Also, a portion of HSV-2 Us2 is tightly associated with the membranes. The membrane association might be contributed by the interaction with ubiquitin that are conjugated to membrane proteins. Nonetheless, the mechanism of HSV-2 Us2-membrane interaction remains to be defined.

5.4.2 Oligomerization

Both PRV Us2 and HSV-2 Us2 are capable of forming oligomers (Chapter 4.1.4 and 4.3.3). PRV Us2 and HSV-2 Us2 share only three N-terminal conserved regions while the C-terminal sequences are very different (Figure 5-2). This may suggest that the formation of oligomers is contributed by the N-terminal sequences. Although PRV Us2 and HSV-2 Us2 share only about 30% of identity in their protein sequences, their tertiary structure might be similar and use same surface to form oligomers.
5.4.3 Interaction with MAPK ERK

Previous studies have shown that PRV Us2 interacts with the MAPK ERK, sequesters ERK in the cytoplasm, and subsequently prevents it from entering the nucleus and activating its nuclear substrates (Chapter 4.1) [11]. Luciferase assays revealed that expression of PRV Us2 in the cells reduced activation of the ERK nuclear substrate, Elk-1 (Figure 4-2D) [11]. Furthermore, inhibition of ERK kinase activity caused virions to accumulate in infected cells, an effect that was more profound in PRV Us2-null infection, suggesting that ERK kinase activity is required for efficient virus egress and that the interaction between PRV Us2 and ERK is important for this process [11]. To determine whether PRV Us2 and HSV-2 Us2 share similar properties, the interaction between HSV-2 Us2 and ERK was examined. Plasmids expressing HSV-2 Us2 and ERK were co-transformed into yeast to determine their interaction. The results show that they did not interact with each other (Figure 5-3A). Interactions were also determined by co-IP experiments and luciferase assays. The co-IP results show that ERK did not co-precipitate with HSV-2 Us2 (data not shown). Interestingly, the luciferase assay data showed that HSV-2 Us2 could partially inhibit nuclear localization of ERK, which subsequently reduced the activity of its nuclear substrate, Elk-1, suggesting that HSV-2 Us2 may interact with ERK with low affinity or indirectly through other proteins (Figure 5-3B). The different binding affinity between PRV Us2 and HSV-2 Us2 toward ERK may suggest that differences in amino acid sequences between individual Us2 orthologs affect binding affinity toward the same proteins or differentiate the mechanisms by which
Figure 5-3. HSV-2 Us2 does not interact with ERK, but partially inhibits its nuclear activity. (A) HSV-2 Us2 does not interact with ERK in yeast. Plasmids expressing PRV Us2 or HSV-2 Us2 were co-transformed with empty vector (EV) or plasmid expressing ERK into yeast. Transformed cells were grown on the selection medium to determine the interaction. Interactions between proteins allowed yeast to express the reporter genes encoding histidine and adenine, and grow on selective medium lacking these amino acids. (B) Elk-1 luciferase reporter assays. NIH 3T3 cells were transfected with plasmids expressing empty vector (EV), PRV Us2 or HSV-2 Us2, constitutively active MEK, GAL4-fused Elk-1, and a luciferase reporter linked to GAL4 binding sequences. Luciferase activity is expressed as relative light units. Combinations of plasmids are indicated under the x axis. Each assay was performed in triplicate, and the error bars represent the standard deviations between replicate assays.
they influence infection through the interaction with distinct proteins. Membrane proteins are synthesized in the ER, and travel to the Golgi where they are sorted to their final destinations. As proteins reach the TGN, vesicular cargoes are formed by fission, from the TGN membranes, and directed to the plasma membrane or various intracellular organelles, such as late endosomes [317]. The transport of cargoes from the TGN to the plasma membrane is regulated by protein kinase D (PKD), a serine/threonine protein kinase [318]. Like cellular proteins, mature virions formed at the TGN are transported to the plasma membrane for release via vesicular cargoes derived from the TGN. Previous studies on HSV-1 show that PKD is required for the trafficking of virions from the TGN to the plasma membrane and virion egress [319, 320]. While the MAPK ERK is suggested to co-operate with PRV Us2 to promote virus egress [11], HSV-2 Us2 may utilize PKD to facilitate this process. However, further investigation is required to support this hypothesis.

5.4.4 Interaction with ubiquitin

From the results of LC-MS/MS analysis (Chapter 4.3.5 to 4.3.9), we discovered that HSV-2 Us2 interacts with ubiquitin. Both HSV-1 Us2 and HSV-2 Us2 were able to pull down ubiquitin-conjugated proteins (Figure 4-17). However, no detectable amount of ubiquitin-conjugated proteins was co-precipitated with Flag-HSV-2 Us2 in transfected cells, which might be due to the relative low level of Us2 expression in transfected cells compared to the infected cells. Low binding affinity may also contribute to the failure of co-precipitating ubiquitin-conjugated proteins. To resolve this problem, the amount of
cell lysates used in the ubiquitin binding assay was doubled to increase Us2 concentration and the results showed that Us2 is capable of interacting with ubiquitin in transfected cells (Figure 4-21), suggesting that Us2 itself is sufficient for ubiquitin binding. The presence of other viral proteins may enhance, but is not essential for, ubiquitin binding of HSV-2 Us2. In contrast, the expression of PRV Us2 was robust in transfected cells, compared to HSV-2 Us2. The difference in expression level appeared on Western blotting might be due to different sensitivity of the antibodies. Nonetheless, no detectable amount of ubiquitin-conjugated proteins was co-precipitated with Flag-PRV Us2 from transfected cells (data not shown). This may suggest that PRV Us2 does not interact with ubiquitin or it requires other viral proteins for ubiquitin binding. As noted previously, protein sequences contained within PRV Us2 and HSV-2 Us2 are quite different, regardless of the conserved regions, which may thereby distinguish the function of PRV Us2 from that of HSV-2 Us2. Although it is still unclear why HSV-2 Us2 interacts with ubiquitin, determination of interaction between PRV Us2 and ubiquitin in infected cells may provide us clues to the significance of the interaction.

5.4.5 Summary

Our data show that PRV Us2 and HSV-2 Us2 share properties in membrane localization and the capability of oligomerization but are different in the proteins they interact with. PRV Us2 showed strong interaction with ERK while HSV-2 Us2 did not. The inhibition of ERK kinase activity caused a delay in the release of PRV extracellular viruses and this defect was more substantial in PRV Us2-null virus infection [11]. In
order to understand Us2 function in the context of viral infection, HSV-2 Us2-deleted and repair mutant viruses were constructed. Growth kinetics were monitored under various conditions (e.g. different cell types) (Appendix A). Like PRV Us2, there were no obvious phenotypes observed in viral replication of HSV-2 Us2-null virus in cultured cells. Nonetheless, treatment with ERK inhibitors did not cause any defects in viral replication and the release of extracellular viruses for both wildtype HSV-2 and Us2-null virus (Appendix B). These findings may suggest that the moderate interaction between HSV-2 Us2 and ERK observed in luciferase assays are not significant for virus egress. Recent studies showed that HSV-1 infection is influenced by PKD [319, 320]. Us2 may be involved in HSV-2 egress through the interaction with PKD. Determination of HSV-2 Us2-PKD interaction and effect of inhibition of PKD activity on HSV-2 Us2-null virus replication and egress may provide us clues to HSV-2 Us2 function.

Other conditions have also been examined to determine the involvement of Us2 in HSV-2 interferon resistance and ER stress responses (Appendix C-D). However, HSV-2 Us2 does not seem to be involved in either of these functions. Interactions with two other PRV Us2 interacting partners were also analyzed. PRV Us2 showed strong binding affinity toward both vimentin and beta-tubulin in co-IP experiments while HSV-2 was unable to pull down these proteins, although this could be due to low expression level of HSV-2 Us2 in transfected cells (Figure 4-12). Interactions between HSV-2 Us2 and vimentin, and beta-tubulin, need further confirmation in both transfected and infected cells.
In the co-IP experiments and LC-MS/MS analysis, ubiquitin was identified as an interacting partner of HSV-2 Us2. Interactions between HSV-2 Us2 and ubiquitin conjugated to membrane proteins may mediate membrane association of HSV-2 Us2. Interactions between PRV Us2 and ubiquitin were also examined. However, PRV Us2 did not appear to interact with any ubiquitin-conjugated proteins in transfected cells. Nonetheless, we cannot exclude the possibilities that PRV Us2 requires other viral proteins to interact with ubiquitin. Alternatively, PRV Us2 may interact with both ubiquitin and the conjugated proteins simultaneously, or that the protein population recognized by PRV Us2 is not ubiquitinated in the absence of other viral proteins. Whether the ubiquitin binding property of PRV Us2 is infection-dependent will require further analysis.
Chapter 6

Summary and Future Directions

The tegument protein Us2 is conserved among alphaherpesviruses, with the exception of VZV. Previous studies have shown that the deletion of the Us2 gene in PRV resulted in an accumulation of virions in the cytoplasm of the infected primary cells [22]. PRV Us2 contains a CAAX motif for prenylation, which targets Us2 to the plasma membrane [21]. Our studies show that PRV Us2 oligmerizes and forms complexes with MAPK ERK. It interacts with ERK, via the CD domain, and sequesters ERK at the plasma membrane without inhibiting its kinase activity [11]. Inhibition of ERK activity caused virions accumulation in the cytoplasm of the infected cells and this defect was more profound in PRV Us2-null infection, suggesting that the ERK kinase activity is required for efficient virus egress and the interaction between Us2 and ERK is important for this process [11]. We speculate that PRV Us2 acts as a scaffold to spatially regulate ERK activity in order to facilitate virus egress.

HSV-2 Us2 was examined to determine whether it shares similar properties with PRV Us2. Regardless of the three N-terminal conserved regions, its amino acid sequence shares only 30% identity with PRV Us2 and lacks post-translational modification signals for membrane targeting. Nonetheless, like PRV Us2, it localizes to the plasma membrane and is associated with lipid rafts, through a mechanism that is not yet defined. Our studies show that HSV-2 Us2 also localizes to recycling endosomes. Different from PRV Us2,
HSV-2 Us2 does not interact with ERK. Instead, it interacts with ubiquitin, a small 76 amino acid protein containing 7 lysine residues, each of which can be utilized to form a polyubiquitin chain. Other than the well-known proteasomal function, ubiquitin has recently been discovered to participate in endocytosis and lysosomal degradation. The fate of a ubiquitinated protein depends on the type of ubiquitin linkage attached to it.

Proteins conjugated with a K48-linked polyubiquitin chain are targeted to the proteasome whereas those tagged with a K63-linked polyubiquitin chain are targeted to LE/MVB, and subsequently transported to lysosomes for degradation [220]. Ubiquitin itself also serves as an endocytic signal at the plasma membrane and a sorting signal at early endosomes [222]. Membrane proteins that are monoubiquitinated are internalized into the endosomes [222]. Final envelopment of alphaherpesvirus has been shown to take place at several sites, including the TGN [131], late endosomes [132] and multivesicular bodies (MVB) [133, 134]. Recent studies on HSV-1 propose a new envelopment site and a novel model of egress, suggesting that viral envelope proteins migrate to the plasma membrane first, and are then endocytosed for final envelopment. Capsids are wrapped in the glycoprotein-containing, plasma membrane-derived endocytic tubules. Considering the properties of recycling endosome localization and ubiquitin binding, we hypothesize that HSV-2 Us2 facilitates the endocytosis of viral envelope proteins, through the interaction with ubiquitin, and participates in the egress of the enveloped virions through recycling endosomes.
From the data obtained from the ubiquitin binding assays, we cannot exclude that possibility that HSV-2 Us2 is involved in the proteasomal degradation pathway. Possible Us2 function may be similar to that of HPV E6, promoting the degradation of host proteins, such as p53, to prevent the initiation of apoptosis [297, 321].

In summary, alphaherpesvirus Us2 may promote viral replication and egress in three ways: 1) by promoting proteasomal degradation of host proteins (Figure 6-1, A), 2) by facilitating endocytosis of viral envelope proteins for final envelopment at endocytic tubules (Figure 6-1, B), and 3) by promoting virus egress, through the recycling endosomes, by interaction with MAPK ERK or lipid rafts, which cycle between intracellular compartments [322, 323] (Figure 6-1, C).

Some questions remain to be addressed to further understand the functions of Us2. Although it is clear that HSV-2 Us2 interacts directly with ubiquitin and shows equal binding affinity toward K48-linkage and K63-linkage, its role in the proteasomal degradation pathway still need to be defined. Furthermore, how does HSV-2 Us2 interact with ubiquitin? It does not contain any known UBDs. In addition, does PRV Us2 interact with ubiquitin? Our co-IP data show that PRV Us2 did not interact with ubiquitin in transfected cells, nonetheless, the interaction in infected cells still needs to be defined, inasmuch as other viral proteins may be required for the interaction. Mapping the interaction sites on both Us2 and ubiquitin may provide us clues to the answers of these questions. Furthermore, HSV-2 Us2 does not interact with ERK and ERK kinase activity is not essential for HSV-2 replication and egress. Recent studies demonstrate that PKD is
Figure 6-1. Hypothesized Us2 functions. (A) Us2 promotes degradation of host proteins through the interaction with K48-linked polyubiquitin chain. (B) Us2 facilitates endocytosis of viral envelope proteins through the interaction with ubiquitin. (C) Us2 promotes viral egress, through either recycling endosomes or MVB, by interacting with ERK or lipid rafts. EE: early endosomes, LE: late endosomes, MVB: multivesicular bodies, RE, recycling endosomes.
required for efficient HSV-1 egress [320]. Does HSV-2 Us2 participate in the egress through the interaction with PKD? Co-immunoprecipitation experiments would help determine the interaction between HSV-2 Us2 and PKD. Answers to these questions will provide insight into the roles of Us2 in virus egress.
Appendix A

Growth kinetics of HSV-2 Us2-deleted virus on different cell types

HSV-2 Us2-deleted and repair viruses were constructed, by our former post-doc, Dr. V. Le Sage, to determine whether the deletion of Us2 gene affects viral replication and egress. Different cell types were used to monitor the phenotypes.

A.1 Vero cells

Cells were infected with wildtype HSV-2, Us2-null and repair viruses at an MOI of 3. At 6, 12 and 24 hours post-infection, supernatants were collected to determine the titre of extracellular viruses. Cells were collected, by scraping into 1ml medium, to determine the titre of cell-associated viruses. Virus titre was determined by plaque assay.

No differences in growth kinetics between wildtype HSV-2, Us2-null and Us2 repair infection were observed, indicating that the absence of Us2 did not cause defects in viral replication and egress in Vero cells.
Figure A.1. Growth kinetics of wildtype HSV-2, Us2-null and Us2 repair viruses in Vero cells. (WT: wildtype HSV-2, Us2-d: Us2-null virus, Us2R: Us2 repair virus.)

A.2 Caco-2 cells

Consistent with the fact that HSV infects the apical side of mucosal cells, previous studies have shown that HSV preferentially infects apical surfaces of epithelial cells [324]. Differences between apical and basolateral membranes may also have impact on virus egress. To be more reflective to in vivo condition and since cells infected by herpesviruses are mostly polarized cells, the involvement of Us2 in virus egress was determined by infecting polarized cells with wildtype HSV-2, Us2-null and Us2 repair recombinant viruses.

Caco-2 cells become polarized when grown on the transwell (Figure A.2.1). To determine the growth kinetics of HSV-2 in the absence of Us2, cells were grown on 0.4μm-pored-size, collagen-coated transwell inserts and infected with wildtype HSV-2, Us2-null and Us2 repair viruses. In the transwell, medium contacting apical and
basolateral surfaces are separated from each other. Prior to infection, the transepithelial electrical resistance (TER) was measured everyday, using an epithelial voltohmmeter, until the TER values were steady for two consecutive days. Cells were infected from the apical membranes at an MOI of 10. After infection, medium from both apical and basolateral sides were collected at the indicated time points to determine virus yield. Similar methods have been described previously by the Herold laboratory [324]. Virus titre was determined by plaque assay on Vero cells and the results show that the deletion of Us2 did not affect viral replication and egress in polarized cells (Figure A2.2).

Figure A.2.1. Transwell. Caco-2 cells were grown on the filter with 0.4µm pores. Medium contacting apical and basal surfaces were separated from each other. Cells were infected from the apical side.
Figure A.2.2 Growth kinetics of wildtype HSV-2, Us2-null and Us2 repair viruses in Caco-2 cells. (WT: wildtype HSV-2, Us2-d: Us2-null virus, Us2R: Us2 repair virus. baso: basolateral.)

A.3 T12 cells

T12 cells are non-transformed human foreskin fibroblasts, whose life span has been extended by telomerase expression. To determine if the deletion of \textit{Us2} gene affects virus replication in T12 cells, cells were infected at an MOI of 0.1. At the indicated time points, supernatants were collected to determine the titre of extracellular viruses. Cells were collected, by scrapping into 1ml medium, to determine the titre of cell-associated viruses. Virus titre was determined by plaque assay. The results from the plaque assay showed that no differences in growth kinetics between wildtype HSV-2, Us2-null and Us2 repair infection were exhibited, suggesting that the deletion of \textit{Us2} gene does not affect viral replication and egress from T12 cells.
Figure A.3. Growth kinetics of wildtype HSV-2, Us2-null and Us2 repair viruses in T12 cells. (WT: wildtype HSV-2, Us2-d: Us2-null virus, Us2R: Us2 repair virus. baso: basolateral.)
Appendix B

Effect of inhibition of ERK kinase activity on HSV-2 replication

To determine whether ERK kinase activity is required for HSV-2 egress, Vero cells were treated with MEK inhibitor, UO126. Since ERK1 and ERK2 are the only known substrates of MEK, inhibition of MEK activity subsequently inhibits activation of ERK. Vero cells were treated with UO126 one hour prior to the infection. Cells were infected with wildtype HSV-2, Us2-null and Us2 repair viruses at an MOI of 2. At the indicated time points, supernatants were collected to determine the titre of extracellular viruses. Cells were collected, by scrapping into 1ml medium, to determine the titre of cell-associated viruses. Virus titre was determined by plaque assay. No differences in growth kinetics were observed between wildtype HSV-2, Us2-null and Us2 repair viruses in the presence and the absence of ERK inhibitor, indicating that ERK kinase activity is not required for viral replication and egress, regardless of the presence of absence of Us2.
Figure B. Effects of the inhibition of ERK kinase activity on HSV-2 growth kinetics. Vero cells were infected with wildtype HSV-2, Us2-null and Us2 repair mutant viruses at an MOI of 2. MEK inhibitor, UO126, was added to the cells 1 hour prior to and during the infection, at a final concentration of 30uM. Cells and supernatant were collected at the indicated time points. Cells were collected by scraping into 1ml medium. Virus titre was determined by plaque assay. CELL: cell-associated viruses, SUP (supernatants): extracellular viruses, (+): with UO126, (-): in DMSO without UO126.
Appendix C
Involvement of HSV-2 Us2 in viral resistance to interferon

To determine if HSV-2 Us2 is involved in interferon resistance, T12 cells were treated with either 0.1% BSA or type I interferon (IFN) at a concentration of 1000U/ml for 24 hours prior to the infection. Cells were then infected with wildtype HSV-2, Us2-null and Us2 repair viruses at an MOI of 0.1. Both supernatants and cells were collected at 24 hours post-infection. Virus titre was determined by plaque assay. The results show that HSV-2 Us2 did not mediate viral resistance to interferon.

Figure C. HSV-2 Us2 is not involvement in interferon resistance. (WT: wildtype HSV-2, Us2-d: Us2-null virus, Us2R: Us2 repair virus.)
Appendix D
Involvement of HSV-2 Us2 in the ER stress response

Proteins identified as interacting partners of HSV-2 Us2 included components of ER stress response, such as BiP (Chapter 5.6), which may suggest an involvement of HSV-2 Us2 in this process. Activation of ER stress results in phosphorylation of eIF-2α. To determine whether HSV-2 Us2 plays a role in the ER stress, HeLa cells were infected with wildtype HSV-2, Us2-null and Us2 repair viruses at an MOI of 5. The ER stress inducing drug, thapsigargin (Tg), was added to the cells at 4 and 8 hours post-infection at a final concentration of 1µM Tg for 1 hour. Cells were collected in SDS-PAGE loading buffer and subjected to Western blotting to determine phosphorylation status of eIF-2α. If Us2 interferes with the ER stress pathway, phosphorylation of eIF-2α could be inhibited in HSV-2 and Us2 repair infected cells when treated with Tg, while it is not in HSV-2 Us2-null infected cells. However, no such phenotypes were observed when treated with Tg, suggesting that HSV-2 Us2 does not interfere with ER stress, signaling to eIF-2α.
Figure D. Phosphorylation status of eIF-2a during HSV-2 infection. HeLa cells were infected with wildtype HSV-2, Us2-deleted and Us2 repair viruses at an MOI of 5. ER stress inducing drug, Tg, was added to the cells at the indicated time points. One hour after the addition of Tg, cells were washed and collected in PBS. SDS loading buffer was added to the cells for Western blotting analysis.
### Appendix E

**Primer sequences**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
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<tbody>
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<td>5'-GCCGAATTACATGGGGGTTGACGCGCCATCACCA'</td>
</tr>
<tr>
<td>PRVUs2 F EcoRV</td>
<td>5'-ATCGAATTCGATGGGGGTTGACGCGCCATCACCA</td>
</tr>
<tr>
<td>PRVUs2 R SalI</td>
<td>5'-GGCCTGACCTAGGAGATGGTACACGCCGAGG</td>
</tr>
<tr>
<td>PRVUs2 R BglII</td>
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<td>PRVUs2 N37</td>
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<td>5'-GTAGTCGACTACACCTTCGCGTGACGAC</td>
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<td>5'-GTAGTCGACTACACCTTCGCGTGACGAC</td>
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<td>PRVUs2 N157</td>
<td>5'-GTAGTCGACTACACCTTCGCGTGACGAC</td>
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<td>PRVUs2 N214</td>
<td>5'-GAAGAGATCTACAGGAGATGGTACACGCCGAGG</td>
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<tr>
<td>PRVUs2 R184K/R185 F</td>
<td>5'-CGCCTGACCTAGGAGAGAAGCCGCGGTCGAGC</td>
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<tr>
<td>PRVUs2 R184A/R185 A F</td>
<td>5'-CGCCTGACCTAGGAGAGAAGCCGCGGTCGAGC</td>
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<tr>
<td>PRVUs2 R184A/ R185 A R</td>
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<tr>
<td>PRVUs2 LxxRR5 A F</td>
<td>5'-CGCCTGACCTAGGAGAGAAGCCGCGGTCGAGC</td>
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<tr>
<td>PRVUs2 LxxRR5 A R</td>
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<td>5'-ATCGATATCG ATGGGGGTTTGTGTTGTTTAATGTTG</td>
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<tr>
<td>ERK2 R XhoI</td>
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<tr>
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<td>CGTTGGTCCTAGGAGGGTAACCACACCTAACAACAAACGTCCTATTTCGTC</td>
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*Italic: Restriction site*
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