University of Alberta

Herpes Simplex Virus Requires VP11/12 to Activate Src Family Kinase-PI3 Kinase-Akt Signalling

by

Melany J. Wagner

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Virology

Medical Microbiology and Immunology

Fall 2010
Edmonton, Alberta

Examining Committee

James R. Smiley, Medical Microbiology and Immunology

Debby N. Burshtyn, Medical Microbiology and Immunology

David H. Evans, Medical Microbiology and Immunology

Jim C. Stone, Biochemistry

Stephen A. Rice, Microbiology, University of Minnesota

ABSTRACT

This thesis defines a novel role for the Herpes Simplex Virus (HSV) tegument protein, virion protein (VP) 11/12 as a modulator of host cell signalling. Studies aimed at examining infection induced lymphocyte inactivation, revealed that VP11/12 is tyrosine phosphorylated in three lymphocyte lineages (T cell, B cell and NK cell) following exposure to HSV-1 or HSV-2 infected fibroblasts. Tyrosine phosphorylation of VP11/12 was greater in lymphocytes compared to fibroblasts or epithelial cells and phosphorylation was enhanced by the lymphocyte specific Src family kinase (SFK) Lck during transfection- or infection-based assays. This suggested that VP11/12 is a substrate of Lck or a kinase activated by Lck. Lck is best known for initiating intracellular signalling downstream of the T cell receptor (TCR) and NK cell receptors. However, VP11/12 null HSV mutants retained the ability to block TCR signalling and NK cell cytotoxicity.

Phosphorylation of VP11/12 occurred in the absence of any known Lck stimulus, like TCR ligation. Infection alone may activate Lck since Lck in infected Jurkat cells displayed features characteristic of activation: a reduced electrophoretic mobility in sodium dodecyl sulphate polyacrylamide gel and a marked increase in phosphorylation at the activation loop tyrosine (Y394). SFK substrates sometimes activate their cognate kinase through high affinity binding of the SFK Src homology (SH) 2 or SH3 domains. VP11/12 may serve this dual

function since it interacts with Lck or Lck signalling complexes and is strictly required for Lck activation during infection.

SFKs including Lck lie upstream of the canonical phosphoinositide 3-kinase (PI3K)-Akt pathway in signalling emanating from immune receptors, growth factor receptors and polyoma middle T antigen (MTAg). In HSV infection of Jurkat T cells and human embryonic lung fibroblasts, we find that VP11/12 interacts with PI3K either directly or indirectly and is required for infection induced activation of the PI3K-Akt signalling pathway. SFK activity is required for tyrosine phosphorylation of VP11/12, VP11/12-PI3K interactions, and Akt activation in infected fibroblasts. This data suggests that VP11/12 orchestrates signalling analogous to that of MTAg. In this model, VP11/12 activates SFKs to induce its own phosphorylation, subsequently allowing for interactions with PI3K and activation of Akt.

ACKNOWLEDGEMENTS

I would like to thank all my friends in the Department of Medical Microbiology and Immunology for your support. Thank you to my mentor Dr. Jim Smiley for guiding my research and for teaching me how to be a better scientist. Thank you to my lab members for sharing your scientific ideas with me and for your camaraderie. Thank you to my committee members Dr. David Evans and Dr. Debby Burshtyn for your help with my science and academics. Thank you to Dr. Hanne Ostergaard and Dr. Rob Ingham for their guidance through the signalling aspect of my project. Finally, thank you to my close personal friends Joelle St. Pierre, Kim Zimmerman, Deanna Hockley, Bindi Ferguson, and Dr. Catherine Ewen for truly making Edmonton my home.

ABREVIATIONS

4EBP1	eukaryotic translation initiation factor 4E (eIF4E) binding protein 1
α	immediate early
AP-1	activator protein 1
β	delayed early
BAC	bacterial artificial chromosome
BCR	B cell receptor
BH	BCR homology
bp	base pair
BSA	bovine serum albumin
CD	cluster of differentiation 3
CHV	cercopithecine herpesvirus
cpm	counts per minute
CREB	cAMP response element-binding protein
CTL	cytotoxic T cell
DAG	1,2-diacylglycerol
DMEM	Dulbecco's modified eagle's medium
DOG	deoxy-galactose
E:T	effector: target
EBV	Epstein-Barr Virus
FBS	fetal bovine serum
FOXO	forkhead box 0
Fyn	fibroblast src/yes novel gene
γ1	leaky late
γ2	true late
GADS	Grb2-related adaptor protein downstream of Shc
gB	glycoprotein B
gC	glycoprotein C
gD	glycoprotein D
GDP	guanosine diphosphate

gE	glycoprotein E
gG	glycoprotein G
gH	glycoprotein H
gI	glycoprotein I
gL	glycoprotein L
gM	glycoprotein M
Grb2	growth factor receptor-bound protein 2
GSK3	glycogen synthase kinase 3
GTP	guanosine triphosphate
HCF-1	host cell factor 1
hCMV	human cytomegalovirus
HEL	human embryonic lung fibroblast
HHV	human herpesvirus
HIV-1	human immunodeficiency virus 1
hpi	hours post-infection
HSV	herpes simplex virus
HVEM	herpes virus entry mediator
ICP	infected cell protein
IFN	interferon
IL	interleukin
IP_3	inositol 1,4,5-triphosphate
IRS-1	insulin receptor substrate-1
ITAM	immunoreceptor tyrosine-based activation motif
KSHV	Kaposi’s sarcoma-associated herpesvirus
L particles	light particles
LAT	latency associated transcripts
LAT	linker for the activation of T cells
Lck	lymphocyte-specific cytoplasmic protein-tyrosine kinase
MAPK	mitogen associated protein kinase
MEF	mouse embryonic fibroblast
MOI	multiplicity of infection

MTAg	middle T antigen
mTORC	mammalian target of rapamycin complex
NK	natural killer
ORF	open reading frame
PBS	phosphate buffer saline
PDGFR-β	platelet derived growth factor receptor β
PDK1	3-phosphoinositide-depedent kinase 1
PFU	plaque forming unit
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-biphosphate
PIP3	phosphatidylinositol 3,4,5-triphosphate
PKC	protein kinase C
PLC-γ	phospholipase C γ
PMA	phorbol myristate acetate
PrV	pseudorabies virus
PTB	phosphotyrosine binding group
PTEN	phosphatase and tensin homologue
Rheb	Ras homologue enriched in brain
Rheb-GAP	Rheb-GTPase activating protein
S6K	p70 ribosomal S6 kinase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFK	Src Family Kinase
SH	Src homology
SLP-76	Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kDa
TAP	transporter associated with antigen processing
TCR	T cell receptor
Th	T helper
TMP	terminal membrane protein
TNF-α	tumour necrosis factor α
TSC2	tuberous sclerosis complex 2

UL	unique long
US	unique short
vhs	virion host shut-off
VP	virion protein
v-Src	viral Src
VZV	varicella-zoster virus
ZAP-70	zeta-chain (TCR) associated protein kinase 70kDa

Table of Contents

Table of Figures

CHAPTER 1: Introduction

1.1 OVERVIEW OF HERPES SIMPLEX VIRUS -1 (HSV-1)

1.1.1 HSV-1: a Brief Introduction

HSV-1 is a highly prevalent human pathogen. Initial infection occurs at the epithelia surrounding the mouth or less commonly through the eye. The virus then enters the innervating neurons and travels retrograde to establish latency in the trigeminal ganglion (48). The incidence of HSV-1 infection increases with age (87, 114, 181). By the age of 60, HSV-1 DNA is found in the trigeminal ganglion of ca. 75-100% of individuals (87, 114, 144). The proficient spread of HSV-1 is mostly due to asymptomatic shedding of the virus. One study demonstrated that 92% of asymptomatic individuals shed HSV-1 in their tears or saliva at least once during a one month period (97). Most HSV-1 infections remain asymptomatic. However, during periods of stress or immunocompromisation reactivation of the lytic life cycle can occur at the initial site of infection or in the central nervous system, leading to “cold sores”, progressive blindness or lethal encephalitis (181).

1.1.2 Taxonomy

HSV-1 is a member of the herpesviridae family within the order of herpesvirales (41). The herpesviridae infect mammals, birds and reptiles and

there are presently over 200 viruses classified within this family (162). Originally herpesvirus classification was based on virion morphology, and more recent herpesvirus classification is based on genomic sequence data (162). The herpesviridae share a common virion structure (described in detail for HSV-1, Section 1.1.2) (162). Herpesviridae infection is most often asymptomatic with the virus establishing a latent infection in which no progeny virions are produced and only a subset of viral genes are expressed. However, all eight herpesviruses known to infect humans have the potential to inflict disease. Potential disease states include chicken pox/shingles induced by varicella-zoster virus (VZV); mononucleosis induced by Epstein-Barr virus (EBV); and tumourogenesis inflicted by EBV or Kaposi's sarcoma-associated herpesvirus (KSHV).

The herpesviridae contains three subfamilies, alphaherpesvirinae, betaherpesvirinae, and gammaherpesvirinae, as well as numerous unassigned viruses (41). The alphaherpesvirinae have a variable host range, multiply rapidly, and establish latency in the sensory ganglia (162). This subfamily is subdivided into four genera (simplexvirus, varicellovirus, mardivirus, and iltovirus), and includes three human viruses, HSV-1, HSV-2 and VZV (1). The betaherpesvirinae have a restricted host range, a long reproductive cycle, and maintain latency in secretory glands, lymphoreticular cells, kidneys, and other tissues (162). This subfamily includes human cytomegalovirus (hCMV), human herpesvirus (HHV)- 6 and HHV-7 (1). Finally, the gammaherpesvirinae primarily

infect T and B cells, and establish latency in lymphoid tissue. This subfamily includes the human viruses, EBV and KSHV (162).

The taxonomic classification of HSV-1 relative to the eight known human herpesviruses is outlined in Figure 1.

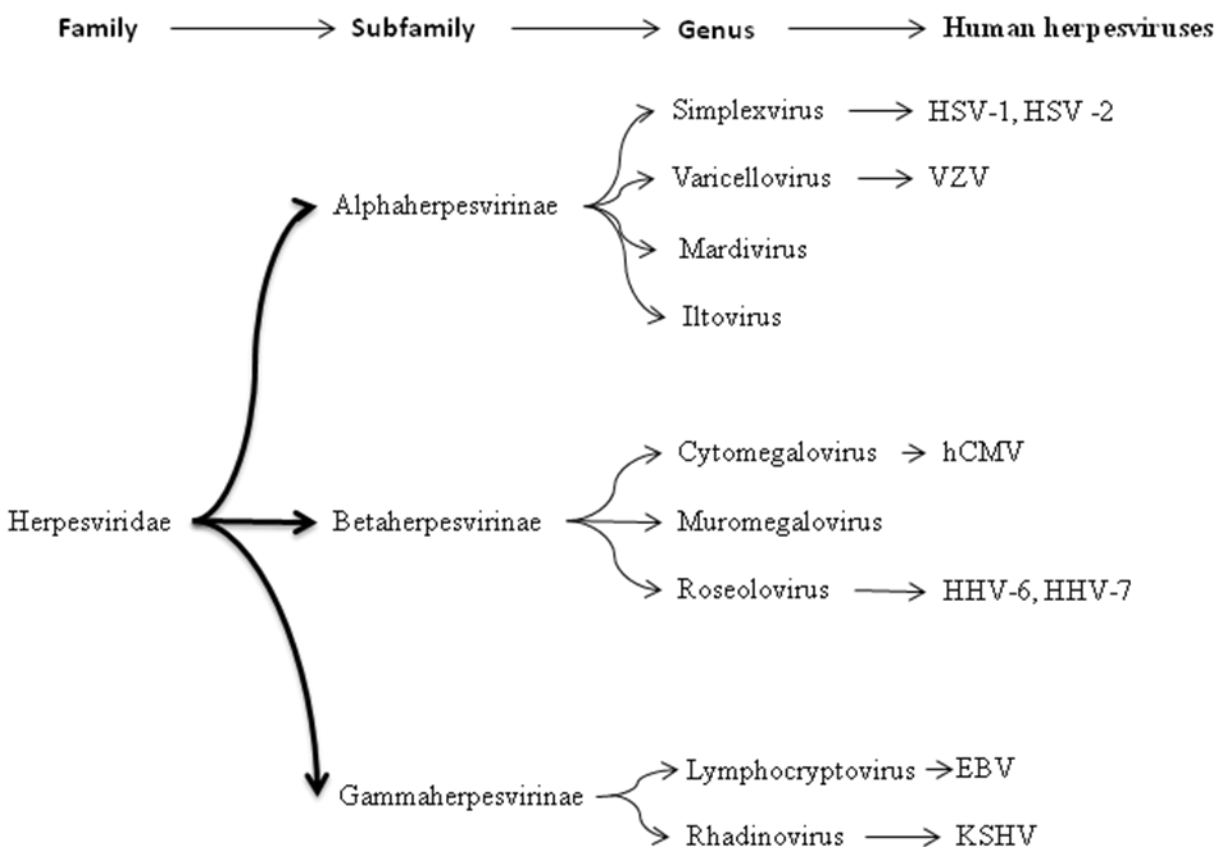

Figure 1. Taxonomy of Herpes Simplex Virus (HSV)-1

Taxonomy of HSV-1 also known as human herpesvirus 1 (HHV-1) relative to the eight known human herpes viruses: Herpes simplex virus 2 (HSV-2/HHV-2), Varicella-zoster virus (VZV/ HHV-3), Epstein-Barr virus (EBV/HHV-4), Human cytomegalovirus (hCMV/HHV-5), HHV-6, HHV-7, Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8).

1.1.3 Virion Structure

At the core of the HSV-1 virion is a 152 261 base pair (bp) double stranded genomic DNA molecule held within a nucleocapsid (Figure 2A). A matrix of proteins known as the tegument surrounds the capsid, and both the capsid and the tegument are encased by a lipid envelope decorated with mostly glycosylated proteins (181). The average size of the entire virion is 225 nm (181), and the size varies based on factors like the variable thickness of the tegument (162).

1.1.3.1 Envelope

The HSV-1 envelope is derived from host cell membrane (206). Although the origin of the virion envelope is a subject of debate, current evidence suggests that it is derived from the trans-Golgi network (136). The envelope contains ca. 9 viral glycoproteins (glycoprotein B (gB), gC, gD, gE, gG, gH, gI, gL, gM) and at least 2 non-glycosylated proteins (unique long 20 (UL20), unique short 9 (US9)) (181). During viral entry the virion envelope fuses with the plasma membrane in a process facilitated by gD, gB, gH and gL (reviewed in (76)).

1.1.3.2 Capsid

The capsid is composed of 162 capsomers arranged with icosahedral symmetry (T=16). The outer shell of the capsid consists of four viral proteins

with virion protein (VP) 5 as the major capsid component forming the penton and hexon capsomers (181). Triplexes made up of two UL18 molecules and one UL38 molecule reside between the connecting capsomers (181), and VP26 forms hexamers attached to the hexameric capsomers (262). At one of the twelve capsid vertices resides the portal, a ring-like dodecamer of UL6, used for packaging viral DNA and possibly used to release the genomic DNA into the nucleus following viral entry (136).

1.1.3.3 Tegument

The tegument layer consists of at least twenty proteins packaged between the envelope and the capsid (reviewed in (99)). Tegument assembly is believed to be an organized process, however any order of assembly has yet to be fully defined. Tegument proteins have been shown to segregate into inner and outer layers and the absence of inner tegument proteins UL36 and UL37 prevents tegument and virion assembly (45, 46). The integrity of tegument protein interactions is further apparent through experiments with partially assembled virion particles which lack capsids, known as light particles (L-particles) (134, 220). The tegument of these particles remains intact upon removal of the virion envelope (134). The tegument may also support virion integrity through interactions with capsid and envelope proteins. In support of this theory, VP22 and VP16 have been shown to interact with the envelope embedded proteins, gE and gM, and gH and gD respectively (136).

In addition to structural functions, tegument proteins are thought to play important roles in promoting virus replication and suppressing host cell defences early during infection. Tegument proteins are ideally situated to perform such a function, since many of these proteins disperse throughout the host cell cytoplasm following viral entry. A basis for this theory is found in the known functions of tegument proteins, virion host shut-off (vhs) protein and VP16. VP16 promotes immediate early gene transcription and is essential for viral replication (reviewed in (247)) and vhs provides a potent block in host protein synthesis through RNA degradation (reviewed in (202)).

1.1.4 Lytic Cycle

The lytic cycle of HSV-1 (Figure 2b) produces progeny virus during primary infection and reactivation (symptomatic and asymptomatic). HSV-1 enters through fusion of the viral envelope and the plasma membrane (reviewed in (76)). Entry requires envelope embedded proteins. The initial binding of HSV-1 to the cell surface is enhanced by the interactions between heparan sulphate of cell surface proteoglycans and gC, (or gB in the absence of gC) (77, 78, 193, 246). Cell surface receptor binding by gD is then essential for virus entry (115). The cell surface receptors involved in this process have not been wholly identified, but are known to include Nectin-1, the herpesvirus entry mediator (HVEM) and heparan sulphate modified by 3-O-sulfotransferases (76). Fusion of the viral envelope and the cellular membrane follows, and this requires gD, gB, and a

heterodimer of gH and gL (226). Upon membrane fusion outer tegument proteins like VP16 are thought to be released into the cytoplasm while inner tegument proteins including UL36 and UL37 remain associated with the capsid (shown for the related alphaherpesvirus, pseudorabies virus (PrV) (70)). The microtubule network and the cellular motor protein dynein transport the capsid and associated tegument proteins to the nucleus, where UL36 aids in the release of viral genomic DNA into the nuclear pore (30, 50, 107, 225).

HSV-1 DNA replication and gene transcription take place in the nucleus. All viral genes are transcribed by host RNA polymerase II (3), and transcriptional regulation segregates the genes into immediate early (α), delayed early (β), leaky late (γ1) and true late (γ2) kinetic classes (81). Transcription of α genes is promoted by a complex consisting of VP16, host transcription factors Oct-1 and host cell factor 1 (HCF-1) (reviewed in (247) and (57)). This step is crucial for viral replication, since most of the α gene products are required for subsequent viral gene expression (82). For example, infected cell protein 4 (ICP4) is essential for the virus to achieve full expression of all post-α genes (44, 171, 240), and ICP0 is required for full expression of α, β and γ genes during infections initiated at a low multiplicity of infection (MOI) (184, 215). Similarly, many β genes protein products enhance subsequent transcription of γ1 genes, and are strictly required for transcription of γ2 genes (82). These β genes products are required for genome replication and include UL9, which binds the origin of replication and

initiates helicase activity; ICP8, which binds single stranded DNA; the helicase-primase complex of UL5, UL8 and UL52; and the DNA polymerase holoenzyme subunits UL30 and UL42 (181). Viral DNA replication facilitated by these delayed early proteins is believed to titre out inhibitory DNA binding elements, which may include ICP4 bound to the γ2 promoter (174, 181). The γ genes encode many of the virion structural proteins and their expression marks the onset of virion assembly and egress from the infected cell.

Assembly (reviewed in (136)) begins in the nucleus where one genomic unit of the replicated, concatemeric viral DNA is loaded into the capsid in place of the scaffold proteins VP21 and VP22a (181). Enveloped HSV virions are present in the perinuclear space, and it was originally thought that the final stage of virion assembly, which encloses the capsid and tegument proteins in a glycoprotein embedded envelope, occurs at the nuclear membrane. Presently, the primary nuclear enveloped virion is thought to represent a transient stage of virion assembly and final virion assembly is thought to occur in the cytoplasm based on several observations. 1) The lipid composition of the extracellular virion envelope differs from the nuclear membrane and resembles a plasma or Golgi-derived membrane (228). 2) An HSV mutant genetically modified to restrict gD localization to the nucleus and the endoplasmic reticulum produces progeny primary virions in the perinuclear space which express gD, and produces mature extracellular virions that lack gD (198). 3) Envelope glycoprotein B, tegument proteins VP16 and VP11/12, and the major capsid protein, VP5, are concentrated

at cytoplasmic foci believed to be sites of virion assembly (154). 4) Capsids have been seen juxtaposed to invaginated cytoplasmic vesicles (209); in theory, capsids entering these vesicles would acquire a Golgi-derived double membrane and would egress from the cell by exocytosis.

How capsids translocate from the nucleus to cytoplasmic sites of virion assembly remains a point of contention. Enlarged nuclear pores have been observed during infection by the related bovine herpesvirus-1 and these pores represent one possible means of capsid nuclear export (242). However, most evidence to date supports an envelopment-deenvelopment model of nuclear egress. In this model, the primary virion envelope is acquired at the inner nuclear membrane by a mechanism that is known to involve lamina restructuring by UL31, UL34 (178, 179, 197), the cellular protein kinase C (161), and the viral serine/threonine kinase, US3 (146). This primary envelope then presumably fuses with the outer nuclear membrane to expel naked capsids into the cytoplasm by a process that is known to require gB (55, 245), gH (55), as well as US3 (179, 245).

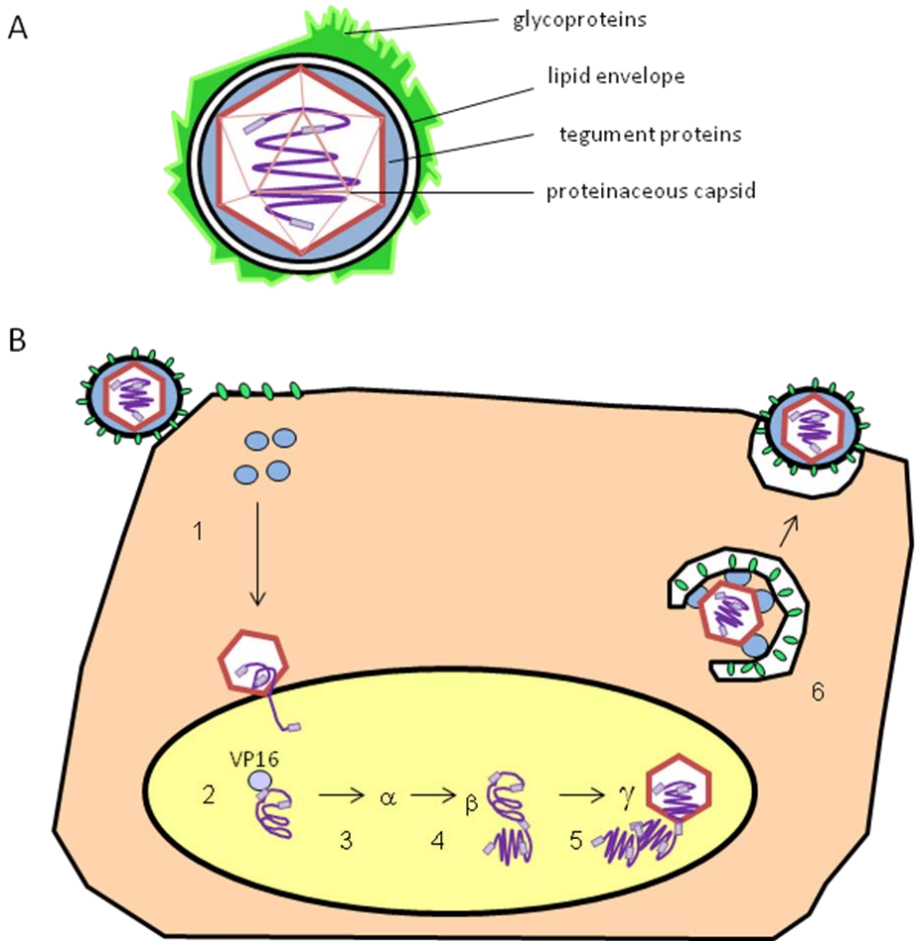

Figure 2. The HSV-1 Virion and Lytic Lifecycle

A) An outline of the structural components of the HSV-1 virion. B) The HSV-1 lytic lifecycle. The virus binds, enters via membrane fusion and injects the viral genome into the nucleus (1). The VP16 transcriptional complex initiates gene transcription (2). Genes are transcribed in kinetic classes (α, β, γ) (3,4,5). β gene products support viral DNA replication (4). γ genes encode virion structural proteins and allow for virion assembly beginning with viral DNA packaging into nucleocapsids (5). It has been theorized that assembly of the virion is completed as the capsid enters Golgi derived vesicles and newly formed virions are then transported to the cell surface (6).

1.1.5 HSV-1 Latency

HSV-1 latent infection in the human trigeminal ganglion and in the sensory ganglia of mouse and rabbit experimental models is characterized by restricted viral gene expression. The only viral gene products expressed in abundance are the latency associated transcripts (LATs): RNA species that are not known to be translated into protein (106, 180, 212, 213).

The mechanisms that restrict viral gene expression and viral replication during HSV-1 latency are not fully understood, but remain an active area of research. The virus may actively induce latency through LAT transcripts, which have been shown to repress lytic gene expression (25, 66). The latent genome associates with nucleosomes, suggesting that chromatinization may play a role in limiting viral gene expression (47). Neuronal cells may express a repressive host factor and/or may lack a host factor required for viral replication. For example, neurons have weak expression of the α gene transcription factor Oct-1 (72), and it has been suggested that, in neurons, the related transcription factor Oct-2 out-competes this small amount of Oct-1 for binding at the α gene promoter (100, 116). Finally, the immune system may repress viral replication. Low levels of HSV proteins have been detected in the trigeminal ganglion (56, 71, 105). It is believed that these viral proteins are the result of a low basal level of reactivation, and that these proteins may activate cluster of differentiation 8 $(CD8)^+$ cytotoxic T lymphocytes (CTL), which have been shown to suppress reactivation in mice (120, 196).

1.1.5 HSV Immune Evasion

To ensure survival and production of progeny virus HSV-1 encodes proteins that prevent detection by the host innate and adaptive immune systems. ICP0, ICP27, ICP34.5, vhs, and US3 inhibit a variety of processes associated with the type I IFN response of innate immunity (reviewed in (159)). gC binds the complement component C3b (61), and inhibits the alternative complement cascade (62), a process that normally results in lysis of infected cells or virus particles. gE and gI form a heterodimer on the infected cell surface which binds host antibodies (9, 91, 92). This viral encoded Fc receptor prevents virus particle neutralization by the classical complement pathway (60), and impedes infected cell lysis by antibody dependent cellular cytotoxicity (51).

1.1.5.1 HSV Evasion of T Cells and NK Cells

In humans, T cells have been shown to reside near HSV-1 lytically infected epithelial cells (36), and latently infected trigeminal ganglion (222). Thus, HSV must evade these adaptive immune cells to allow for progeny virion production. Natural killer (NK) cells and CTL eliminate cells that pose a danger to the host and as such play a crucial role in limiting the severity of disease caused by HSV (49, 158). CTL recognize virus infected cells through T cell receptor (TCR) binding of a specific viral peptide presented within a specific MHC I molecule. Many viruses evade CTL detection by downregulating cell surface expression of MHC I (4). NK cells complement CTL in viral immunity by

specifically killing cells that lack cell surface MHC I (95). HSV has evolved at least three distinct mechanisms to evade NK cell and CTL killing. 1) HSV-1 infected cells are innately resistant to CTL and NK cell killing due to virus encoded anti-apoptotic proteins, like US3 (22, 90). 2) The tegument protein vhs inhibits synthesis of MHC I (224), and the immediate early protein ICP47 binds the transporter associated with antigen processing (TAP) to interfere with peptide loading of MHC I and trap MHC I in the endoplasmic reticulum (252). Collectively, these processes prevent viral peptide presentation to CTL (224, 252). 3) CTL and NK cells exposed to HSV infected cells become infected through cell to cell spread of the virus (8), and infection renders these lymphocytes incapable of killing immunological targets including virus infected cells, allogeneic cells and cells with low MHC I expression (28, 166-168, 251).

1.1.5.2 TCR Signalling

Upon TCR binding of a viral peptide presented in the context of an MHC molecule, the T cell becomes active and able to respond to the presence of this intracellular pathogen (Figure 3). The TCR complex consists of a variable α and β chain that recognize specific peptide-MHC complexes, and CD3 dimers (CD3ε/γ, CD3 ε/δ and CD3 ζ/ζ) which collectively contain 10 immunoreceptor tyrosine-based activation motifs (ITAMs; consensus sequence D/EXXYXXI/LX$_{(6\text{-}8)}$YXXI/L; (177)) capable of propagating intracellular signalling (108). The co-receptors CD8 (for CTL) and CD4 (for T helper (Th)

cells) bind the invariant region of MHCI and MHCII, respectively. The Src family kinase (SFK) lymphocyte-specific cytoplasmic protein-tyrosine kinase (Lck) is recruited to the immune synapse through non-covalent interactions with CD4 and CD8 and initiates intracellular signalling following TCR ligation (216, 231). Lck phosphorylates the CD3 ζ ITAMs to allow for ITAM binding by ζ-associated protein of 70 kDa (ZAP-70) (24, 88, 229). Active ZAP-70 phosphorylates the transmembrane adaptor protein, linker for the activation of T cells (LAT) (259), followed by the cytosolic adaptor protein Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) (19). Upon tyrosine phosphorylation, these adaptors bind a myriad of signal transducing proteins which activate the Ras-mitogen associated protein kinase (MAPK) pathway; the phosphoinositide 3-kinase (PI3K)-Akt pathway; and the phospholipase C γ (PLC-γ)– inositol 1,4,5-triphosphate(IP_3)/1,2-diacylglycerol (DAG) pathway, which results in activation of protein kinase C (PKC) and intracellular Ca^{2+} signalling (203). In response to a viral antigen, this signalling allows for viral clearance by instructing $CD4^+$ Th cells to produce and secrete pro-inflammatory Th1 cytokines and by instructing $CD8^+$ CTL to kill the infected cell through secretion of cytotoxic granzymes and perforin.

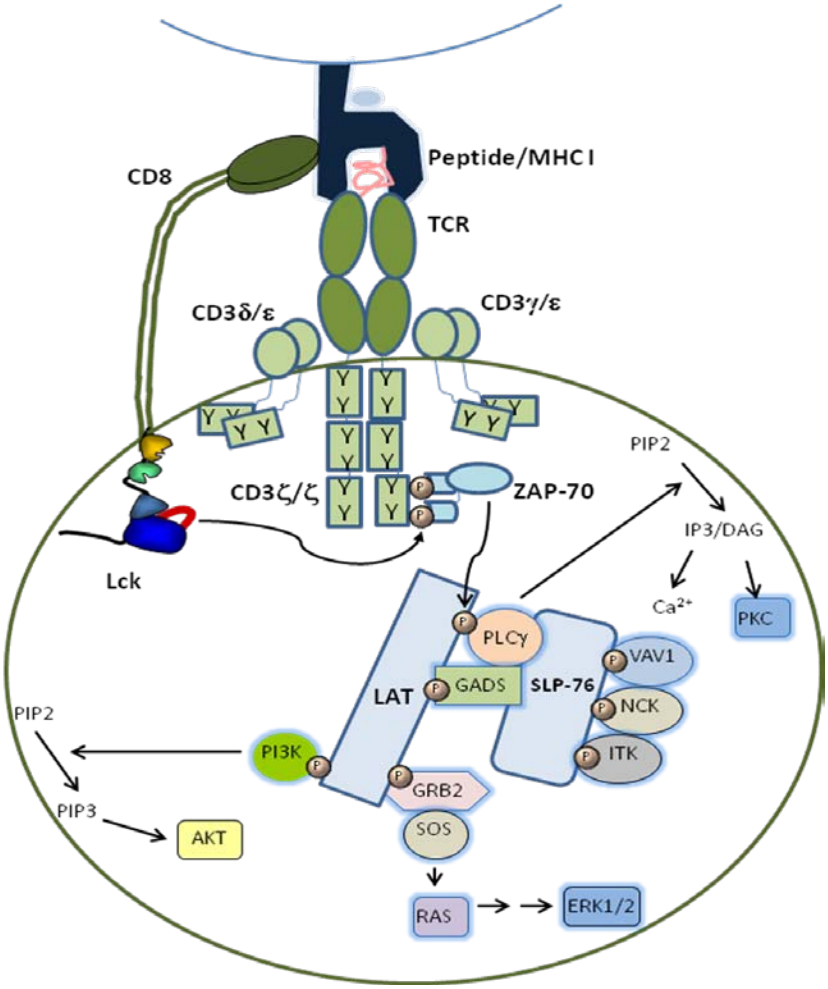

Figure 3. The TCR Signalling Pathway.

The TCR binds to a specific peptide presented on the cell surface by MHC. The CD8 co-receptor binds MHCI and concomitantly recruits Lck to the immune synapse. Lck phosphorylates the CD3 ITAMs. ZAP-70 binds the phosphorylated ITAM and phosphorylates the downstream adaptor, LAT (note: LAT is not drawn as a transmembrane protein due to spatial restraints). Phosphorylated LAT recruits a critical adaptor protein SLP-76 through their mutual binding partner GADS. As well, phosphorylated LAT recruits the signalling effectors, PLCγ1, PI3K, and the Grb2-SOS complex. PI3K activates the PI3K-Akt signalling pathway; SOS activates the Ras-ERK1/2(MAPK) pathway; and PLCγ1 allows for intracellular Ca^{2+} signalling and activation of PKC.

1.1.5.3 Infection-induced Modulation of TCR Signalling

T lymphocyte inactivation by HSV-1 is due in part to active remodeling of signalling downstream of the TCR. Sloan et. al. have shown that a direct infection by HSV inhibits TCR mediated Ca^{2+}signalling and activation of the MAPKs ERK1 and ERK2 in the immortalized $CD4^{+}$ Jurkat T cell line (199). HSV does not induce a global inhibition of cellular signalling. Phorbol myristate acetate (PMA) and ionomycin can bypass the HSV signalling blockade to trigger ERK1/2 activation, indicating that HSV specifically targets TCR-mediated activation of ERK1/2 (199). HSV also targets only specific intracellular signalling events in the TCR pathway. Lck mediated activation of ZAP-70 remains intact, however signalling is inhibited prior to phosphorylation of LAT and the association of LAT with downstream effectors growth factor receptor-bound protein 2 (Grb2), Grb2-related adaptor protein downstream of Shc (GADS) and PLC-γ (199). The nature of the HSV TCR signalling blockade allows for a specific cytokine response upon TCR ligation. Production of the pro-inflammatory Th1 cytokines IFN-γ, tumor necrosis factor α (TNF-α) and interleukin 2 (IL-2) is abrogated, however the Th2 (tolerance-inducing) cytokine IL-10 is produced at a level comparable to that of uninfected cells (200). This remodeling of TCR signalling may be facilitated by viral modulation of several signalling effectors. For example, it has been suggested that the specific induction of IL-10 requires infection induced activation of p38 and JNK kinases (200).

HSV-1 virions seem to be sufficient to inhibit TCR signalling. Infection by UV-killed HSV and infection in the presence of the protein synthesis inhibitor cycloheximide indicates that *de novo* virus protein production is not required for inhibition of TCR signalling (199). This suggests a potential role for the HSV-1 tegument proteins. Notably, the potent shut off of host protein translation by the tegument protein vhs is not required for inactivation of CTL (166). One report deemed the tegument protein US3 to be necessary (201), however no mechanism of action was defined and no subsequent reports have implicated US3 in this process. Further experimentation is required to fully define the contribution of US3 and other virion components to the TCR signalling blockade. During our studies of the HSV-1 induced blockade of TCR signalling we obtained evidence that the viral tegument protein VP11/12 is able to modulate the activity of Lck. These studies form the basis of this thesis.

1.2 VIRAL AND CELLULAR ACTIVATION OF SRC FAMILY KINASES

Lck and other SFKs (Src, Fyn, Yes, Fgr, Hck, Blk, Lyn) are non-receptor tyrosine kinases that have been studied extensively for their roles in cancer, phagocytosis, cell adhesion, and signalling through growth factor receptors and immune receptors (reviewed in (11, 17, 123)). The N-terminus (50-70 amino acids) of each SFK consists of a sequence that is unique to the individual SFK, but always encompasses a myristoylation or palmitoylation site that allows for constitutive membrane association (103, 176). This allows SFKs to initiate

intracellular signalling downstream of membrane embedded receptors. While viruses are known to impair immune receptor signalling by disrupting SFK activation (27, 63, 85, 94, 138, 223), active SFKs are also needed to promote cellular functions that benefit the virus, like enhanced cell survival or enhanced viral spread (discussed in detail in section 1.2.2).

1.2.1 SRC Family Kinase Activation

On a biochemical level, SFK activity is enhanced as the enzyme switches from a "closed" inactive conformation to an "open" active configuration (Figure 4). This conformational change is regulated by the phosphorylation state of a C-terminal inhibitory tyrosine residue, and by the binding of ligands to the Src homology (SH) 2 and SH3 domains of the SFK (reviewed for Src in (182)). Crystal structures of inactive SFKs indicate that the closed conformation is characterized by intramolecular interactions between the SH2 domain and the C-terminal inhibitory phosphotyrosine motif, and between the SH3 domain and the linker region connecting the SH2 domain and the catalytic domain (195, 249). Dephosphorylation of the inhibitory tyrosine is sufficient to trigger an open conformation and enzyme activation (5, 29, 102, 248). Kinase activity can also be triggered by signalling adaptors and SFK substrates which bind to the SFK SH2 and/or SH3 domains and disrupt their interactions with the intramolecular inhibitory motifs (reviewed in (17, 54)). The affinity of SH2 domains for phosphotyrosine depends on the amino acid sequence surrounding the

phosphotyrosine residue (204). High affinity motifs found in a variety of signalling proteins are able to out-compete the relatively low affinity SFK C-terminal inhibitory tyrosine for binding to the SH2 domain, leading to enzyme activation (121). In some circumstances, this allows an SFK substrate to activate its cognate kinase. Similarly, high-affinity ligands of the SH3 domain are able to induce activation by disrupting inhibitory interactions of the SH3-SH2/kinase linker region (54).

In the open and active conformation, the key regulatory tyrosine residue in the SFK activation loop is exposed for phosphorylation (195, 248, 249). This modification is symptomatic of activation, and significantly enhances kinase activity by reinforcing the open conformation (37, 250). Phosphorylation of the activating tyrosine plays a dominant role in enzyme activation *in vitro*, since it blocks the negative effect of phosphorylating the C-terminal inhibitory tyrosine (218). The activation loop tyrosine also appears to be dominant *in vivo*, as documented by observations that SFKs activated by hydrogen peroxide or the SH3 ligand human immunodeficiency virus 1 (HIV-1) Nef display enhanced phosphorylation of the activating tyrosine and no measurable decrease in phosphorylation at the inhibitory tyrosine (73, 111).

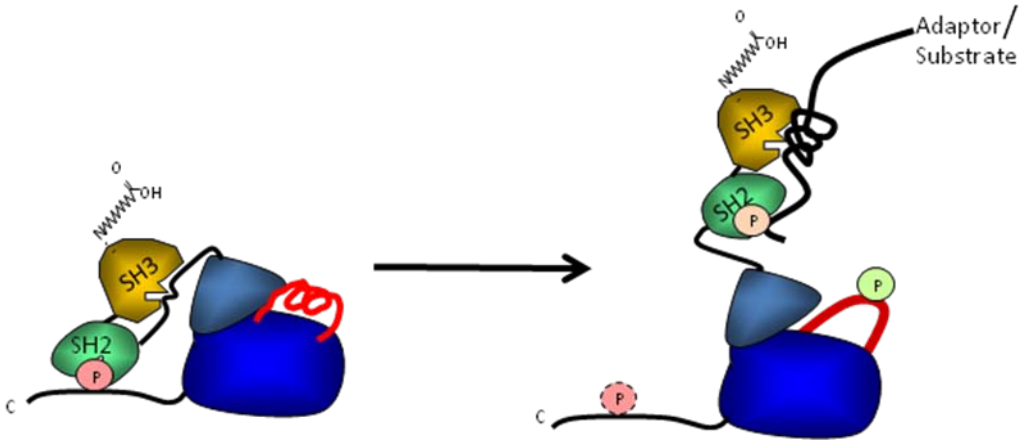

Figure 4. SFK Activation.

SFKs are held in a "closed" and inactive conformation by intramolecular interactions of the SH2 and SH3 domains. Dephosphorylation of the inhibitory SH2 binding motif in the C-terminal tail is sufficient to "open" and activate an SFK. Adaptors and substrates of SFKs activate the kinase through high affinity binding of the SH2 and/or SH3 domains. In the active conformation, a regulatory tyrosine in the kinase loop is exposed for phosphorylation. Phosphorylation of this residue reinforces the active conformation in a manner that supersedes any effect of the inhibitory phosphotyrosine.

1.2.2 Viral Modulation of SFK-mediated Signalling

Many viruses activate SFKs to initiate cellular signalling processes that benefit the virus. Polyoma middle T antigen (MTAg) stimulates Src to trigger cell cycle entry and DNA replication, thereby providing the environment needed for viral genome replication (reviewed in (84, 188)). Vaccinia virus produces cell-associated progeny virus particles that initiate outside-in signalling to activate Src. Src then phosphorylates the vaccinia protein A36R, triggering the formation of actin tails which facilitate virus spread by thrusting the cell-associated virus particles toward neighbouring uninfected cells (reviewed in (147)).

In the gammaherpesvirinae, genes next to the terminal repeats of the genome encode a family of signal transducing proteins called terminal membrane proteins (TMPs). TMPs physically interact with SFKs, to facilitate SFK activation while also acting as SFK substrates (reviewed in (16)). TMPs orchestrate signalling pathways that can regulate lytic or latent infection, and alter cell survival and/or immune function in infected lymphocytes (16). For example, the TMP LMP2A of EBV interacts with the SFK Lyn to impair immune function, maintain latent infection, and promote survival of latently infected B cells (reviewed in (160)). LMP2A promotes degradation of Lyn to prevent B cell receptor (BCR) signalling (137). This facilitates immune evasion and simultaneously suppresses viral reactivation from latency (63, 137, 138). However, B cells normally require tonic BCR stimulation to survive (141). For

this reason, LMP2A-Lyn interactions also induce pro-survival signals by activating the PI3K-Akt signalling pathway (64, 219).

1.3 CELLULAR AND VIRAL INITIATION OF PI3K-AKT SIGNALLING

As discussed in detail in section 1.3.3, the PI3K-Akt signalling axis is found downstream of SFK activation in many pathways. This includes signalling emanating from immune receptors, TCR and BCR (reviewed in (75, 203)); growth factor receptors, platelet derived growth factor receptor β (PDGFR-β) and insulin receptor (reviewed in (151)); and the viral protein MTAg (reviewed in (26)). Modulation of PI3K-Akt signalling through SFKs or by any other means allows a virus like HSV-1 to control a diverse array of biological functions including cell motility, growth, proliferation, transcription, translation and metabolism (reviewed in (126)).

1.3.1 PI3K-Akt Signalling

Active Class IA PI3K initiates signalling through the PI3K-Akt signalling module (Figure 5). There are three classes of PI3Ks, Class I PI3Ks are subdivided into class IA PI3Ks, activated downstream of receptor tyrosine kinases, G protein coupled receptors and Ras, and class IB PI3Ks, activated exclusively by G protein coupled receptors; the function of class II PI3Ks is unknown; and class III PI3Ks are important regulators of vesicle trafficking (119). Class IA PI3K (here forth referred to as PI3K) consists of a catalytic subunit

p110, and a regulatory subunit p85. The regulatory subunit known as p85 has five isoforms encoded by three genes: p85 β, p55γ and p85 α as well as its splice variants p55α and p50α (119). The p85 α and p85 β subunits contain one SH3, one BCR homology (BH) domain and two SH2 adaptor domains, while the p55α, p55γ and p50α subunits contain only SH2 adaptor domains.

PI3K activity is initiated as it is brought to its lipid substrate by interactions of the p85 adaptor subunit and membrane associated proteins, like the activated PDGFR-β receptor (253), the insulin receptor adaptor protein, IRS-1 (149), and the viral MTAg (221). Once proximal to its substrate, PI3K converts phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3), a secondary messenger that leads directly to Akt activation (119). This activity may be antagonized by the lipid phosphatase and tensin homologue (PTEN), an important negative regulator of PI3K-Akt signalling (119).

The formation of PIP3 allows for Akt activation since PIP3 specifically binds the pleckstrin homology (PH) domains of Akt and the Akt activator, 3-phosphoinositide-depedent kinase 1 (PDK1) (152). Co-localization of Akt and PDK1, and a conformational change in Akt induced by PIP3 binding allows PDK1to phosphorylate Akt at threonine 308 (T308) of the activation loop (210, 214). Full Akt kinase activity also requires phosphorylation at serine 473 (S473) of the Akt hydrophobic motif by an enzyme known as PDK2 (186). Several kinases have been proposed to act as PDK2, and the mammalian target of

rapamycin complex 2 (mTORC2) is currently the favoured candidate (186). The final biological outcome of this pathway depends on the specific substrate(s) targeted by active Akt (reviewed in (126)).

Active Akt phosphorylates numerous substrates to prevent apoptosis and promote cell survival, metabolism, growth, and proliferation. Among the known Akt substrates are the proapoptotic BH3-only protein BAD (40, 43); the proapoptotic forkhead box 0 (FOXO) transcription factors (18); GSK3, a kinase implicated in the control of transcription factors activator protein 1 (AP-1) and cAMP response element-binding protein (CREB) as well as glycogen and protein synthesis (35); and tuberous sclerosis complex 2 (TSC2), a negative regulator of mTORC1 which plays a critical role in promoting translation (outlined in detail in section 1.3.2) (86, 127, 169).

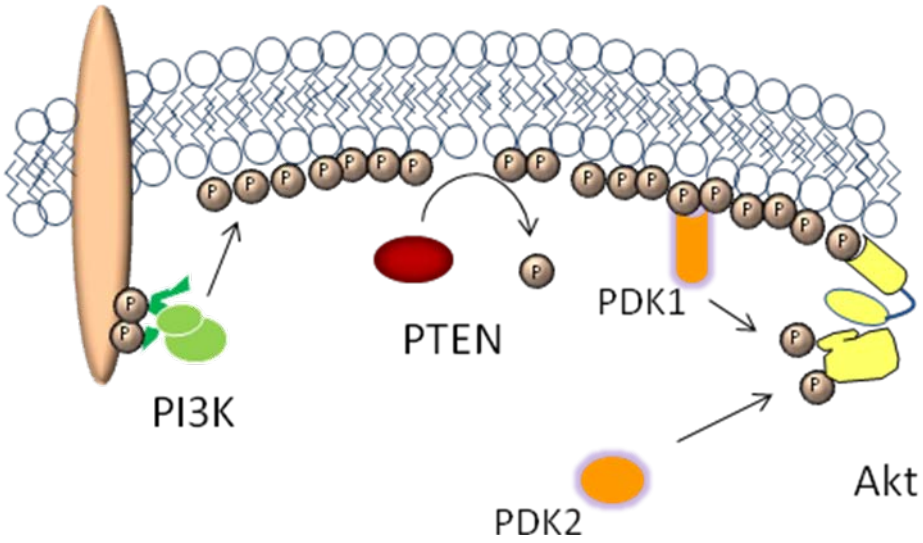

Figure 5. PI3K-Akt Signalling

Upon recruitment to a membrane PI3K converts PIP2 to PIP3. PTEN phosphatase antagonizes this activity to act as a negative regulator of PI3K-Akt signalling. The PH domains of Akt and its activator, PDK1 bind PIP3. This allows PDK1 to phosphorylate T308 of the Akt activation loop. For full kinase activity, Akt must also be phosphorylated at S473 of the hydrophobic motif by PDK2.

1.3.2 Viral Modulation of Akt Signalling

The importance of Akt signalling in viral infection is highlighted by the discovery of Akt as a viral oncogene with cellular homologues (208). In this instance Akt activity prevents apoptosis and promotes infected cell survival. Many viruses have now been shown to manipulate Akt to the same end. This ensures infected cell survival for viruses like EBV (42, 190, 219), human papilloma virus (257), and hepatitis B virus (194).

In addition to promoting infected cell survival, viruses and viral proteins initiate a number of biological functions through activation of PI3K-Akt signalling. The EBV protein BRLF1 activates signalling to promote viral gene transcription and subsequently initiate lytic replication in EBV positive cell lines (39). Akt signalling is critical for entry of Zaire ebolavirus (185), and HIV-1 uses at least two viral proteins to enhance virion production through Akt signalling. HIV-1 gp120 initiates signalling as it binds the virion to the cell surface during viral entry, and HIV-1 Nef participates in the assembly of a PI3K signalling complex (59, 117).

Viruses are also thought to activate mTORC1 signalling downstream of the PI3K-Akt pathway (Figure 6), to enhance viral protein translation (reviewed in (20)). The canonical mTORC-1 pathway leads to phosphorylation and activation of p70 ribosomal S6 kinase (S6K), and hyperphosphorylation and inhibition of eukaryotic translation initiation factor 4E (eIF4E) binding protein 1

(4EBP1). These two events converge to enhance translation. It is of note that active mTORC1 also supports cellular functions other than translation like the enhancement of viral DNA replication observed during adenovirus infection (155). During hCMV (93), and adenovirus infection (155) mTORC1 activation is known to require the activity of PI3K. However, in other viral infections the mTORC1 pathway may be activated either directly by a virus-encoded protein or it may be targeted through an alternate cellular mechanism, like the ill defined pathway linking excess amino acids and mTORC1 activation (reviewed in (52) and (165)).

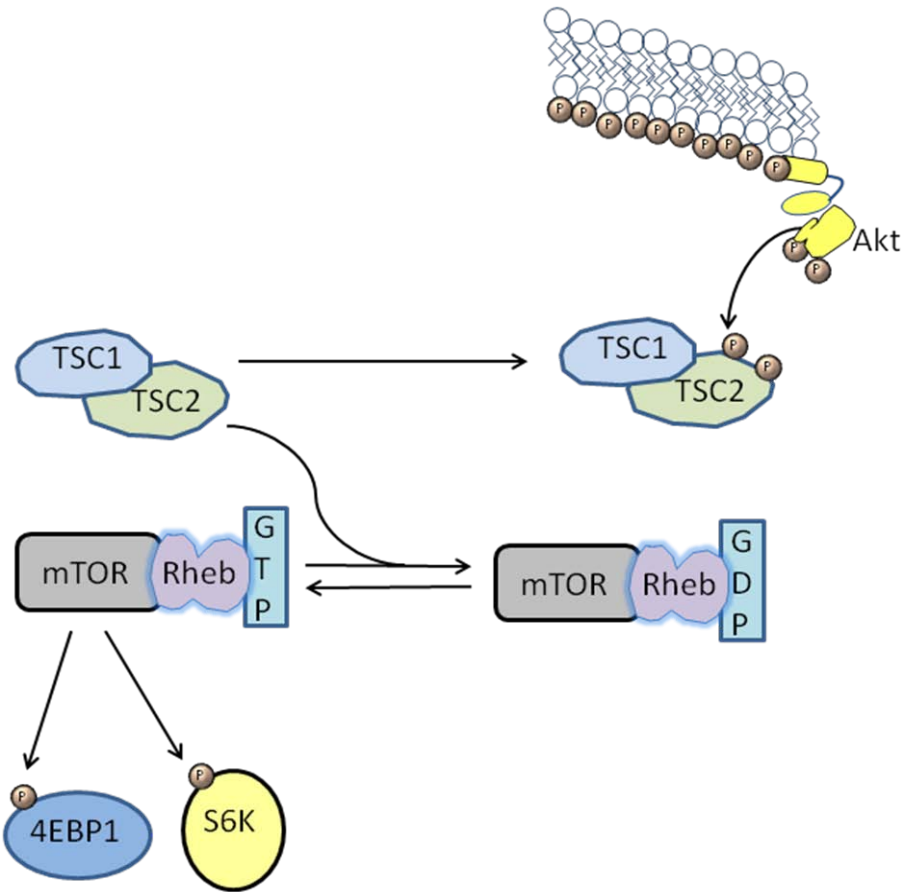

Figure 6. Akt-mediated Activation of mTORC1 Signalling.

Akt phosphorylates TSC2 to inactivate the mTORC1 inhibitor, TSC1/TSC2. mTORC1 is active when bound to Ras homologue enriched in brain (Rheb)-guanosine triphosphate (GTP) and is inactive when bound to Rheb-guanosine diphosphate (GDP). TSC1/TSC2 acts as a Rheb-GTPase activating protein (Rheb-GAP), to hydrolyze Rheb-bound GTP and inactivate mTORC1. Active mTORC1 enhances translation through two phosphorylation events. Phosphorylation of 4EBP1 causes the release of the translation initiation factor eIF4E; phosphorylation of S6K activates this kinase and stimulates translation through phosphorylation of S6K substrates including the ribosomal protein S6.

1.3.3 SFKs Upstream of Akt

Several lines of evidence suggest that SFKs influence the PI3K-Akt signalling axis; however the underlying mechanisms of this link have not been clearly defined. The strongest correlation between SFK activity and Akt signalling is provided by studies of viral Src (v-Src) from Rous sarcoma virus. Minor differences in amino acid sequence makes v-Src a constitutively active form of cellular Src (reviewed in (131)). Transformation of cells by v-Src is accompanied by the formation of a v-Src-PI3K complex and an increase in PI3K activity (65). This suggests that an active SFK is sufficient to induce PI3K-Akt signalling. Early studies suggested that the mechanism responsible for this activation involves direct binding of v-Src and PI3K since the SH3 domains of v-Src (122), Lck (235), and Fyn (170) bind PI3K *in vitro*. However, *in vivo* data supporting this theory seem to be lacking.

SFKs are found upstream of the PI3K-Akt signalling module in many pathways, including signalling initiated by the TCR (203) ; the BCR (75); PDGFR-β (10, 142, 254); the insulin receptor (149, 151, 254); and polyoma virus MTAg (221). In these signalling pathways SFKs control PI3K activation by mediating phosphorylation of a PI3K p85 SH2 binding motif(s) (consensus sequence YXXM). This was most clearly demonstrated in the PDGFR-β pathway (Figure 7). Upon receptor ligation PDGFR-β binds the Src SH2 domain at one of

two compensatory juxtamembrane motifs, and this high affinity binding activates Src (142, 227). A conservative tyrosine to phenylalanine mutation in these motifs ablates Src binding and activation (142, 227). As a consequence tyrosine phosphorylation of the PDGFR-β receptor is severely reduced and phosphotyrosine mediated binding of downstream effectors (including of PI3K) is ablated (10). The above experiments clearly outlined the mechanism of PDGFR-β-Src-PI3K signalling. However, in this pathway it is unclear whether Src phosphorylates the YXXM motif directly or indirectly through activation of a downstream kinase.

Signalling initiated by MTAg is thought to result in Src directly phosphorylating two PI3K p85 SH2 binding motif. MTAg contains a putative high affinity SFK SH2 binding motif (YEEI) that is believed to bind and activate Src. Evidence suggests that active Src bound to MTAg directly phosphorylates the YXXM motif found within MTAg (14, 33, 34, 187), and this phosphorylated motif facilitates PI3K binding and PI3K-Akt signalling *in vivo* (217, 221).

In signalling downstream of the insulin receptor Src activates an intermediate kinase to mediate phosphorylation of the YXXM motif and elicit PI3K activity. Downstream of insulin receptor ligation Src phosphorylates the insulin receptor to enhance the receptor's kinase activity (254), and the insulin receptor phosphorylates YXXM in the membrane associated adaptor protein IRS-1 to initiate PI3K-Akt signalling (149).

During TCR signalling the mechanism of SFK mediated PI3K activation is more complex. Lck phosphorylates the ITAM motif of the CD3ζ chain of the TCR complex, and this indirectly leads to activation of the ZAP-70 (reviewed in (203)). ZAP-70 in turn phosphorylates the membrane associated adaptor protein, LAT. PI3K associates with LAT, however this interaction may involve additional unidentified adaptor proteins since LAT does not contain a YXXM motif (258).

One report suggested an alternative *in vivo* mechanism for SFK-mediated activation of PI3K-Akt signalling whereby active SFKs inhibit PTEN activity (124). Although, this second *in vivo* mechanism is not well established, it is an intriguing possibility that SFKs may simultaneously active PI3K and suppress PTEN to induce Akt activity *in vivo*.

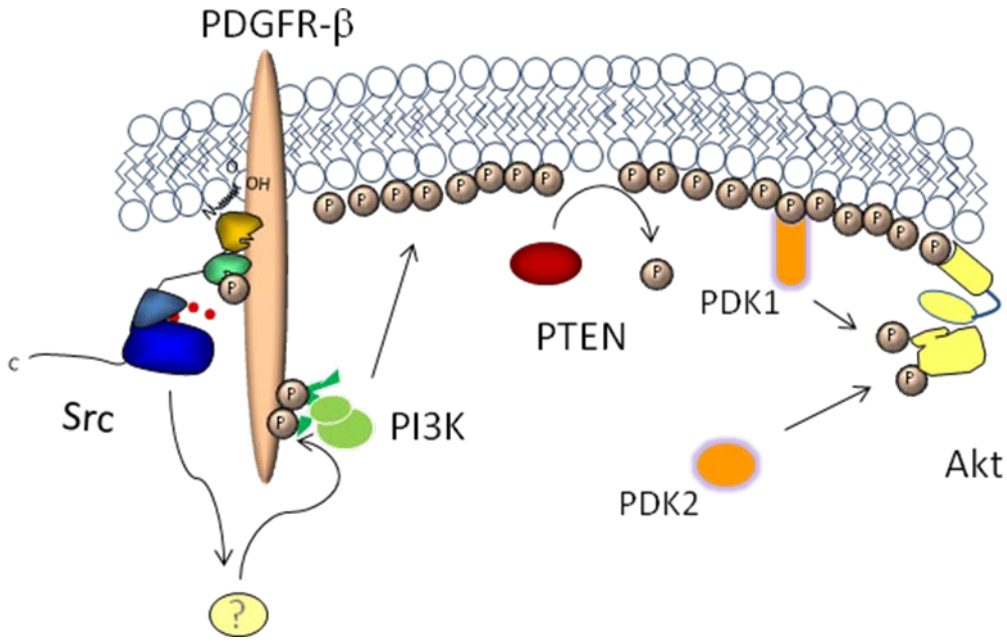

Figure 7. PDGFR-β Signalling

Ligand-occupied PDGFR-β autophosphorylates to create SH2 docking sites for downstream effectors. The Src SH2 domain binds with high affinity in the juxtamembrane region. Src mediates phosphorylation of SH2 binding motifs of downstream effectors including PI3K either directly or indirectly through activation of a separate kinase. SH2-mediated binding of PI3K to PDGFR-β initiates the PI3K-Akt pathway.

1.4 HSV INFECTION INDUCED ACTIVATION OF SFKs AND AKT

HSV infection is known to activate a number of signal transducing proteins including p38 (135, 255), JNK (135, 255), SFKs (113), Akt (12) and mTORC1 (237). HSV induced activation of p38 and JNK in T cells and non-lymphocyte cell lines is well established (135, 200, 255). In non-lymphocyte cells ICP27 is responsible for p38 and JNK activation (74), and it has been suggested that this signalling stabilizes the expression of a subset of host mRNA transcripts (31). In T cells this signalling is implicated in enhancing the production of the Th2 cytokine, IL10 (200). HSV infection-induced activation of SFKs, Akt and mTORC1 has not been the subject of much investigation. The HSV encoded signalling modulators activating these kinases, the cellular signalling pathways involved, and the biological consequences of activation have yet to be fully explored.

1.4.1 HSV Infection-Induced Activation of SFKs

Src isolated from HSV infected fibroblasts has a slightly elevated level of *in vitro* activity compared to Src isolated from mock infected cells (113). Consistent with SFK activation, SFKs in an infected cell display a small but distinct decrease in electrophoretic mobility through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and an increase in phosphorylation at the activating tyrosine (113). Three viral encoded proteins

have been implicated in the activation of SFKs. The change in electrophoretic mobility was influenced by the viral kinases US3 and UL13, while the increase in activating phosphorylation required the viral immediate early protein ICP0 (113). ICP0 was also shown to interact with the isolated SH3 domains of the SFKs Src, Yes, Fyn and Fgr suggesting that it directly influences SFK activity (113). The mechanism by which US3, UL13, and ICP0 activate SFKs, any subsequent cellular signalling, and any associated biological functions have yet to be fully explored.

1.4.3 HSV Infection-Induced Activation of Akt and mTORC1

Although HSV is capable of activating Akt and the prototypical Akt downstream target mTORC1; it is not known whether these active kinases constitute a common pathway during HSV infection. In the epithelial cell line, HEp-2, HSV-1 strain F activates Akt early in infection (12). Late in infection Akt activation is suppressed by the viral serine-threonine kinase US3, since activation of Akt is only induced by US3 null mutants of HSV-1 (12). It is possible that US3 negatively regulates Akt by directly phosphorylating cellular proteins that participate in Akt signalling. Any viral or cellular component(s) acting with US3 to suppress Akt activation and those activating Akt in the absence of US3 have yet to be defined.

HSV-1 infection has also been shown to induce activation of the Akt target mTORC1 in primary fibroblasts and several cell lines (237). In these experiments

activation of mTORC1 was measured as a rapamycin sensitive hyperphosphorylation of 4EBP1 (237). Whether HSV-induced Akt signalling enhances mTORC1 activation has yet to be investigated.

1.5 VIRION PROTEIN 11/12

This document describes the HSV-1 tegument protein VP11/12 as a modulator of SFK-PI3K-Akt signalling. VP11/12 is an outer tegument protein which should disperse into the cytoplasm upon viral entry and is then free to translocate to various cellular locations for immediate manipulation of host cell functions (99). VP11/12 is one of the most abundant HSV-1 tegument proteins (260). Despite the implied importance of VP11/12 in HSV infection, VP11/12 lacked a well established function at the onset of my doctoral studies.

VP11/12 was originally named according to its migration relative to other virion proteins in SDS-PAGE. It was named as two proteins (VP11 and VP12) since it sometimes forms a doublet of proteins at the molecular weights of ca. 87 and 93 kDa (207). Subsequently, VP11/12 was shown to be one protein encoded by the open reading frame UL46 (260).

Transient transfection assays originally suggested that VP11/12 enhances α gene transcription in collaboration with VP16 and suppresses α gene transcription in the absence of VP16 (96, 133). Consistent with this hypothesis, VP11/12 and VP16 have been shown to physically interact (96, 234). However,

the role of VP11/12 in modulating immediate early gene expression during infection was brought into question upon construction of viral mutants bearing null mutations in the UL46 open reading frame. The VP11/12 null virus is viable in cultured cells and displays no detectable defect in α gene expression (261). Thus, the primary function of VP11/12 during infection had yet to be discovered.

Tegument proteins like VP11/12 are thought to guide the capsid through the various stages of virion assembly. Studies of the US3 protein have established that this protein enhances primary envelopment (145, 179, 245). Other tegument proteins are also thought to navigate capsids through virion assembly, based on observed interactions between capsids and the tegument protein. For example, VP11/12, UL37 and vhs associate with capsid in the nucleus, suggesting a role in the early stages of virion assembly (21, 175). As well, VP16 associates with capsid found between the outer and inner nuclear membranes, suggesting a role in the nuclear egress of capsids (150).

Some evidence suggests that VP11/12 may guide capsids through virion assembly in the cytoplasm. The bulk of VP11/12 localizes to the perinuclear cytoplasmic foci thought to be sites of virion assembly (96, 154, 243); VP11/12 has been shown to rapidly translocate in the cytoplasm (243); and VP11/12 displays strong interactions with capsids *in vitro* (148). Collectively, these observations suggest that VP11/12 may accompany capsids to various sites of assembly in the cytoplasm. Importantly, VP11/12 is not essential for virus

replication in cell culture (261). Therefore any role for VP11/12 in virion assembly must be compensated by some other viral or cellular protein.

The sub-cellular localization of VP11/12 suggests that VP11/12 serves a function at the plasma membrane or other cellular membranes. Subcellular fractionation experiments indicate that VP11/12 associates with membranes in the infected cell but not within mature virions (148), suggesting that membrane association is reversible and occurs before the final stages of virion assembly. There is also evidence suggesting that VP11/12 delivered by the infecting virion localizes to the plasma membrane shortly following viral entry (243). This suggests that VP11/12 may function at membranes, either upon viral entry or at later stages prior to virion assembly.

This document outlines an investigation into the function of the HSV-1 tegument protein VP11/12. Our studies began with the discovery that VP11/12 is heavily tyrosine phosphorylated in B, T and NK lymphocytes. From this initial observation we found that the lymphocyte specific SFK Lck enhances tyrosine phosphorylation of VP11/12 in the context of an infection or a transfection, suggesting that VP11/12 is a substrate of Lck or a kinase downstream of Lck (Chapter 3). We discovered that HSV-1 infection activates Lck, and that this activation strictly requires VP11/12. Furthermore, we demonstrated an interaction between VP11/12 and Lck during infection which suggests that VP11/12 activates Lck directly (Chapter 4). Finally, we investigated signalling downstream of VP11/12-Lck interactions. We found that VP11/12 interacts with PI3K and that

VP11/12 is required to induce SFK-dependent signalling through the PI3K-Akt signalling axis. Interestingly, VP11/12 is not required for infection-induced activation of mTORC1, implying that mTORC1 signalling is independent of the PI3K-Akt module in the context of HSV-1 infection (Chapter 5).

CHAPTER 2: Materials and Methods

2.1 Cells and Viruses

NK-92 cells (ATCC) and 721.221 B cells (98) were a gift from Dr. Debby Burshtyn (University of Alberta, Edmonton, AB). NK-92 cells were grown in α minimal essential medium (αMEM Gibco) supplemented with 12.5% horse serum (Sigma), 12.5% fetal bovine serum (FBS) (PAA laboratories Inc.), 0.2 mM inositol (Sigma), 0.1 mM 2-mercaptoethanol (BDH), 0.02 mM folic acid (Sigma), 2 mM l-glutamine (Gibco), 20μg/mL gentamycin(Gibco) and 100U/mL IL-2 (Hoffmann-LaRoche Inc.). 721.221 B cells were maintained in Iscove's modified Dulbecco's medium with $NaHCO_3$ and without l-glutamine (Sigma) supplemented with 10% FBS, 4mM l-glutamine and 100U/mL penicillin/streptomycin (Gibco).

Jurkat 6.8 and JCAM1.6 (69) were donated by Dr. Hanne Ostergaard and were originally from ATCC. Jurkat and JCAM cells were maintained in RPMI 1640 medium with l-glutamine (Gibco) supplemented with 10% FBS, 200 mM l-glutamine, 100 mM sodium pyruvate (Gibco) and 100U/mL penicillin/streptomycin.

Human embryonic lung (HEL) fibroblasts, human U2OS osteosarcoma cells, and Vero cells were obtained from ATCC. HEL fibroblasts and U2OS cells

were maintained in Dulbecco's modified eagle's medium (DMEM, Gibco) supplemented with 10% FBS, 100 mM sodium pyruvate and 100 U/mL penicillin/streptomycin, while Vero cells (obtained from ATCC) were maintained in DMEM supplemented with 5% FBS and 100 U/mL penicillin/streptomycin.

$CD8^+$ T cells were kindly donated by Dr. Chris Bleakley (University of Alberta, Edmonton, AB). $CD8^+$ T cells were derived from human peripheral blood lymphocytes, isolated as described previously (6) and selected based on CD8 expression using the RosetteSep human $CD8^+$ T cell enrichment cocktail (Stem Cell Technologies). The $CD8^+$ T cells were maintained as described previously for unsorted human peripheral blood lymphocytes (6).

Cells were kept at 37°C and 5% CO_2 in a humidified chamber except during viral stock preparations. Most virus stocks were prepared from infected Vero cells maintained at 34°C and 5% CO_2 in 199 medium supplemented with 5% FBS and 100 U/mL penicillin/streptomycin. For experiments involving the n212 virus and its parental strain KOS, viruses were prepared from infected U20S cells.

The HSV-1 strain KOS ICP0 mutant n212 was a kind gift from Dr. Priscilla Schaffer. HSV-1 strain F and the F-derived mutants R7356 ($UL13^-$), R7041 ($US3^-$), and R7306 (US3 repair virus) were generously provided by Dr. Bernard Roizman (University of Chicago, Chicago, IL). The HSV-1 KOS recombinants, GHSV-UL46 (243), and KOS-G (139) have been described previously.

2.2 Virus Infection

Jurkat and JCAM cells were directly infected at a multiplicity of infection (MOI) of 10 plaque forming units (PFU) per cell in a small volume of serum free RPMI (4 million cells/200 μL), at 37^{o}C for 1 hour. HEL and Vero were similarly directly infected at an MOI of 10 in a small volume of serum free DMEM (1 million cells/500 μL), at 37^{o}C for 1 hour. Infected cells were harvested at 4-20 hours post-infection (hpi), as indicated in the figure legend. Where indicated, the PI3K inhibitors Wortmannin (Sigma-Aldrich) and Ly294002 (Sigma-Aldrich); the Src-family kinase inhibitor PP2 (Calbiochem); and the inactive analogue of Ly294002, Ly303511 (EMD Chemicals) were added to the infection at a final concentration of 1 μM, 20μM, 10μM and 20 μM, respectively.

In some experiments lymphocytes were infected indirectly by co-culture with infected HEL fibroblasts. HEL fibroblasts were infected at an MOI of 10 PFU/cell and the infection was allowed to proceed for 12 hours. Infected cells were then washed with phosphate buffered saline (PBS). Lymphocyctes were added to the fibroblasts at a 1:1 ratio unless otherwise stated, and the culture was incubated for 3-4 hours. Where indicated, the Src-family kinase inhibitor PP2 (Calbiochem) was added to a final concentration of 10 μM during the co-culture period. The lymphocytes were then collected and for most experiments they were used directly for a lytic assay or western blot analysis. In the analysis of SFK

activation in infected CD8+ T cells, the lymphocytes were cultured for an additional 5 hours prior to western blot analysis.

2.3 Construction of UL46 Null HSV

The HSV-1 bacterial artificial chromosome (BAC), KOS-37, a gift from Dr. David Leib (University of Washington, St. Louis, MO), was mutated at the UL46 locus (67). Mutagenesis was performed using a form of bacterial recombination called recombineering (reviewed in (32)) according to the public protocol (http://web.ncifcrf.gov/research/brb/protocal.aspx). Specifically, *E. coli* SW102, which contains a lambda prophage tightly controlled by a temperature sensitive repressor was used (239). SW102 also contains a deletion of the galK gene in an otherwise intact galactose operon. When provided in trans galK allows for galactose metabolism and consequently positive selection in the presence of galactose and negative selection in the presence of the galactose derivative, 2-deoxy-galactose (DOG).

The UL46 open reading frame (ORF) of the KOS-37 BAC was initially replaced by the galK gene (ΔUL46galK). GalK was then precisely removed to form a seamless deletion of UL46 (ΔUL46) or was repaired by replacement of the UL46 ORF (RUL46). For the construction of ΔUL46galK a linear galK expression cassette was amplified by PCR of pgalK (239) using primers consisting of sequence homologous to 50 bp immediately upstream of the UL46

start codon and 54 bp immediately downstream of the UL46 stop codon (Primer 1 and Primer 2; see Table 1).

For the construction of ΔUL46, a linear oligo homologous to the regions immediately flanking UL46, 50 bp immediately upstream and 54 bp downstream of the UL46 ORF was used (oligo 1; Table 1).

To construct RUL46, a linear cassette of the UL46 ORF was amplified from KOS-37, primed 52 bp upstream of the UL46 start codon and 57 bp downstream of the UL46 stop codon (Primer 3 and 4; Table 1).

Recombineering was performed as follows. KOS-37 was electroporated into SW102, and transformants were grown at 32^{o}C in the presence of 12.5μg/mL chloramphenicol. Recombination machinery was induced by growth in liquid culture at 42^{o}C for 15-30 minutes. Following induction, the appropriate linear cassette was transformed into cells by electroporation. The galK expression cassette was used first to replace the UL46 ORF of KOS-37(ΔUL46galK) and positive selection was performed by growth on M63 minimal agar containing galactose and subcloning on MacConkey agar (Becton Dickinson) with galactose. Subsequently transformation of the linear oligo replaced galK to form a seamless deletion of the UL46 ORF (ΔUL46), and, in parallel, the UL46 cassette replaced galK (RUL46). ΔUL46 and RUL46 recombination events were negatively selected on M63 minimal media containing 2-DOG (Sigma). All transformed candidate colonies were screened by PCR of BAC DNA with primers internal to

the galK ORF (Primer 5 and 6; Table 1), primers internal to the UL46 ORF (Primer 7 and 8; Table 1) and primers flanking the UL46 ORF (Primer 9 and 10; Table 1). BACs were isolated using a large construct purification kit (Qiagen). Viruses were derived by transfection of BACs into the Cre-Vero epithelial cell line using lipofectamine (Invitrogen) (previously described (67)).

2.4 Plasmid Construction

A plasmid containing the HSV-1 KOS UL46 ORF (pUL46) was made by inserting into the EcoRI and XhoI restriction sites of pcDNA3.1, PCR product spanning from 10bp upstream of the UL46 start codon to 30bp downstream of the stop codon (Invitrogen) (Primer 11 and 12; Table 1).

A plasmid containing flanking sequences of HSV-1 KOS UL46 ORF (pL1R1) was made by first inserting a PCR product beginning 18 bp downstream of the UL46 stop codon and spanning 455 bp of downstream sequence (Primer 13 and 14; Table 1) into the XbaI and NotI restriction sites of pBluescript SK+ (Fermentas). A PCR product of 350bp immediately upstream of the UL46 start codon was then inserted into the resulting plasmid at the NotI and SacI restriction sites (Primer 15 and 16; Table 1).

For construction of pGUL46 a GFP tag was inserted into pUL46 10 amino acids from the C terminus of VP11/12 (at K709), to give a VP11/12-GFP fusion protein analogous to that of GHSV-UL46 (243). First, a portion of the multiple

cloning site of pUL46, containing a HindIII restriction site was removed by excision of the NheI-EcoRI fragment. GFP cDNA was amplified from pEGFP-N1 (Clontech) (Primer 17 and 18; Table 1). GFP cDNA was then inserted into the remaining HindIII restriction site within UL46.

Table 1. Table of Primers

	5' → 3' (capitals indicate HSV sequence)
Primer 1	GACAAACAGGGGGAAAGGGGCGTGGTCTAGCGACGGCAGCACGGGCGGAGGCGT-cctgttgacaattaatcatcggca
Primer 2	GGACGCGGCATAACTCCGACCGGCGGGTCCCGACCGAACGGGCGTCACC-tcagcactgtcctgctcctt
Oligo 1	GACAAACAGGGGGAAAGGGGCGTGGTCTAGCGACGGCAGCACGGGCGGAGGCGT/GGTGACGCCCGTTCGGTCGGGACCCGCCGGTCGGAGTTATGCCGCGTCC
Primer 3	GTCGACAAACAGGGGGAAAG
Primer 4	CTGGACGCGGCATAACTC
Primer 5	ccattgtcgcacatgaaaac
Primer 6	tccagtgaagcggaagaact
Primer 7	GGACTCAGCCGGTGACATAC
Primer 8	AAGTACCTGCAGACGGTGGT
Primer 9	GACAAACAGGGGGAAAGG
Primer 10	GGACGCGGCATAACTCC
Primer 11	ttgaattcGGGCGTCACCATGCAG
Primer 12	tttctcgagGTCTAGCGACGGCAGCAC
Primer 13	tctctagaGTCCAGTCGGCAAGATCCTC
Primer 14	ttttgcggccgCACGCCTCCGCCCGTGCTGC
Primer 15	gtttgcggccgcGGTGACGCCCGTTCGGT
Primer 16	ttgagctcCGGAGCACGTGGATCTGC
Primer 17	gtcaaagcttAAGATGGTGAGCAAGGGCGA
Primer 18	cttgaagcttCTTGTACAGCTCGTCC

2.5 Southern Blot

BAC DNA and viral DNA of each UL46 mutant was digested by NotI, BamHI, ApaLI, HincII and MluI. For probes, the UL46 ORF from pUL46 was isolated by digestion with EcoRI and XhoI, the galK ORF was isolated from pgalK by digestion with EcoRI and NotI and the 5'and 3' flanking regions of UL46 were isolated from pL1R1 by digestion with XbaI, NotI and SacI. P^{32} labeling of the UL46, galK and flank probes and southern blotting of the BAC and viral DNA were performed as previously described (139).

2.6 Lytic Assay

Target 721.221 B cells were labeled with 100 μCi $Na_2{}^{51}CrO_4$ (PerkinElmer) for 1.5-3 hours at 37^{o}C and were then washed and resuspended in medium for NK-92 cells. NK-92 and 721.221 cells were co-incubated at the indicated effector: target (E:T) ratios for 4-5 hours at 37^{o}C. Radioactivity was measured using a 1450 Wallac Microbeta TriLux liquid scintillation counter. Lysis was calculated as follows: percentage of lysis = (radioactivity of sample (counts per minute; cpm) – radioactivity of minimum target lysis (cpm))/ radioactivity of maximum target lysis (cpm). The radioactivity of minimum target lysis was determined by radioactivity measurements of 721.221 cells in the

absence of NK-92 effectors. The radioactivity of maximum target lysis was measured by radioactivity measurements of 721.221 cells in the presence of 5% IGEPAL CA-630 (Sigma).

2.7 Immunoprecipitation

Cell extracts of 16 million Jurkat or 4 million HEL were prepared at 4^{o}C by a 20 minute incubation in lysis buffer (1% Nonidet, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EGTA, 10mM NaF, 10mM β-glycerol phosphate, 1mM Na_3VO_4, 50mM Tris-HCl pH 7.4) supplemented with a complete protease inhibitor cocktail (Roche). All steps were carried out at 4^{o}C. Cell lysate was cleared of debris by centrifugation at 16, 000 x g for 15 min. The clarified lysate was preabsored to protein G Sepharose during 30 min incubation. The appropriate amount of antibody was bound to protein G Sepharose during 2 hour or overnight incubation in PBS. These conjugates were washed twice with lysis buffer. After preabsorption 100 μL was retained as a lysate control and the remaining lysate was incubated with the protein G Sepharose conjugated antibody and 0.1% BSA for 1-2 hours. Precipitates were washed three times with lysis buffer.

2.8 Western Blot

Cell extracts were collected and cleared of debris as described for immunoprecipitation samples in section 2.7. Cell extracts or immunoprecipitation samples separated by 8%, 10% or 12.5% SDS-PAGE were transferred to a nitrocellulose membrane (Hybond ECL, Ambersham Pharmacia). Specific proteins were detected using HRP-conjugated secondary antibodies (Promega) and an ECL plus detection system (Amersham Biosciences), or were detected and quantified using fluorochome-conjugated secondary antibodies, an Odyssey infrared imager (LI-COR Biosciences), and Odyssey application software v. 1.2. For detection by ECL, membranes were incubated for 1 hour in a 4% bovine serum albumin (BSA) TBS-T (25 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween-20) solution. For detection by the Odyssey infrared imager, Odyssey blocking buffer (LI-COR Biosciences) diluted 1:1 in TBS-T was used in place of BSA TBS-T. Primary antibodies were incubated with the membrane for 1 hour or overnight at 4^{o}C. Secondary antibodies were incubated with the membrane for 1 hour or overnight at 4^{o}C. In some cases membranes were stripped with stripping buffer (62.5 mM Tris pH 6.7, 2% SDS, 0.7% β-mercaptoethanol) for reprobing.

2.9 Antibodies

Immunoprecipitation was performed using anti-phosphotyrosine (4G10, 4 μg, Upstate), anti-GFP (2μL, kind gift of Dr. Luc Berthiaume (University of

Alberta, Edmonton, AB)), anti-Lck (3A5, 1 μg, Santa Cruz Biotechnology Inc.), anti-phospho-Src family (Tyr416) (20 μL, Cell Signalling) anti-p85 (4 μg, Millipore), and anti-rabbit IgG (4 μg, Sigma).

Antibodies used for primary detection by western blot included antibodies directed against phosphotyrosine (1/20,000), anti-UL46 (1/30,000), Lck (1/1000), p85 (1/10,000), GFP (1/3000), VP16 (LP1, 1/32, 0000, provided by Dr. Tony Minson (University of Cambridge, Cambridge, UK)), ICP27 (#1113, 1/2500, Goodwin Institute), and actin (1/1000, Sigma). Antibodies directed against the following were also used for primary detection by western blot (1/1000, Cell Signalling): phospho-Src family (Tyr 416), phospho-Akt (Thr308), phospho-Akt (Ser473), Akt, phospho-p38(Thr180/Tyr182), p38, phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK, phospho-p70 S6 Kinase (Thr389), p70 S6 Kinase , 4E-BP1, phospho-GSK-3β (Ser9) , GSK-3β (27C10). For detection of the Lck protein in immunoprecipitates rabbit IgG TrueBlot (1/1000, eBioscience) and mouse TrueBlot ULTRA (1/1000, eBioscience) secondary antibodies were used. Other secondary antibodies included donkey anti-mouse IR800 (1:15,000, Rockland inc.), goat anti-rabbit Alexa Fluor680 (1:15, 0000, Invitrogen), goat anti-mouse Alexa Fluor680 (1:15, 0000, Invitrogen), goat anti-rabbit HRP (1/3000, Promega), goat anti-mouse HRP (1/5000, Promega), and donkey anti-goat HRP (1/15, 000, Jackson ImmunoResearch).

TCR stimulation was performed by incubation with soluble anti-CD3 (10 μg/mL, OKT3, eBioscience) for 15 minutes at 4^{o}C, followed by incubation with anti-mouse IgG2a (15 μg/mL, Sigma-Aldrich) for 15 minutes at 4^{o}C. Signalling was then initiated through incubation of the cells at 37^{o}C for 10 minutes.

2.10 Transfection

Vero cells were transfected with empty vector pcDNA3.1 (Invitrogen), plasmid for Lck expression (pSX-SR Lck Y505F), plasmid for VP11/12 expression (pUL46) or plasmid for expression of a GFP tagged version of VP11/12 (pUL46-GFP) using Lipofectamine (Invitrogen) according to the manufacturer's instructions. pSX-SR Lck Y505F has been described previously (238), and was kindly donated by Dr. Debby N. Burshtyn (University of Alberta, Edmonton, AB).

CHAPTER 3: Lymphocyte-specific Tyrosine Phosphorylation of VP11/12: a role for the SFK, Lck

This chapter is based on the unique observation of a ca. 90 kDa tyrosine phosphorylated protein in lymphocytes exposed to HSV infected fibroblasts. This original observation was made by Dr. George Zahariadis in the laboratory of Dr. Keith R. Jerome (University of Washington, Seattle, WA). Dr. George Zahariadis contributed to the experimental planning of the work in this chapter. Rosalyn Doepker, and Jessica M. Maciejko executed the experiments planned by Dr. George Zahariadis. Experiments performed by these individuals are indicated in the figure legends. Most of the work in this chapter was published in the Journal of Virology (256).

3.1 RATIONALE

As reviewed in the introduction (section 1.1.5), HSV infects CTL and NK cells by cell to cell spread of the virus (7), and the virion itself is sufficient to inactivate these cells from within so that they are unresponsive to multiple immunological targets (28, 166-168, 199, 251). We aimed to define common changes in T cell and NK cell signalling following exposure to HSV under conditions that allow for killer lymphocyte inactivation. We hypothesized that HSV alters phosphotyrosine mediated signalling in T cells and NK cells to induce lymphocyte inactivation. To address this hypothesis we examined total cellular protein tyrosine phosphorylation in HSV inactivated lymphocytes. As described in this chapter, we unexpectedly discovered tyrosine phosphorylation of the tegument protein VP11/12 in lymphocytes exposed to HSV infected cells and in directly infected lymphocytes. During the course of our research, studies by Sloan *et. al.* highlighted the importance of phosphotyrosine modulation in HSV lymphocyte evasion. Sloan *et. al.* demonstrated that HSV inhibits TCR signalling at or prior to the phosphorylation of LAT, and that HSV induced T cell inactivity is attenuated by phosphotyrosine phosphatase inhibitors (199). Therefore we investigated whether VP11/12 is the component of the HSV-1 virion responsible for attenuating TCR signalling and NK cell cytotoxicity.

3.2 RESULTS

3.2.1 Exposure to HSV Induces Pronounced Tyrosine Phosphorylation of a 90 kDa Protein in Lymphocytes

To broadly examine HSV induced changes in phosphotyrosine mediated signalling we examined protein tyrosine phosphorylation in NK, T, and B cell lines following exposure to HSV-infected fibroblasts under conditions that allow for lymphocyte inactivation (Figure 8). To this end, HEL fibroblasts were mock infected, infected with HSV-1 strain F and infected HSV-2 strain HG52 for 12 hours. Lymphocytes of the $CD4^+$ T cell line, Jurkat, the B cell line, 721.221 and the NK cell line, NK-92 were then exposed to the HEL fibroblasts for 4 hours. Lysates from these cells were analyzed by western blot with an antibody against phosphotyrosine. All three lymphocyte lineages displayed low levels of tyrosine phosphorylated proteins following exposure to mock infected fibroblasts. By contrast, cells of each lineage displayed marked tyrosine phosphorylation of a ca. 90 kDa protein upon exposure to HSV-1 or HSV-2 infected fibroblasts. In some experiments, exposure of Jurkat T cells to HSV-1 and HSV-2 infected fibroblasts also induced the appearance of tyrosine phosphorylated proteins of ca. 35-40 kDa, 55-60 kDa and 60-65 kDa (Figure 8 and Figure 11). However, detection of these phosphoproteins was less consistent. Overall, our result show that cells from three distinct lymphocyte lineages express a ca. 90 kDa tyrosine phosphorylated protein following exposure to HSV infected fibroblasts.

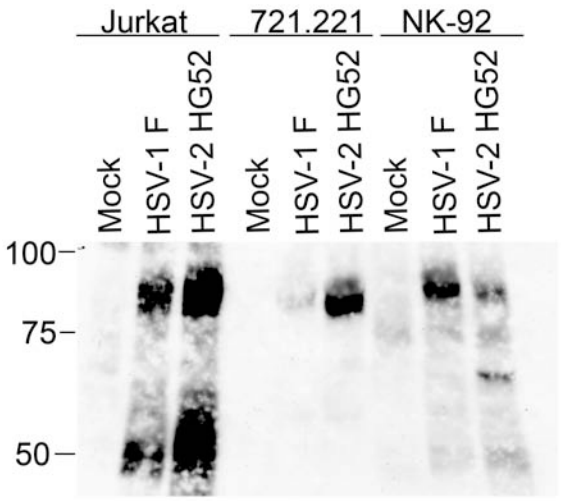

Figure 8. Lymphocytes exposed to HSV display tyrosine phosphorylation of a 90 kDa protein.

HEL fibroblasts were mock-infected or infected by HSV-1 F or HSV-2 HG52 for 12 hours. Lymphocyte cell lines (Jurkat T cells, 721.221 B cells and NK-92 NK cells) were incubated with mock and HSV infected fibroblasts as indicated for 4 hours. Protein was collected from the non-adherent lymphocytes and tyrosine phosphorylated proteins were detected by western blot analysis using an anti-phosphotyrosine antibody. * Experiment performed by G. Zahariadis and R.C. Doepker * Experiment repeated by G. Zahariadis and J. Maciejko

3.2.2 Jurkat T cells Phosphorylate VP11/12

We aimed to identify the 90 kDa tyrosine phosphorylated protein present in lymphocytes exposed to HSV infected fibroblasts, under the assumption that this phosphoprotein may play a role in HSV-mediated lymphocyte inactivation. Jurkat T cells and NK-92 cells were exposed to HSV-2 HG52 infected fibroblasts (as described for Figure 8). The ca. 90 kDa and 60 kDa phosphoprotein bands were isolated by anti-phosphotyrosine immunoprecipitation and separation through SDS-PAGE. Mass spectrometry was used to make a crude identification of the ca. 90 and 60 kDa phosphoproteins. The HSV-2 HG52 protein, VP11/12 was among the proteins identified in both the 60 and 90 kDa fractions (unpublished data of Rosalyn Doepker).

VP11/12 was originally identified as two protein species of ca. 87 kDa and 93 kDa (207). Therefore, it was possible that our 90 kDa phosphoprotein of interest is full length VP11/12, while the 60 kDa phosphoprotein observed less frequently is a degraded, cleaved or truncated product of VP11/12. To corroborate the initial identification of VP11/12 as a tyrosine phosphorylated protein, Jurkat T cells were exposed to infected fibroblasts and were then subjected to immuprecipitation/western blot analysis of phosphotyrosine and VP11/12 (Figure 9). Jurkat T cells were exposed to HEL fibroblasts infected by HSV-2 HG52, HSV-1 KOS, and the HSV-1 KOS derivative GHSV-UL46 which

encodes a VP11/12-GFP fusion protein (243). Immunoprecipitation of anti-phosphotyrosine (pY) and western blot analysis using antisera reactive to HSV-2 VP11/12 (96) demonstrated detectable levels of phospho-VP11/12 (ca. 90 kDa) in Jurkat cells exposed to HSV-1 KOS and HSV-2 HG52 and detectable levels of phospho-VP11/12-GFP (ca. 120 kDa) in Jurkat cells exposed to GHSV-UL46 (Figure 9A). Similarly, anti-GFP immunoprecipitates of Jurkat T cells exposed to GHSV-UL46 yielded a large amount of tyrosine phosphorylated protein corresponding to the molecular weight of VP11/12-GFP (ca. 120 kDa) (Figure 9B). Interestingly, immunoprecipitation of VP11/12-GFP also pulled down tyrosine phosphorylated proteins at molecular weights below that of VP11/12-GFP (< 120 kDa). Further investigation is required to determine if these proteins are associated with VP11/12 during infection or are the product of VP11/12 degradation, truncation or cleavage. Collectively, this data strongly suggests that Jurkat T cells exposed to HSV infected fibroblasts acquire a tyrosine phosphorylated form of VP11/12.

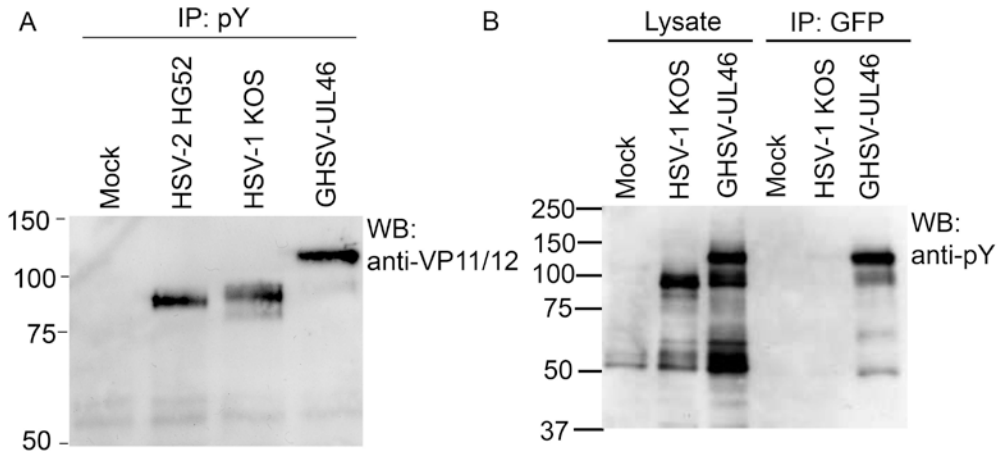

Figure 9. The HSV tegument protein VP11/12 is tyrosine phosphorylated in Jurkat T cells.

Jurkat T cells were exposed to mock-infected HEL fibroblasts or HEL fibroblasts infected by HSV-2 HG52, HSV-1 KOS, or a KOS derivative containing a GFP-tagged VP11/12, GHSV-UL46. Protein was collected from the Jurkat T cells. A) Western blot of anti-UL46 reactive proteins in anti-phosphotyrosine immunoprecipitate. *Similar results seen Jurkat cells exposed to HSV-1 KOS and HSV-2 HG52 and tested by G. Zahariadis and R.C. Doepker. B) Western blot of anti-phosphotyrosine reactive proteins in cell lysate and anti-GFP immunoprecipitate. Results confirmed through reciprocal immunoprecipitation using anti-phosphotyrosine followed by anti-GFP western blot.

3.2.3 Construction of VP11/12 Null HSV

To investigate whether VP11/12 represents all or a fraction of the ca. 90 kDa anti-phosphotyrosine band that we observed in lymphocytes exposed to HSV, we constructed a VP11/12 null mutant of HSV. To this end the VP11/12 open reading frame of the UL46 gene was precisely removed from the HSV-1 BAC KOS-37 using a bacterial homologous recombination system known as recombineering (reviewed in (32) and outlined in detail in chapter 2). Briefly, the bacterial selection gene galK was inserted in place of the VP11/12 open reading frame (ΔUL46galK), and recombinants were positively selected. The galK gene was then replaced with either a seamless deletion of the VP11/12 open reading fame (ΔUL46) or a repaired open reading frame (RUL46), and recombinants were negatively selected. Candidate colonies were initially identified through a PCR screen for the desired mutation, and a restriction enzyme digest was performed to assay the BAC for any large and undesired insertions or deletions.

The accuracy of recombination at the UL46 ORF in the mutant BACs was tested by Southern blot of infected cell DNA (Figure 10). Southern blots were probed with DNA homologous to the VP11/12 open reading frame (UL46), the galK open reading frame (galK) and the sequence flanking UL46 (Flanks ; 350 bp 5' and 455 bp 3' of the UL46 ORF). DNA of infected cells was digested with BamHI or MluI, which cut within the VP11/12 ORF or the galK gene,

respectively (Probes and cut sites illustrated in Figure 10A). The wild type KOS-37 infected cell DNA bound the flanks and UL46 probes to demonstrate the 8055 bp and 717 bp fragments generated by digestion with BamHI, as well as the 3027 bp fragment generated by digestion with MluI (Figure 10B). Consistent with replacement of the VP11/12 ORF by the galK gene, ΔUL46galK infected cell DNA gave rise to a single 7933 bp fragment upon digestion with BamHI and two fragments of 1317 bp and 784 bp upon digestion with MluI, all of which bound the flanks and the galK probes, but not the UL46 probe. Digestion of ΔUL46 infected cell DNA by both BamHI and MluI generated a single fragment which only bound the flanks probe and not the galK or UL46 probes. Furthermore, digestion of the ΔUL46 sample by MluI generated an 870 bp fragment which is consistent with a seamless deletion of the VP11/12 ORF (2157 bp) when compared to the fragment in the KOS-37 sample (3027 bp). The Southern blot of RUL46 infected cell DNA is identical to that of wild type KOS-37 indicating efficient repair of the VP11/12 ORF. Collectively, this data indicates that the galK expression cassette was inserted specifically into the VP11/12 ORF in ΔUL46galK and was subsequently removed to form a seamless deletion (ΔUL46) or a repaired UL46 ORF (RUL46).

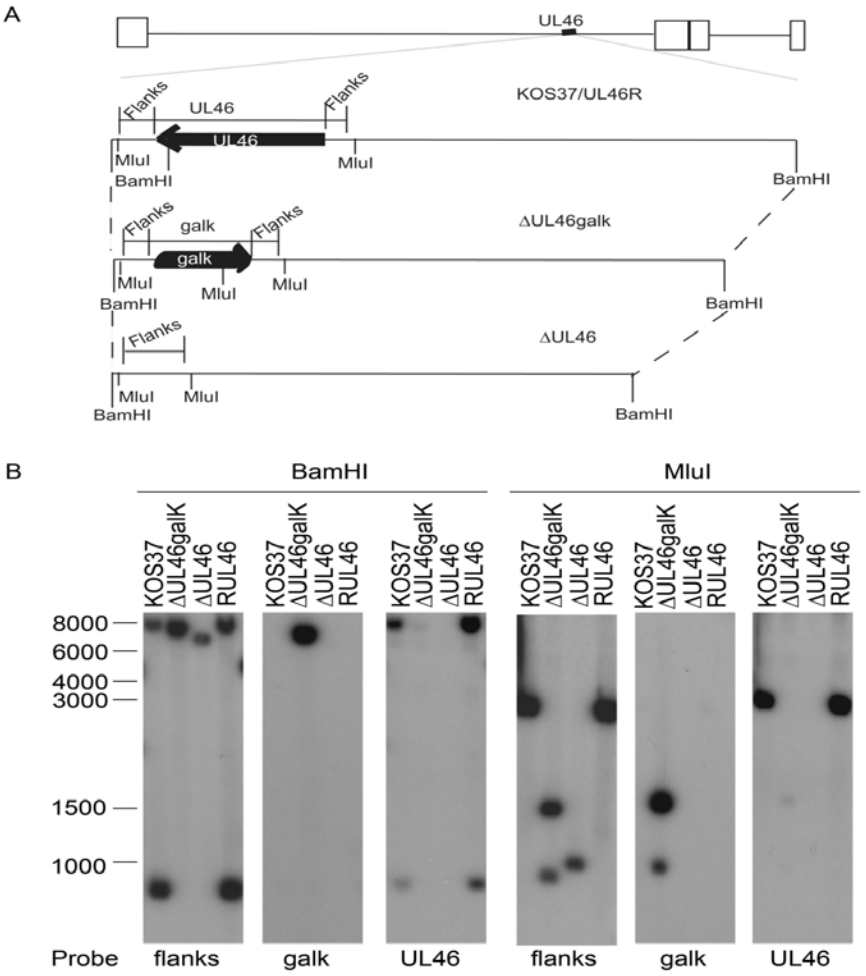

Figure 10. Validation of UL46 targeted recombination.

A) A schematic of a southern blot comparison of the wild-type HSV-1 KOS37, UL46 null mutant (ΔUL46galk and ΔUL46) and UL46 repair virus (UL46R) genomes. DNA cleavage by BamHI, or MluI and annealment of probes for the UL46 ORF, the galK ORF or the sequence flanking UL46 (less than 500 bp 5' and 3') are illustrated. B) Southern blot (outlined in A) of DNA from wild-type HSV-1 KOS37, ΔUL46galk, ΔUL46 and UL46R infected Vero. This experiment was repeated with DNA isolated from the corresponding bacterial clone. *Viruses constructed by M.J. Wagner and J. Maciejko

3.2.4 VP11/12 is the Main Component of the 90 kDa Tyrosine Phosphorylated Protein(s) in Jurkat T cells Exposed to HSV

The UL46 null viruses (ΔUL46galK, ΔUL46) lack the genetic sequence for VP11/12 expression. We wanted to confirm the desired mutation at the protein level by examining VP11/12 expression in infected HEL and Vero, using antisera reactive to HSV-2 VP11/12 (96)(Figure 11A). Examining lysates from infected HEL or Vero (data not shown), we found expression of VP11/12 in fibroblasts infected by HSV-2 HG52, wild-type KOS-37 and RUL46, but not in fibroblasts that were infected by ΔUL46galK and ΔUL46. Importantly, the absence of VP11/12 in ΔUL46galK and ΔUL46 is not due to a general impairment of HSV protein expression, since the VP16 protein which has similar expression kinetics to VP11/12 is found at comparable levels in all HSV infected samples. This confirms that VP11/12 is efficiently expressed in RUL46 and is not expressed in ΔUL46galK or ΔUL46.

We further wanted to assess whether VP11/12 accounted for all or part of the 90 kDa tyrosine phosphorylated protein(s) that we observe in lymphocytes exposed to HSV. Jurkat T cells and NK-92 cells were exposed to HEL fibroblasts infected by HSV-2 HG52, wild-type KOS-37, VP11/12 null viruses, ΔUL46galk and ΔUL46, and the repaired virus, RUL46. Exposure to HSV-2 HG52, HSV-1 KOS37 and RUL46 resulted in the appearance of a ca. 90 kDa tyrosine phosphorylated protein in Jurkat cells (Figure 11B) and NK-92 cells (data not

shown). By contrast, this phosphoprotein was notably absent in cells exposed to VP11/12 null virus (ΔUL46galK and ΔUL46). Interestingly, KOS-37 and RUL46 samples displayed bands corresponding to lower molecular weight phosphoproteins (ca. 35-40 kDa, 55-60 kDa, and 60-65 kDa) which were weak or absent in ΔUL46galK and ΔUL46 samples. These phosphoproteins may be VP11/12 degradation, truncation or cleavage products or they may be indicative of VP11/12 directly or indirectly promoting cellular tyrosine phosphorylation events. Since the 60 kDa tyrosine phosphorylated protein(s) was predicted by mass spectrometry to contain VP11/12, the absence of the ca. 60-65 kDa band does argue for the existence of VP11/12 cleavage or degradation products. Along with the data from Figure 9, this experiment indicates that VP11/12 is tyrosine phosphorylated in lymphocytes exposed to HSV infected fibroblasts.

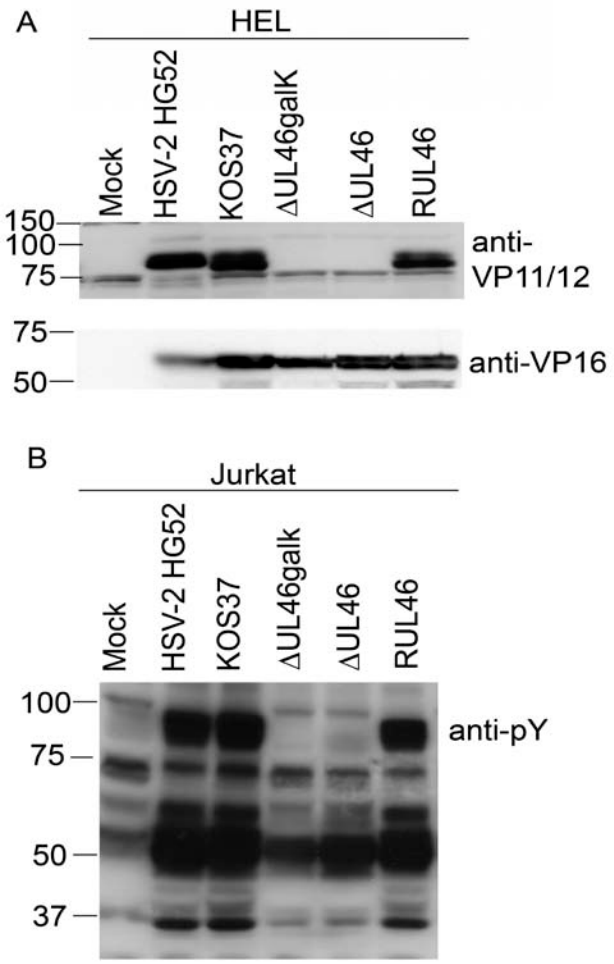

Figure 11. VP11/12 is the main component of the 90kDa phosphoprotein in HSV exposed Jurkat cells.

A) Western blot for the indicated proteins in HEL infected by KOS37, ΔUL46galK, ΔUL46 and RUL46. Similar results were obtained in infected Vero cells. B) Western blot of phosphotyrosine in Jurkat cells exposed to mock-infected HEL fibroblasts and HEL fibroblasts infected by HSV-2 HG52, HSV-1 KOS 37, UL46 null viruses ΔUL46galk, or ΔUL46 and UL46 repair virus UL46R. This experiment was repeated three times (twice using direct infection). * Experiment in B performed by M.J. Wagner and J. Maciejko

3.2.5 Tyrosine Phosphorylation of VP11/12 is Enhanced in Lymphocytes

Our data indicates that multiple lineages of lymphocytes tyrosine phosphorylate the viral protein VP11/12 of HSV-1 and HSV-2. Previously, as much as eight tyrosine phosphorylated proteins were observed in HSV-1 infected HeLa epithelial cells and 3T3 mouse embryonic fibroblasts (13). We wanted to determine if tyrosine phosphorylated VP11/12 is present in multiple infected cell types or if VP11/12 tyrosine phosphorylation is a lymphocyte-specific event.

We observed tyrosine phosphorylated VP11/12 in lymphocytes following co-cultured with HSV infected HEL fibroblasts. Therefore, we first asked whether VP11/12 was tyrosine phosphorylated in the HEL fibroblasts of these co-cultures. HEL cells were mock infected, infected by wild-type HSV-1 KOS and infected by GHSV-UL46. Jurkat T cells and NK -92 cells were exposed to some of the fibroblast cultures for 4 hours (at 12-16 hpi). Tyrosine phosphorylated proteins in HEL fibroblasts (16 hpi) were compared to that of Jurkat T cells and NK-92. The ca. 90 kDa VP11/12 and the ca. 120 kDa VP11/12-GFP displayed a much higher level of tyrosine phosphorylation in Jurkat T cells and NK-92 cells (Figure 12A). Upon over-exposure of the western blots we were able to observe anti-phosphotyrosine band(s) corresponding to the molecular weight of VP11/12 and VP11/12-GFP in fibroblast samples, but the intensity of these bands was

much weaker than that observed in lymphocyte samples. This result suggests that VP11/12 tyrosine phosphorylation is more prominent in lymphocytes.

To further examine the extent to which VP11/12 tyrosine phosphorylation is lymphocyte specific, we examined this phosphorylation in equivalent levels of infected cell protein from Jurkat T cells, Vero and HeLa epithelial cells (Figure 12B). Jurkat T cells, unlike primary T cells and some lymphocyte cell lines, can be directly infected by HSV. Thus, each cell type was directly infected by HSV-1 KOS and GHSV-UL46 for 9 hours. Anti-phosphotyrosine bands corresponding to VP11/12 (ca. 90 kDa) and VP11/12-GFP (ca. 120 kDa) were apparent in the samples of Vero and Jurkat cells; however, the intensity of these bands was much greater in the Jurkat T cell sample. Collectively, the data in Figure 12 suggests that a greater number of VP11/12 molecules or a greater number of VP11/12 tyrosine residues are phosphorylated in lymphocytes.

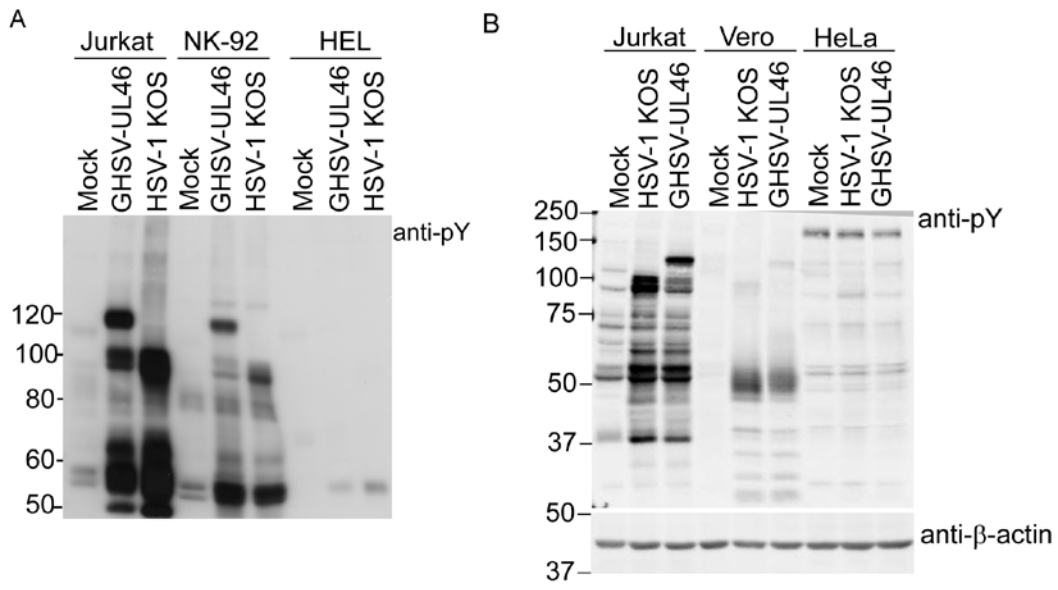

Figure 12. Tyrosine phosphorylation of VP11/12 is enhanced in lymphocytes.

A) HEL fibroblasts were mock infected, infected by HSV-1 KOS or the KOS mutant containing a GFP-tagged VP11/12, GHSV-UL46 for 12 hours then some cultures were exposed to Jurkat or NK-92 cells for 6 hours. HEL fibroblasts at 18 hours post infection and in Jurkat and NK-92 cells exposed to infected HEL fibroblasts at 12-18 hours post infection were analyzed for phosphotyrosine by western blot. *Experiment in A performed by R. Doepker and G. Zahariadis and repeated twice, using Jurkat and HEL cells. B) Jurkat, Vero and HeLa cells were directly infected by cell-free HSV-1 KOS and GHSV-UL46 for 9 hours. Phosphotyrosine was detected by western blot in infected cell lysates normalized for protein content.

3.2.6 Phosphorylation of VP11/12 Requires the Lymphocyte Specific SFK, Lck

The cell type-specific nature of VP11/12 tyrosine phosphorylation suggested the involvement of a lymphocyte-specific kinase. As one possibility we examined the lymphocyte specific SFK Lck, which is known to play a critical role in TCR signalling. To determine if Lck is necessary for VP11/12 tyrosine phosphorylation, we assessed phosphorylation in the Lck deficient Jurkat derivative cell line, JCAM1.6 and in Jurkat cells exposed to the SFK specific inhibitor PP2 (Figure 13A). Marked tyrosine phosphorylation of VP11/12 was evident in untreated Jurkat T cells exposed to HSV-1 KOS and HSV-2 HG52 infected HEL fibroblasts. By contrast, JCAM1.6 cells and Jurkat T cells exposed to PP2 demonstrated low levels of tyrosine phosphorylated VP11/12 despite consistent expression of VP11/12 between samples (Figure 13B). In separate experiments VP11/12 expression was decreased in JCAM1.6 samples or in samples where Jurkat T cells had been exposed to PP2 (data not shown). However, decreases in total VP11/12 were slight compared to the decrease in VP11/12 tyrosine phosphorylation. This suggests that the activity of the SFK Lck is required for VP11/12 tyrosine phosphorylation.

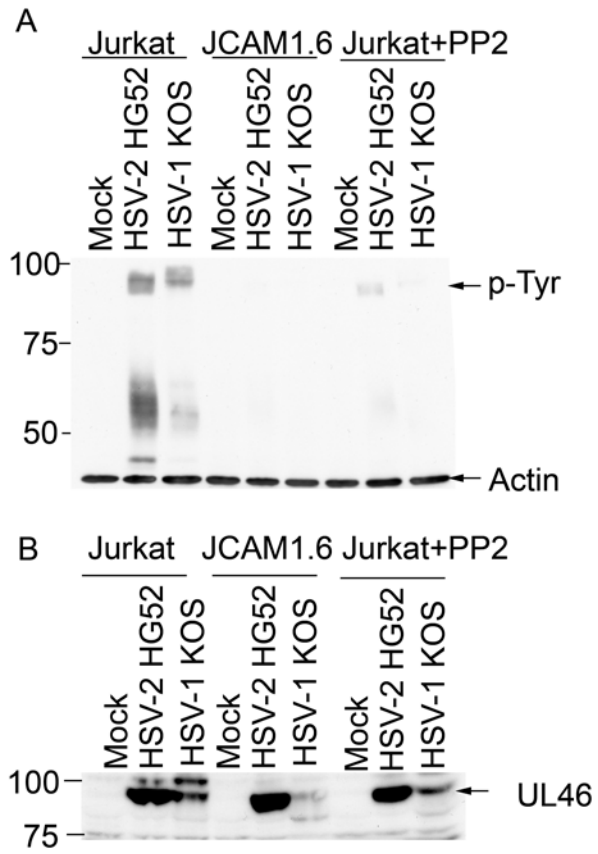

Figure 13. Lck and SFK activity are necessary for tyrosine phosphorylation of VP11/12.

Jurkat T cells and cells of the Lck deficient Jurkat derivative cell line, JCAM1.6, were exposed to mock, HSV-2 HG52 and HSV-1 KOS infected HEL fibroblasts in the presence or absence of the SFK inhibitor, PP2, as indicated. Phosphotyrosine and the indicated proteins were detected by western blot. *Experiment performed by R. Doepker and repeated once.

3.2.7 Lck Enhances VP11/12 Tyrosine Phosphorylation

The preceding experiment suggests that VP11/12 may be a substrate of Lck or a tyrosine kinase activated by Lck. To further investigate this possibility we asked if overexpression of Lck is sufficient to induce tyrosine phosphorylation of VP11/12 in Vero cells (Figure 14). Cells were transfected with plasmid(s) encoding VP11/12, a VP11/12-GFP fusion protein (VP11/12-GFP), and constitutively active Lck (Y505F Lck). Constitutively active Lck encoding a Y505F mutation was used in this experiment to ensure activation of Lck in the absence of the leukocyte specific phosphatase and activator of Lck, CD45 (104, 164, 191). It is well known that Lck Y505F enhances tyrosine phosphorylation of cellular proteins, including the autophosphorylation of Lck (5, 128). Accordingly, all cells expressing Lck demonstrated a tyrosine phosphorylated protein of ca. 56 kDa that we assume is Lck. Unique anti-phosphotyrosine reactive bands were observed in samples that contained Lck and VP11/12 or VP11/12-GFP compared to samples containing Lck alone. This included evidence of a tyrosine phosphorylated VP11/12 seen as a doublet of ca. 90 kDa, and tyrosine phosphorylated VP11/12-GFP which resolved as a single band of ca. 120 kDa. Samples expressing VP11/12 or VP11/12-GFP alone also contained anti-phosphotyrosine reactive bands corresponding to ca. 90 kDa and ca. 120 kDa, respectively. However, the intensity of these bands was much weaker in the

absence of Lck. Thus, Lck greatly enhanced tyrosine phosphorylation of VP11/12 and VP11/12-GFP.

The unique anti-phosphotyrosine reactive bands observed in samples containing Lck and VP11/12 or VP11/12-GFP also included proteins of lower molecular weight. This included the 56-60 kDa protein that we assume to be phospho-Lck as well as unidentified proteins of ca. 60 kDa (seen in both samples) and ca. 30 kDa and 40 kDa (seen in samples containing VP11/12). Analysis of GFP in the VP11/12-GFP containing samples indicates that VP11/12-GFP gives rise to degradation products and suggests that the low molecular weight phosphoproteins may be the result of VP11/12 and VP11/12-GFP degradation. Alternatively, these phosphoproteins may be indicative of cellular tyrosine phosphorylation events induced by exogenous expression of Lck and VP11/12. Additional experiments are required to identify these proteins and the purpose of their tyrosine phosphorylation. Overall, this data shows that Lck is sufficient to enhance VP11/12 tyrosine phosphorylation *in vivo*.

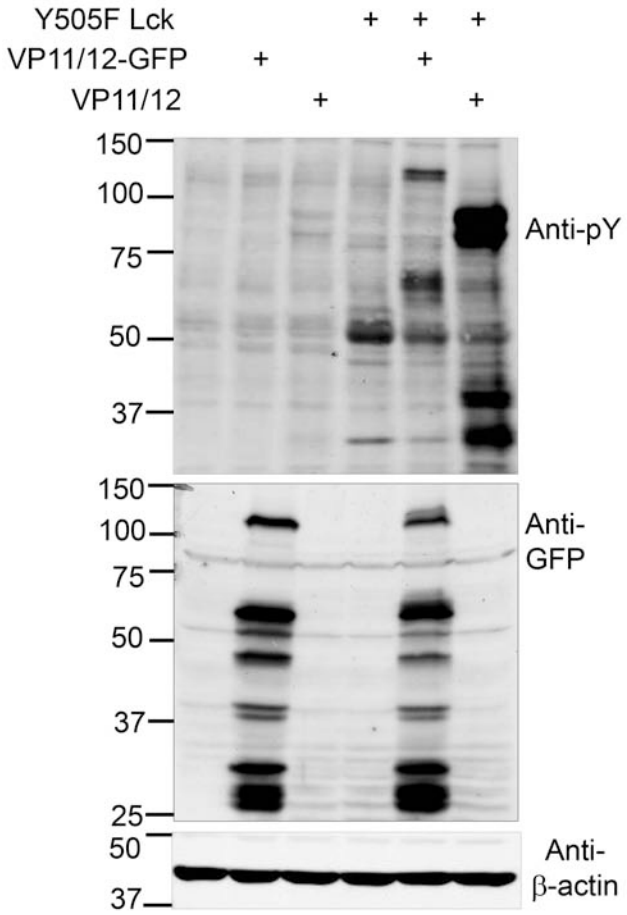

Figure 14. Lck is sufficient to enhance tyrosine phosphorylation of VP11/12.

Vero cells were transfected with plasmids encoding constitutively active Lck, (Lck Y502F), VP11/12 (UL46) and a VP11/12 GFP fusion protein (UL46-GFP), as indicated. Phosphotyrosine, GFP and β-actin were detected by western blot of cell lysates collected 48 hours post-transfection. Similar results were seen in one repeat of this experiment.

3.2.8 VP11/12 is Not Required for HSV Induced Inhibition of TCR Signalling or NK cell Cytotoxicity

As reviewed in the introduction (section 1.1.5), CTL and NK cells are functionally inactivated by HSV. As a first step in examining the role of VP11/12 in lymphocyte inactivation, we asked if VP11/12 is required for the HSV-induced blockade to TCR signalling in Jurkat T cells (199). Jurkat T cells were directly infected by wild-type KOS-37, the UL46 null mutant ΔUL46 and the UL46-repaired virus RUL46. At 8 hpi, half of the cells from each infected culture were stimulated by cross-linking the TCR with an anti-CD3 antibody (OKT-3). To assay for efficient TCR signalling, phosphorylation of the signalling effector ERK1/2 was measured by Western blot (Figure 15a). As previously report by Sloan *et. al.* (199), HSV infection severely impairs ERK1/2 phosphorylation in response to TCR ligation. Infection by wild type KOS-37, the repaired virus, RUL46 and the VP11/12 null virus ΔUL46 efficiently attenuated ERK1/2 phosphorylation (Figure 15A and B). Thus, VP11/12 is not required for the HSV-induced blockade of TCR signalling in Jurkat T cells.

We further investigated whether or not VP11/12 is required for HSVinfection-induced attenuation of NK cell killing. NK-92 cells were exposed to mock and HSV infected HEL fibroblasts. NK-92 cytotoxicity toward a ^{51}Cr-labelled MHC I-low B cell line 721.221 was then measured in a ^{51}Cr release assay

(Figure 16). Compared to mock exposure, exposure to KOS37, ΔUL46galK, ΔUL46, and RUL46 inhibited NK-92 killing of 721.221 cells. Therefore VP11/12 is not necessary for HSV-induced inactivation of the NK-92 cell line.

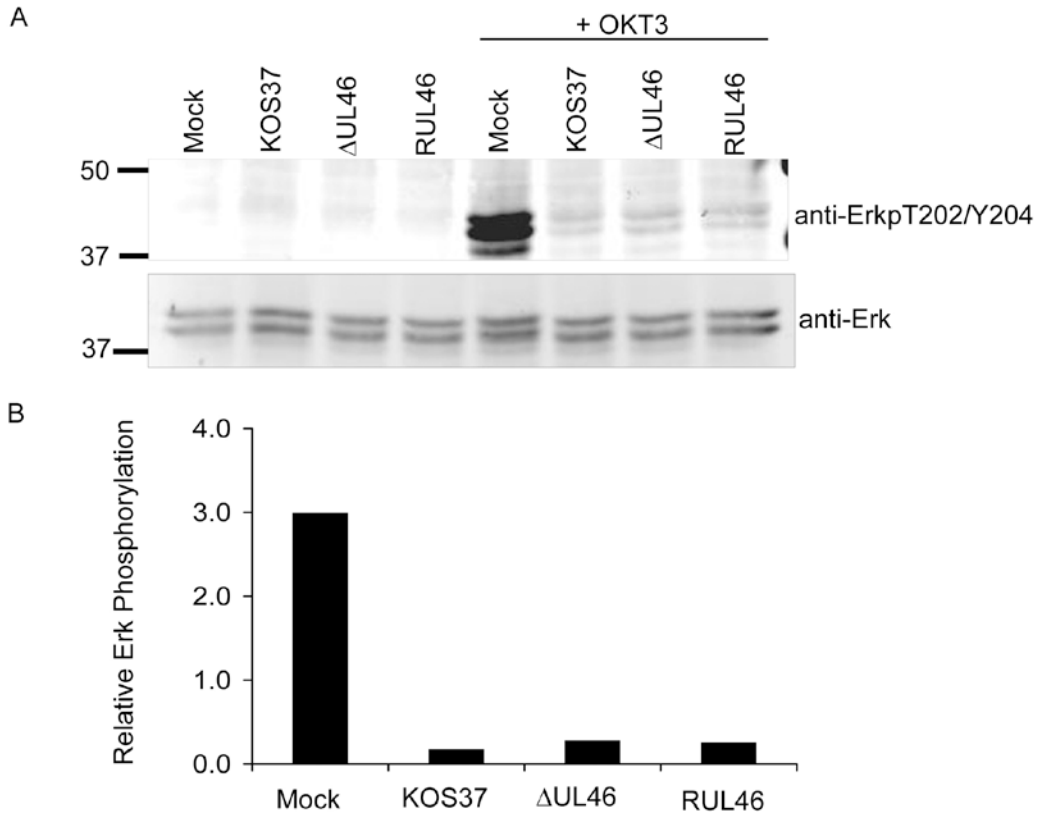

Figure 15. VP11/12 is not necessary for HSV-mediated inhibition of TCR signalling.

Jurkat T cells were exposed to mock, KOS37, ΔUL46 and RUL46 infected HEL fibroblasts. TCR signalling was then stimulated with anti-CD3 (OKT3) as indicated. Signalling was measured by western blot analysis of the active form of Erk1/2. A) Western blot detection of the active, phosphorylated form of Erk 1/2 (anti-ErkpT202/Y204) and total Erk1/2 (anti-Erk). B) The infrared signal obtained with the anti-ErkpT202/Y204 antibody in (A) was divided by the signal obtained with the anti-Erk antibody. This experiment was repeated once.

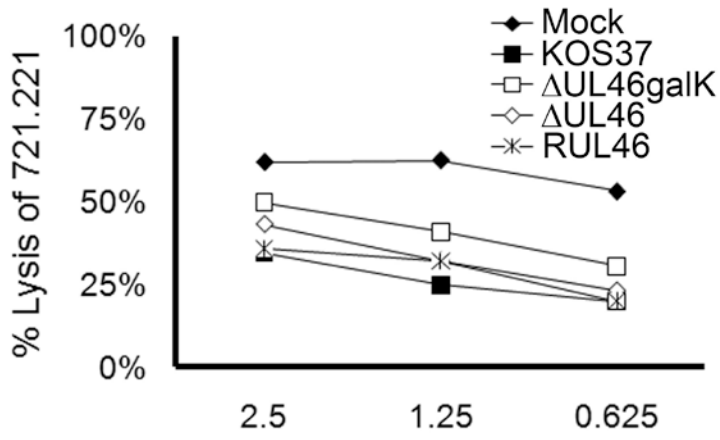

Figure 16. VP11/12 is not necessary for HSV-mediated inhibition of NK cell cytotoxicity.

NK-92 cells were exposed to mock, KOS37, ΔUL46galK, ΔUL46 and RUL46 infected HEL fibroblasts and were then removed and assessed for their ability to kill chromium labeled target cells (B cell lymphoma cell line, 721.221) during a 4 hour incubation. NK-92 cytotoxicity is expressed as a percentage of lysis, determined by measurement of chromium release. Mean values of quadruplicate samples ± standard error is shown. *Experiment performed by M.J. Wagner and J. Maciejko. This experiment was repeated once, using Mock , KOS37, ΔUL46galK and RUL46 viruses.

3.2.9 VP11/12 is Not Required for Immediate Early Gene Expression

Previously, transient transfection studies suggested that VP11/12 enhances VP16 transactivation of α gene transcription (reviewed in section 1.5) (96, 133). However, VP11/12 null virus failed to demonstrate altered α gene expression in a non-lymphoid cell line (260, 261). The tyrosine phosphorylation of VP11/12 that we observe in lymphocytes might influence VP11/12 function. To test whether tyrosine phosphorylated VP11/12 enhances α gene expression during infection, we infected Jurkat cells with KOS-37, ΔUL46 and RUL46 at MOI of 1, 10 and 100, and we compared the level of the immediate-early protein ICP27 between samples (Figure 17A). The relative level of ICP27 (normalized to β-actin) in ΔUL46 was comparable to that of KOS37 and RUL46 (Figure17B), indicating that VP11/12 has little if any effect on the expression of this α gene under the conditions of our assay.

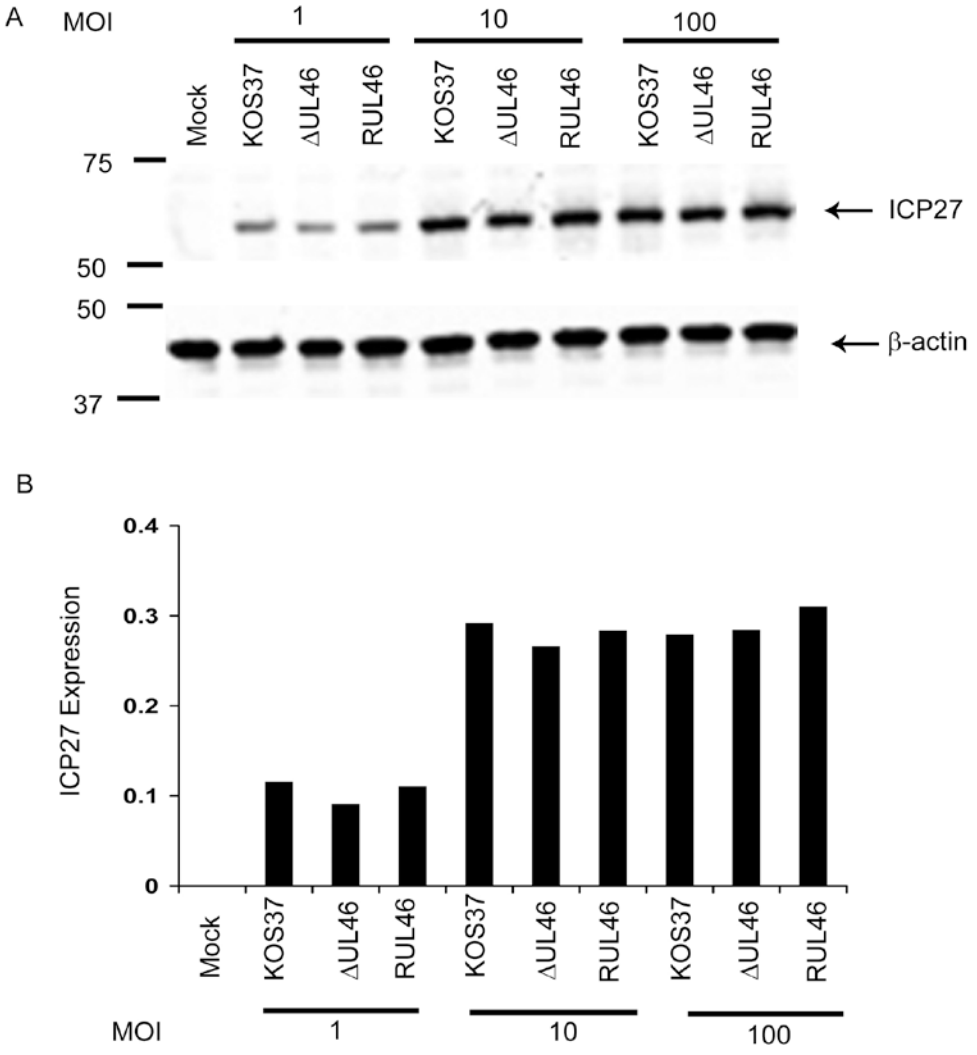

Figure 17. VP11/12 is not necessary for immediate early protein expression.

Jurkat T cells were directly infected with KOS37, ΔUL46 and RUL46 at an MOI of 0.1, 1, or 10 as indicated. At 4 hpi the accumulation of the immediate early protein ICP27 was assessed by western blot. A) Western blot of the immediate early HSV protein ICP27 and protein β-Actin. B) The infrared signal from the anti-ICP27 antibody quantitated relative to that of the anti-β-Actin antibody. Similar results seen in four replicate experiments.

3.3 SUMMARY OF RESULTS

The results outlined in this chapter suggest that the HSV tegument protein VP11/12 is tyrosine phosphorylated by Lck directly or is tyrosine phosphorylated by a kinase activated downstream of Lck during lymphocyte infection.

In this study, we observe protein(s) of ca. 90 kDa which form an anti-phosphotyrosine reactive band in western blot analysis of multiple lymphocyte lineages (B, T and NK cells) following exposure to HSV-1 and HSV-2 infected fibroblasts (Figure 8). Our data show that VP11/12 is tyrosine phosphorylated during HSV infection (Figure 9) and identify VP11/12 as the major component of this band (Figure 11). Tyrosine phosphorylation of VP11/12 is enhanced in an infected lymphocyte cell line (Jurkat) compared to infected fibroblast (HEL) or epithelial (Vero) cell lines (Figure 12). Consistent with the lymphocyte specific nature of tyrosine phosphorylation, the lymphocyte specific SFK, Lck enhances VP11/12 tyrosine phosphorylation in the context of HSV infection (Figure 13), and VP11/12/Lck transfection (Figure 14).

Lck plays an integral role in lymphocyte signalling, however VP11/12 is not necessary for infection-induced functional inactivation of NK cells (Figure 16) or abrogation of TCR signalling (Figure 15). We conclude that Lck mediated phosphorylation of VP11/12 may inhibit immune related signalling, however if this is the case then other HSV proteins must also impede these pathways to compensate for VP11/12 in its absence.

VP11/12 was previously reported to augment VP16-mediated expression of α genes in transfection based assays (96, 133). However, VP11/12 null virus expresses normal levels of α proteins in non-lymphoid cells (261). We find that VP11/12 is also dispensable for proper expression of α gene products during infection of Jurkat T cells (Figure 17). Therefore the molecular and cellular functions of tyrosine phosphorylated VP11/12 have yet to be determined.

CHAPTER 4: HSV-1 Requires VP11/12 to Induce Phosphorylation of the Activating Tyrosine (Y394) of Lck in T cells

The work in this chapter was published in the Journal of Virology (236).

4.1 RATIONALE

In cellular signalling Lck activity is stimulated by activated receptors including the TCR (203), the IL-2 receptor (53), and ephrin-A (79, 80). In chapter 3, the data suggested that VP11/12 is a substrate of Lck or a kinase activated downstream of Lck. However, tyrosine phosphorylation of VP11/12 occurred in the absence of any known Lck stimulus. Therefore, we hypothesized that HSV-1 infection activates Lck.

HSV encodes four viral proteins that may activate Lck during infection. ICP0, US3, and UL13 are candidate activators of Lck since these proteins were previously implicated in the activation of non-lymphocyte SFKs (113). VP11/12 may also activate Lck. As reviewed in the introduction (section 1.2.1), substrates of SFKs can activate their cognate kinase when they contain high affinity SH2 binding motifs (121). Analysis of the VP11/12 sequence indicates the presence of the preferred SFK SH2 binding sequence YEEI (156, 204). This sequence is predicted to bind, "open" and activate SFKs. Thus, the second objective of my PhD studies was to evaluate the activation status of Lck during infection, and (if Lck is active) define virus encoded modulators of Lck activity.

4.2 RESULTS

4.2.1 HSV Infection Enhances Phosphorylation of the Activation Loop Tyrosine of a 56 kDa SFK in Jurkat T Cells

As reviewed in the introduction (section 1.2.1), the kinase activity of Lck and other SFKs is regulated by the phosphorylation status of two key tyrosine residues (183). Dephosphorylation of the C-terminal inhibitory tyrosine (Y505 in Lck) partially activates Lck (5, 128), while phosphorylation of the activation loop tyrosine (Y394 in Lck) serves as a signature of the activated state and stabilizes the active conformation irrespective of the phosphorylation status of Y505 (2, 37, 73, 128). We examined phosphorylation of these regulatory tyrosine residues during HSV-1 infection. Jurkat T cells were mock infected or directly infected with HSV-1strain F for 9 hours. Cell lysates were then analyzed by western blot using antibodies that detect total Lck and Lck phosphorylated at the two regulatory tyrosine residues. We used an antibody specific for Lck phosphorylated at Y505 to evaluate the phosphorylation status of the C-terminal inhibitory tyrosine residue (anti-LckPY505, Figure 18A). An antibody specific for the phosphorylated activation loop tyrosine residue of Lck (Y394) was not available. Therefore, we used an antibody that detects any SFK phosphorylated at the activating tyrosine (phospho-Y416 for the prototypical SFK, Src) to examine this residue (anti-active SFK, Figure 18B).

Two forms of Lck with apparent molecular weights of ca. 56 and 60 kDa were detected using the anti-Lck antibody in both infected and uninfected cells (Figure 18 A and B). Although HSV-1 infection had no significant effect on total Lck levels, in some but not all experiments the proportion of Lck displaying reduced mobility (ca. 60 kDa) was markedly increased in infected cells (Figure 18 A and B). A similar shift from ~56 kDa to ~60 kDa has been described previously following T cell activation through stimulation by mitogens, interleukin-2, antigen presenting cells or anti-TCR antibodies (83, 129, 232, 233). The precise modification(s) responsible for this change in apparent molecular weight has not been defined, but it has been linked to a number of phosphorylation events including compulsory phosphorylation of two serine residues in the N-terminal region (83, 125, 205, 232, 233, 241, 244). Using two channel infrared detection, we found a perfect overlap in the migration patterns of Lck and the proteins detected by the anti-phospho Lck Y505 and the anti-active SFK antibodies, with a ~56 and ~60 kDa species reacting with each antibody. Thus, the mobility shift from ~56 to 60 kDa is not caused by phosphorylation of either of these tyrosine residues.

We quantified the 56 and 60 kDa signals obtained with all three antibodies to determine the effects of infection on phosphorylation of the inhibitory and activating tyrosines (Figure 18C and D). The ratio of phospho-Y505 Lck relative to total Lck did not change, indicating that HSV infection does not alter the fraction of Lck phosphorylated at the inhibitory Y505 (Figure 18C). By contrast,

the 56/60 kDa SFK phosphorylated on the activation loop tyrosine increased in HSV-1 infected samples relative to mock infected samples (Figure 18D). This increase was apparent when the active SFK signal was quantified relative to total Lck (Figure 18 D) or β-Actin (data not shown). These data suggested that one or more SFKs that precisely co-migrate with Lck display a greater than four-fold increase in the levels of phosphorylation of the activation loop tyrosine during HSV-1 infection. However, additional experiments were required to positively identify Lck as the active 56/60 kDa phospho-SFK detected in these experiments.

Virions inactivated by UV irradiation were unable to enhance activating phosphorylation of the ca. 56/60 kDa SFK and time course analysis indicates that enhancement is only apparent at 6 hpi, and reaches a maximum at ca. 9 hpi (Figure 19A and B). Thus, virion components are unable to induce a measureable increase in the activating phosphorylation of SFKs and the onset of a measurable increase in SFK activation at 6 hpi suggests the involvement of newly synthesized β or γ viral gene products.

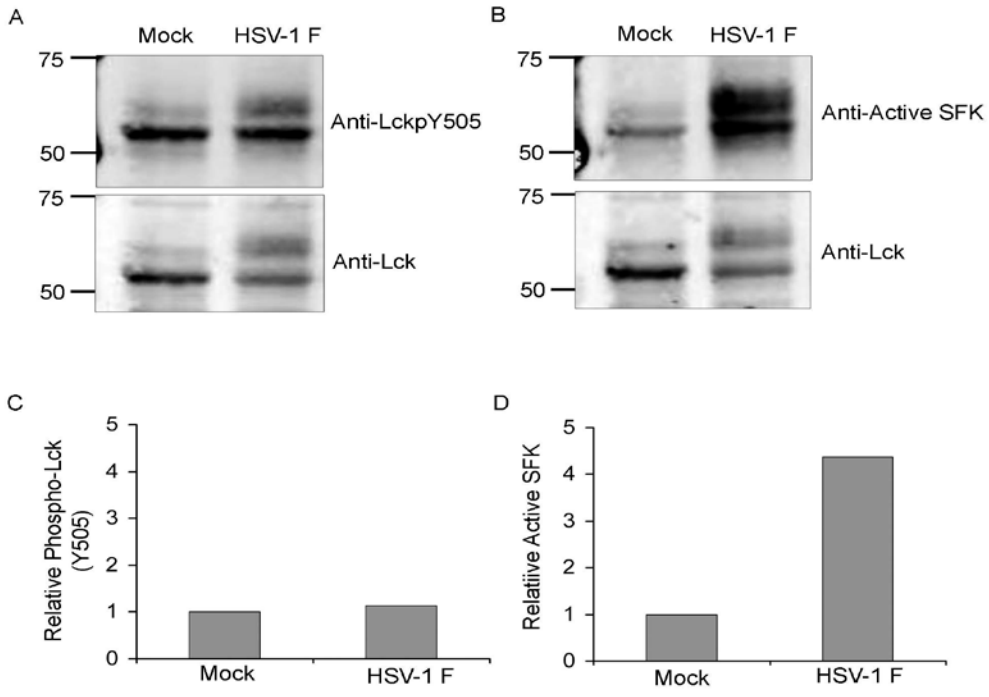

Figure 18. HSV-1 infection enhances phosphorylation of the activation loop tyrosine of a 56/60 kDa SFK in Jurkat T cells.

Jurkat T cells were mock infected or infected with HSV-1F for 9 hours. Cell lysates were analyzed by western blotting with the indicated antibodies, using two channel infrared detection. A) Western blot analysis using antibodies that detect Lck phosphorylated at the inhibitory tyrosine residue (Y505) and total Lck. B) Western blot using antibodies that detect SKFs phosphorylated on the activating tyrosine residue (the equivalent of phospho-Y416 of Src, anti-active SFK) and total Lck. C) The infrared signal obtained with the anti-Lck phospho-Y505 antibody in (A) was divided by the signal obtained with the anti-Lck antibody, and the ratios were normalized to yield a value of one for the mock-infected sample. D) The infrared signal obtained with the anti-active SFK antibody in (B) was divided by the signal obtained with the anti-Lck antibody, and the ratios were normalized to yield a value of one for the mock-infected sample. Experiment repeated once.

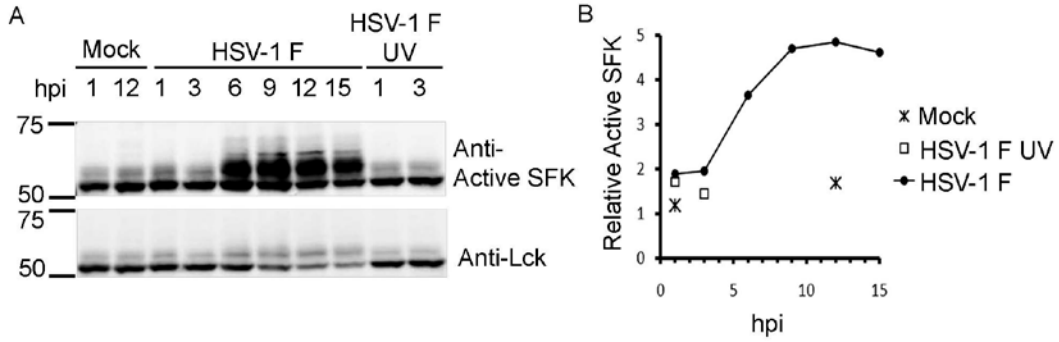

Figure 19. Time course analysis of infection induced activation of a 56/60 kDa SFK.

Jurkat cells were mock infected, infected by HSV-1 F and infected by UV-inactivated HSV-1F. Cell lysate was then collected at the indicated times (hours post-infection, hpi). A) Western blot analysis using the indicated antibodies. B) The signals obtained with the anti- active SFK antibody were divided by those obtained with anti-Lck.

4.2.2 HSV increases the fraction of Lck phosphorylated at Y394

The data displayed in figure 18 demonstrated that HSV infection enhances phosphorylation of the activation loop tyrosine of an SFK that co-migrates with Lck. To directly address whether Lck represents some or all of this 56/60 kDa phospho-SFK population, we examined the effects of HSV-1 infection on SFKs in the Lck-deficient Jurkat derivative, JCAM1.6 (Figure 20A). The active SFK detected in Jurkat cells was not present in JCAM1.6 cells. To verify comparable viral gene expression in both cell lines, we examined accumulation of the immediate early gene product ICP27 and the late gene product VP16. Robust expression of these proteins was observed following infection of both Jurkat and JCAM1.6 cells, indicating that Lck is not required for viral protein synthesis. These data indicated that Lck is the main component of the 56/60 kDa SFK population detected by the anti-active SFK antibody in Jurkat cells.

To further corroborate that Lck undergoes phosphorylation at the activating tyrosine (Y394) during HSV infection of Jurkat cells, we asked if Lck reacts with the anti-active SFK antibody. SFK members phosphorylated at the activating tyrosine were immunoprecipitated using the anti-active SFK antibody and the immunoprecipitates were then analyzed by western blot using the anti-Lck

antibody (Figure 20B). The results demonstrated that Lck was precipitated by the anti-active SFK antibody. Conversely, the anti-Lck antibody precipitated an SFK that reacts with anti-active SFK antibody (Figure 20C). Thus, Lck was phosphorylated at Y394 in all samples tested. As well, the proportion of Lck that reacts with the anti-active SFK antibody appeared to be higher in HSV-1F infected samples compared to mock infected samples (Figure 20B and C). Taken in combination, these data indicate that HSV-1 infection triggers an increase in the fraction of Lck phosphorylated at the activation loop tyrosine, residue Y394.

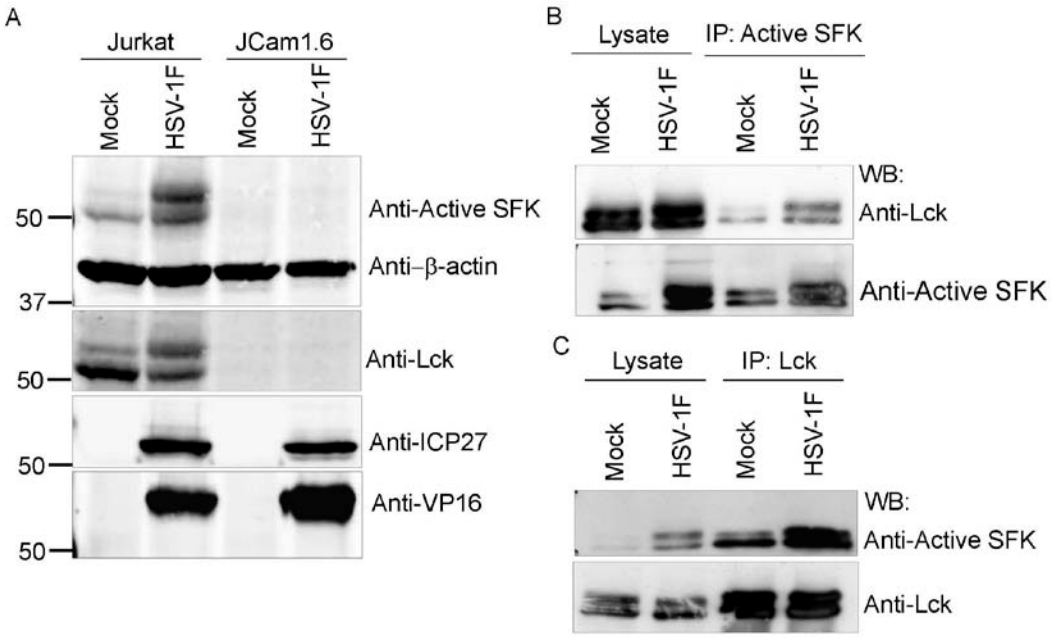

Figure 20. HSV enhances phosphorylation of Lck residue Y394.

Jurkat and JCAM1.6 cells were mock infected or infected with HSV-1F for 9 hours and cell extracts were prepared. A) Western blot analysis using the indicated antibodies. Experiment repeated once. B) Western blot analysis of immunoprecipitates obtained with the anti-active SFK antibody. Lysate controls were analyzed directly. Experiment repeated twice. C) Western blot analysis of immunoprecipitates obtained with the anti-Lck antibody. Lysate controls were analyzed directly. Experiment repeated three times.

4.2.3 ICP0 is Not Required For Enhanced Phosphorylation of Lck at Y394

As described in the introduction (section 1.4), a previous report presented evidence that the HSV-1 immediate early protein ICP0 is required for virus induced phosphorylation of the activating tyrosine residue of SFKs in HEL fibroblasts and a human kidney cell line, HEK 293 cells (113). This conclusion was based on assays of an ICP0 null virus, R7910. However, in these assays, cells infected with R7910 displayed reduced expression of the viral thymidine kinase (113), raising the possibility that the failure of this mutant to stimulate phosphorylation of SFKs may have been due to the reduced expression of one or more viral proteins other than ICP0, rather than loss of ICP0 *per se*. The defect in viral gene expression displayed by ICP0 null viruses can be overcome by using a sufficiently high MOI (184, 215). To differentiate the effects of the ICP0 protein from any symptoms of ICP0 null virus attenuation, we examined phosphorylation of the activation loop tyrosine of Lck during ICP0 null virus infections of increasing MOI.

Jurkat cells were mock infected or infected with the parental wild type HSV-1 KOS and the ICP0 null mutant n212 at an MOI of 1, 10 and 100 (Figure 21a). At all MOIs tested, n212 produced less of the immediate early gene product ICP27 and the late gene product VP16 compared to the wild type KOS virus. Similarly, the signal obtained with the anti-active SFK antibody was reduced in the n212 infected samples relative to KOS at each MOI. However, n212 infected

samples displayed an MOI dependent increase in both viral protein expression (ICP27 and VP16) and the anti-active SFK signal (Figure 21B). This indicates that enhanced phosphorylation of Lck at Y394 is not strictly dependent on the presence of ICP0, but rather correlates with production of viral proteins during n212 infection.

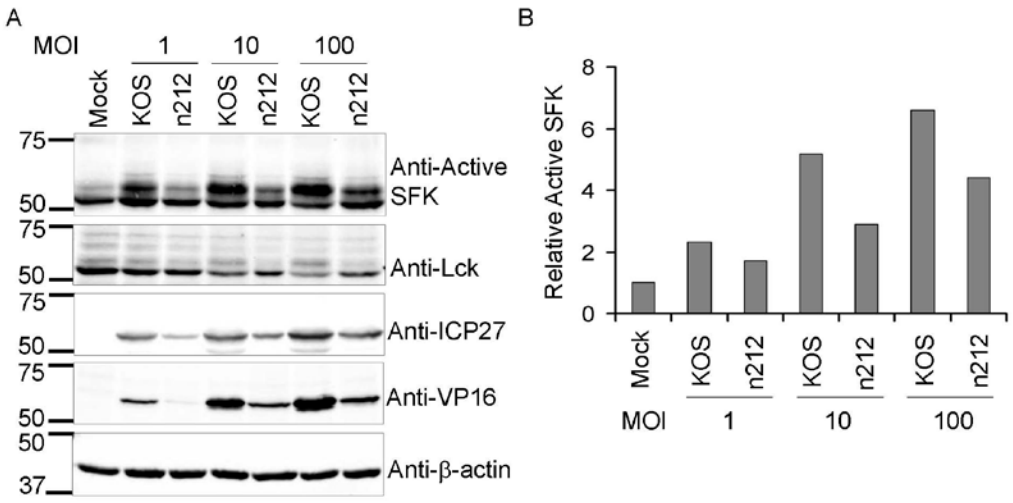

Figure 21. ICP0 is not directly required for enhanced phosphorylation of Lck Y394.

Jurkat cells were infected with HSV-1 KOS and the ICP0 mutant n212 at the indicated multiplicities of infection (PFU/cell) and cell lysates were prepared for western blotting. A) Western blot analysis using the indicated antibodies. B) The signals obtained with the anti- active SFK antibody were divided by those obtained with anti-Lck, and the ratios were normalized to yield a value of one for the mock-infected sample. This experiment was repeated once.

4.2.4 UL13 May Play a Role in Post-translational Modifications of Lck

Like ICP0, the viral serine/threonine kinases US3 and UL13 have been implicated in SFK activation during HSV-1 infection (113). Specifically these two proteins were shown to be necessary for the post-translational modifications that result in an increase in the apparent molecular weight of SFKs in fibroblasts. We therefore examined HSV-1 mutants null for US3 or UL13 to assess the role of these proteins in HSV-1 induced post-translational modifications of Lck in Jurkat T cells.

The UL13 (R7356) and US3 (R7041) null viruses were comparable to the parental HSV-1 F and the US3-repaired virus (R7306) in their ability to enhance phosphorylation of the activating tyrosine of Lck, Y394 (Figure 22A and B). The US3 null virus and its US3-repaired derivative were also indistinguishable from HSV-1F in their ability to enhance the fraction of Lck displaying reduced electrophoretic mobility (most readily visible using the anti-active SFK antibody, Figure 22A). The UL13 null virus, R7356, also gave rise to a slowly migrating form of Lck, but the apparent molecular weight of this form appeared to be less than ~60 kDa (Figure 22A), implying that some of the posttranslational modifications that give rise to the ~60 kDa form of Lck were impaired during this infection. The UL13 null HSV also demonstrated defective expression of the late viral protein VP16 (Figure 22A), which is likely a consequence of the cell-type dependent attenuation of UL13 null virus infection that has been described

previously (172). Further experimentation is therefore required to determine whether altered post-translational modifications stem directly from loss of UL13, or indirectly from reduced expression of another viral protein. Overall, these data indicated that UL13 and US3 are not strictly required for the virus-induced posttranslational modifications that give rise to a high molecular weight form of phospho-Y394Lck.

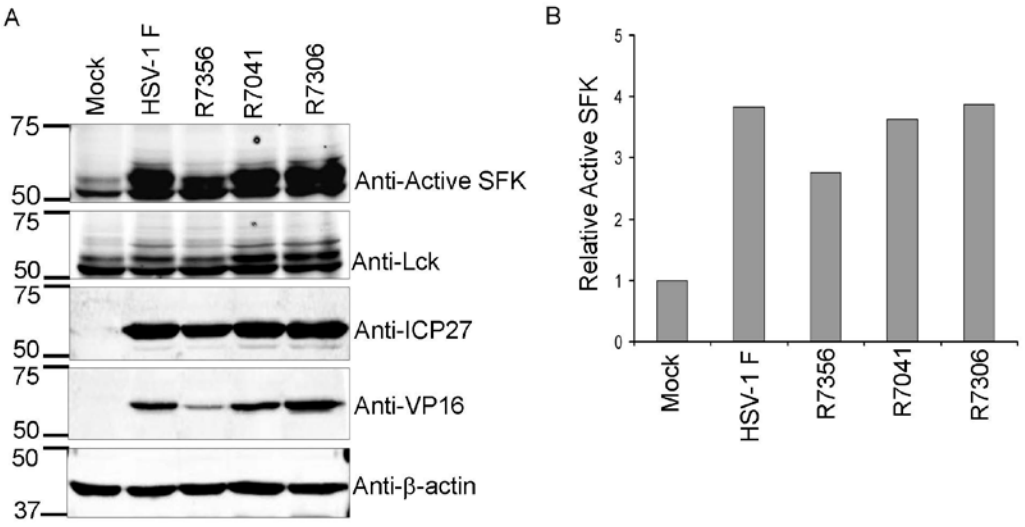

Figure 22. US3 and UL13 are not required for enhanced phosphorylation of Lck Y394.

Jurkat cells were infected with HSV-1 F, the UL13 null mutant R7356, the US3 null mutant R7041, and the US3 repaired virus R7306. A) Western blot analysis of cell lysate using the indicated antibodies. B) The signals obtained with the anti- active SFK antibody were divided by those obtained with anti-Lck, and the ratios were normalized to yield a value of one for the mock-infected sample. This experiment was repeated once.

4.2.5 VP11/12 is Necessary for Enhanced Phosphorylation of Lck Residue Y394

Tyrosine phosphorylation of HSV VP11/12 in T lymphocytes is largely dependent on Lck (Chapter 3). Because some SFK substrates can activate the cognate kinase, we asked whether VP11/12 is necessary for enhanced phosphorylation of Lck residue Y394 during HSV infection (Figure 23). Jurkat cells infected with two mutants lacking VP11/12, ΔUL46galK and ΔUL46, displayed severely reduced levels of phospho-Y394 Lck compared to cells infected with the parental HSV-1 KOS37 or the VP11/12-repaired virus RUL46 (Figure 23 A and B). Indeed, the level of phospho-Y394 Lck during infection by a VP11/12 null virus was indistinguishable from that of mock infected cells. Both mutants directed the accumulation of normal levels of ICP27 and VP16 (Figure 23A), arguing that the defect does not stem from reduced expression of viral proteins other than VP11/12. These data indicate that VP11/12 is necessary for HSV-induced enhancement of phosphorylation of Lck residue Y394 in Jurkat T cells.

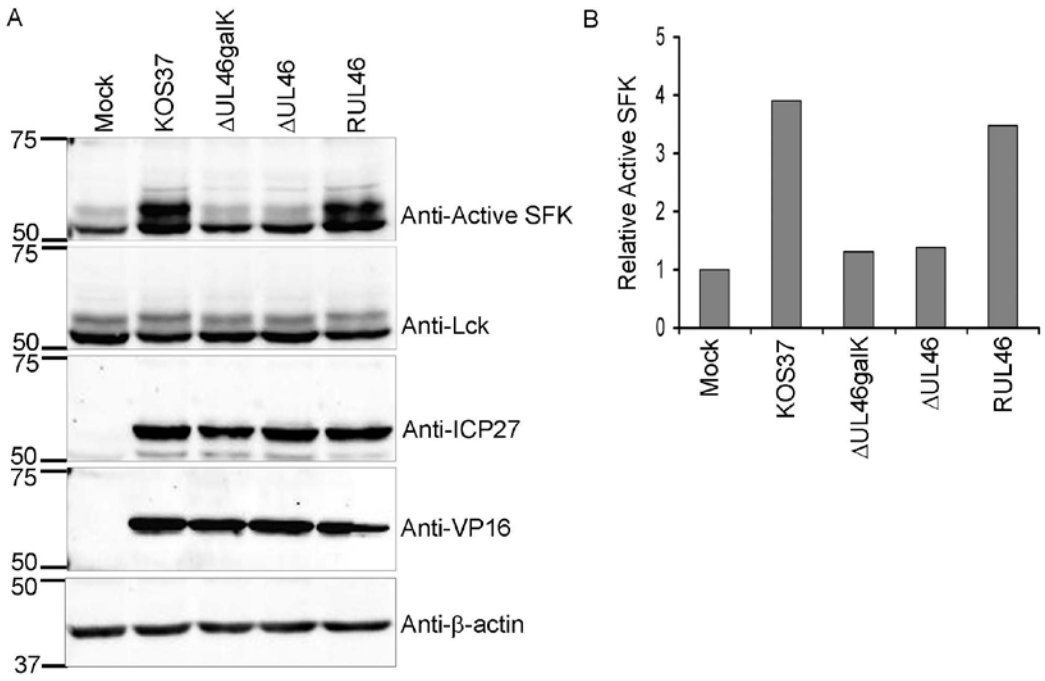

Figure 23. VP11/12 is required for enhanced phosphorylation of Lck Y394.

Jurkat cells were infected with HSV-1 KOS37, the VP11/12 null mutants ΔUL46galK and ΔUL46, and the VP11/12- repaired virus, RUL46. A) Western blot analysis using the indicated antibodies. B) The signals obtained with the anti- active SFK antibody were divided by those obtained with anti-Lck, and the ratios were normalized to yield a value of one for the mock-infected sample. This experiment was repeated three times.

Jurkat cells are an immortalized $CD4^+$ T line derived from EBV-negative T cell leukemia (189). To determine if HSV infection enhances phosphorylation of Lck residue Y394 in normal T cells, we examined $CD8^+$ T cells isolated from human peripheral blood. Others have observed that primary human T cells are resistant to infection by cell-free HSV, and that the efficiency of infection can be enhanced by exposing the T cells to infected fibroblasts or by activating the T cells with mitogens (8, 101, 167). Therefore infection of $CD8^+$ T cells was facilitated through exposure to HEL fibroblasts that had been previously infected with HSV-1, using a 5:1 ratio of T cells to fibroblasts. The presence of SFKs phosphorylated at the activating tyrosine was examined in HEL fibroblasts alone and in $CD8^+$ T cell/HEL fibroblast co-cultures. The anti-active SFK antibody displayed little or no reactivity with samples of mock and HSV-1 KOS37 infected HEL fibroblasts, indicating that signals obtained in co-cultures are derived from SFKs specific to the T cells. In the samples containing $CD8^+$ T cells, HSV enhanced Y394 phosphorylation of Lck and reduced the electrophoretic mobility of phospho-Y394 Lck in a VP11/12 dependent manner (Figure 24A and B).

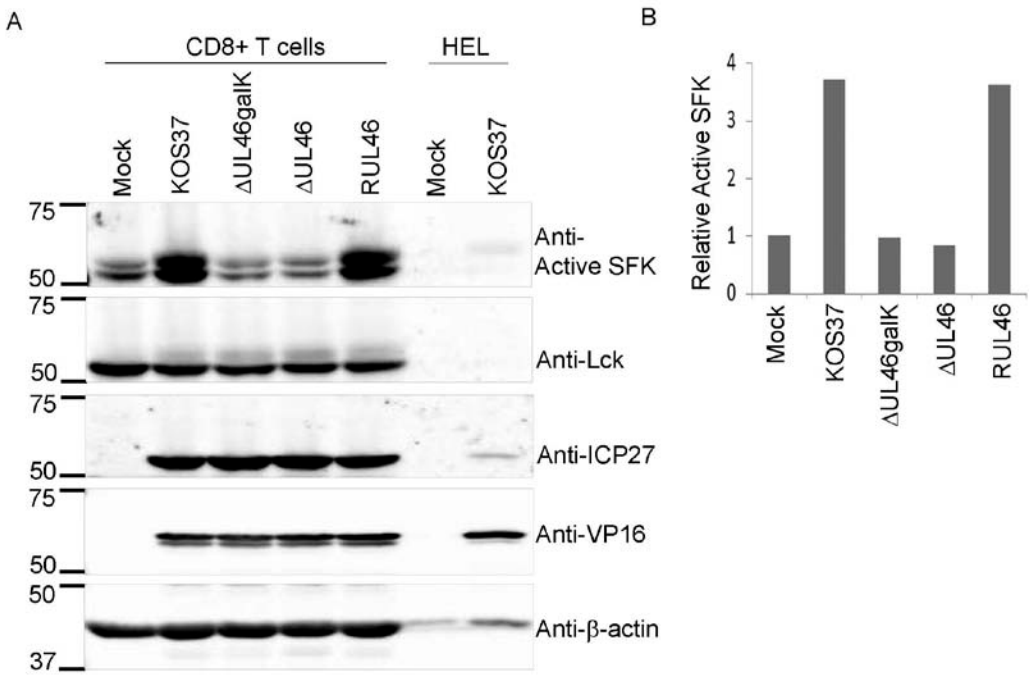

Figure 24. VP11/12 is required for enhanced phosphorylation of Lck Y394 in primary human CD8+ T cells.

HEL fibroblasts were either mock infected or infected with HSV-1 KOS37, the VP11/12 null mutants ΔUL46galK and ΔUL46, and the VP11/12- repaired virus RUL46. At 12 hours post-infection, $CD8^+$ T cells were added to some of the cultures at a 5:1 ratio. Cell lysate was prepared 8 hrs later and analyzed by western blot. A) Western blot analysis using the indicated antibodies. $CD8^+$ T cells: HEL cells overlaid with a 5-fold excess of $CD8^+$ T cells; HEL: HEL cells incubated in the absence of T cells. B) The signals obtained with the anti- active SFK antibody in the samples containing $CD8^+$ T cells were divided by those obtained with anti-Lck, and the ratios were normalized to yield a value of one for the mock-infected sample. This experiment was repeated twice, testing only $CD8^+$ T cell:HEL co-cultures.

4.2.6 VP11/12 Interacts with Lck in Infected T Cells

The data presented in the preceding sections establish that VP11/12 is necessary for infection enhanced phosphorylation of the activation loop tyrosine of Lck, raising the possibility that VP11/12 might interact with Lck or Lck signalling complexes *in vivo*. To determine if this is the case, Jurkat T cells were infected with wild type HSV-1 KOS or GHSV-UL46, a KOS-derived mutant virus expressing a GFP tagged VP11/12 (243). Potential interactions between VP11/12-GFP and Lck were then assessed by immunoprecipitation/western blot assays, using an antibody directed against GFP to precipitate and detect VP11/12-GFP. Detectable levels of Lck phosphorylated on Y394 co-precipitated with VP11/12-GFP (Figure 25A). Conversely, VP11/12-GFP (Figure 25B) was co-precipitated by the anti-Lck antibody. In chapter 3, we described a prominent Lck-dependent tyrosine phosphorylation of VP11/12 in Jurkat T cells (Chapter 3). Anti-phosphotyrosine analysis of Lck immunoprecipitates further demonstrated the presence of phosphorylated VP11/12 (ca. 90 kDa) and VP11/12-GFP (ca. 120 kDa) in the HSV-1 KOS and GHSV-UL46 samples, respectively. Taken together, these data demonstrate that phospho-Y394 Lck forms a complex with tyrosine phosphorylated VP11/12 in infected Jurkat T cells.

A previous report suggested that ICP0 interacts with the SH3 domain of Src through a putative SH3-binding motif (113). We therefore asked if ICP0 detectably interacts with Lck during infection. Immunoprecipitates of Lck from

KOS and GHSV-UL46 infected Jurkat T cells did not contain detectable amounts of ICP0 (Figure 25B). Therefore, our data provide no indication that ICP0 interacts with either Lck or Lck signalling complexes.

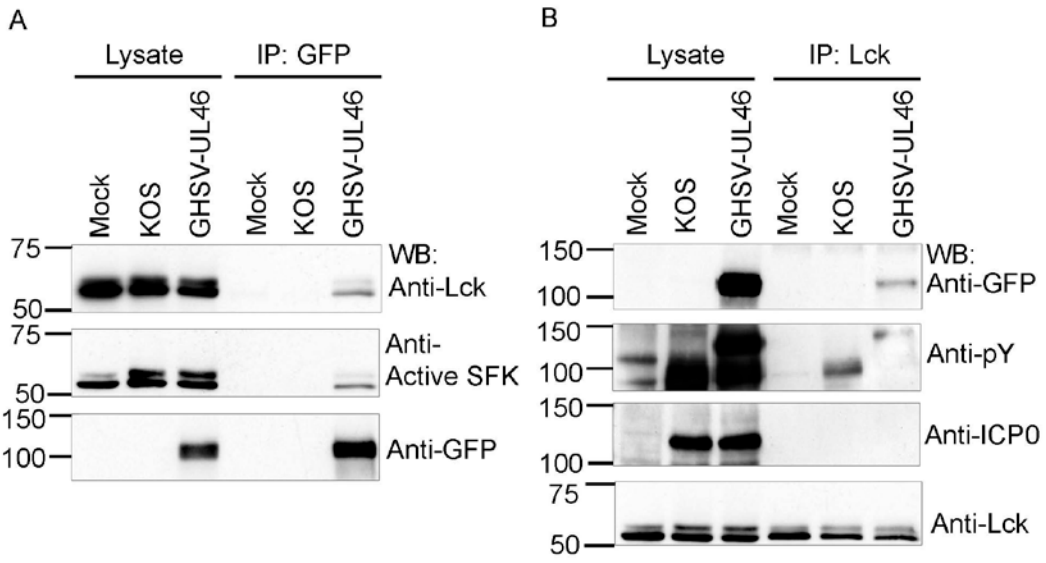

Figure 25. Lck interacts with GFP-tagged VP11/12 during HSV infection.

Jurkat cells were mock infected, infected by HSV-1 KOS or infected by a KOS mutant encoding a VP11/12-GFP fusion protein, GHSV-UL46. Cell extracts were used for immunoprecipitation/western blot analysis. A) Western blot analysis of immunoprecipitates obtained with an anti-GFP antibody. Lysate controls were analyzed directly. This experiment was repeated three times. B) Western blot analysis of immunoprecipitates obtained with the anti-Lck antibody. Lysate controls were analyzed directly. Interactions between Lck and a ca. 90 kDa tyrosine phosphorylated protein were observed in two separate experiments.

4.3 SUMMARY OF RESULTS

The results presented in this chapter show that HSV-1 infection enhances phosphorylation of the activating tyrosine (Y394) of the lymphocyte-specific SFK Lck in T lymphocytes. Although a previous report implicated ICP0, US3 and UL13 in SFK activation in non-lymphoid cells, these proteins are not strictly required for the effects of HSV-1 on Lck. By contrast, VP11/12 is required for the virus to induce activating tyrosine phosphorylation of Lck and *in vivo* interactions of VP11/12 and Lck imply that activation occurs via a direct mechanism.

We observe that HSV infection enhances the phosphorylation of the activating tyrosine of Lck (Y394) (Figure 18 and 20), and this implies that Lck is activated in infected T cells. Phosphorylation of Y394 indicates that Lck has been activated since the inactive conformation of an SFK buries the activating tyrosine, preventing phosphorylation (195, 248, 249). The phosphorylated activating tyrosine is also capable of significantly enhancing SFK activity (37), and preventing deactivation by a phosphorylated inhibitory tyrosine (73, 111, 218). Thus, despite no observable change to the phosphorylation of the inhibitory tyrosine (Y505), the increased phosphorylation of Y394 alone implies that the virus activates Lck.

Liang and Roizman have implicated ICP0, US3 and UL13 in the activation of SFKs other than Lck (113). We have found that ICP0 and US3 are not required for HSV induced activation of Lck (Figure 21 and 22). UL13 was necessary, in part, for HSV to reduce the electrophoretic mobility of phospho-Y394 Lck. However, the absence of UL13 during T cell infection resulted in lower expression of late viral proteins suggesting that the deficiency of a late viral protein, like VP11/12, could have caused this effect.

VP11/12 appears to play a direct role in enhancing Lck activation during T cell infection. Deletion of VP11/12 does not affect viral replication (261), and had no apparent effect on viral protein expression, but it does prevent induction of the active phenotype of Lck in Jurkat and primary T cells (Figure 23 and 24) implying that VP11/12 itself is necessary to enhance Lck activity. Consistent with this hypothesis, measurable increases in the level of phospho-Y394Lck are apparent at 6 hpi and later in infection, a time line that suggests the involvement of newly synthesized VP11/12 (Figure 19). Furthermore, our immunoprecipitation studies suggest that VP11/12 is interacting either directly with Lck or with an Lck-signalling complex (Figure 25) to influence kinase activity. Thus, HSV requires VP11/12 to induce activating phosphorylation of Lck.

Although our data suggest that VP11/12 binds and activates Lck, it seems that only a fraction of total cellular Lck is sequestered and activated by VP11/12

during infection. We are unable to detect enhanced Lck kinase activity in *in vitro* kinase assays of infected cell lysates (data not shown), suggesting that only a small number of Lck molecules are activated during infection. Consistent with this interpretation in many experiments the majority of Lck had an apparent molecular weight of 56 kDa while the active form of Lck, phospho-Y394Lck was distributed equally into ~56 and ~60 kDa fractions (Figure 23,24 and 21 KOS MOI=1). It is also apparent that VP11/12 does not globally activate Lck signalling, since HSV infection inhibits rather than activates TCR signalling (Figure 15 and (199)). VP11/12 could activate specific Lck mediated signalling by localizing to a sub-cellular location that is associated with specific signalling effectors or by simultaneously interacting with Lck and specific downstream signalling protein(s).

CHAPTER 5: HSV-1 Requires VP11/12 to Induce Signalling Through the PI3K-Akt Pathway

5.1 RATIONALE

Lck and VP11/12 interact in a number of ways during infection. 1) Lck enhances tyrosine phosphorylation of VP11/12 (Chapter 3), suggesting that VP11/12 is a substrate of Lck or a kinase activated downstream of Lck. 2) VP11/12 is required for HSV infection-induced activation of Lck and VP11/12 interacts physically with Lck or Lck signalling complexes during infection (Chapter 4).

We hypothesized that the observed VP11/12-Lck interactions were part of a cellular signalling pathway controlled by the virus during HSV infection. Thus, we sought to identify effectors downstream of VP11/12-Lck interactions.

5.2 RESULTS

5.2.1 VP11/12 is Required for Akt Activation During Jurkat T Cell Infection

We sought to identify signalling events downstream of VP11/12-Lck interactions. Previous studies have documented that HSV infection activates several host signalling effectors including p38 (109, 135, 200, 255), JNK (109, 135, 200, 255), Akt (12) and the Akt target, mTORC1 (237). Since HSV-induced activation of p38 and JNK has been described in Jurkat T cells (200), we first asked whether activation of these kinases requires VP11/12. To address this

question, the activating phosphorylation of p38 and JNK was measured in Jurkat T cells infected by wild type HSV-1 KOS37, two VP11/12 null HSV-1 recombinant viruses, ΔUL46galk and ΔUL46, and a ΔUL46galK recombinant with a repaired VP11/12 open reading frame, RUL46 (Figure 26). HSV infection did not require VP11/12 to enhance the activating phosphorylation of these kinases.

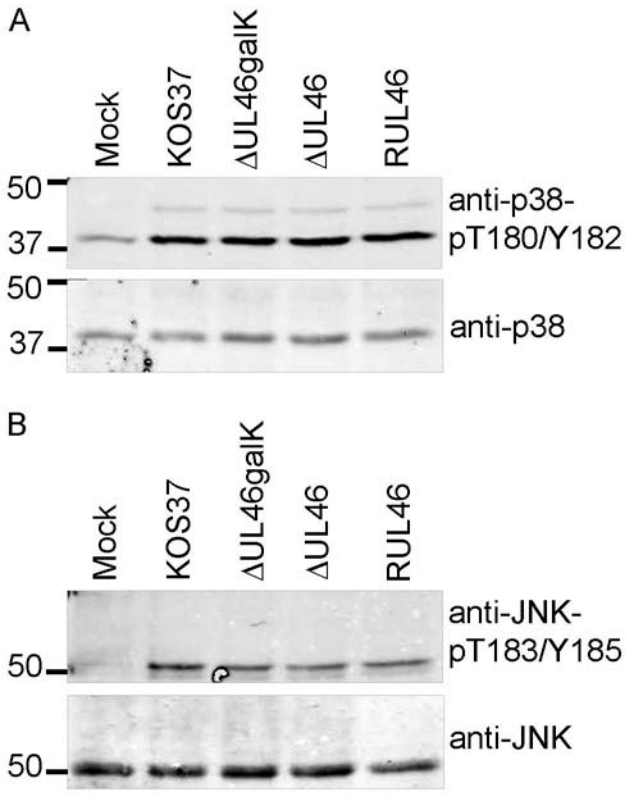

Figure 26. HSV does not require VP11/12 to activate the signalling proteins, p38 and JNK.

Jurkat T cells were mock infected, and infected by HSV-1 KOS37, the VP11/12 null mutants, ΔUL46galK and ΔUL46, and the VP11/12- repaired virus RUL46. Cell lysate was collected at 9 hours post-infection and analyzed by western blot. A) Western blot analysis of activating phosphorylation of p38 at threonine 180 and tyrosine 182 (anti-p38pT180/Y182) and total p38. B) Western blot of active JNK phosphorylated at threonine 183 and tyrosine 185 (anti-JNKpT183/Y185) and total JNK.

SFKs, like Lck, have been found upstream of PI3K-Akt signalling. Therefore, we asked whether HSV infection activates Akt in a VP11/12-dependent manner. Active Akt was detected by western blot, using an antibody specific for Akt phosphorylated at the activating serine of the hydrophobic motif (anti-pS473) (Figure 27A). A ratio of the infrared signal generated by anti-pS473 relative to that of anti-Akt was used to quantitatively compare Akt activation between samples (Figure 27B). As a leukemic cell line, Jurkat T cells have a PTEN deficiency which promotes survival through constitutive activation of Akt (192). Accordingly, mock infected Jurkat T cells displayed a large amount of active phospho-S473 Akt. Despite the constitutive activation of Akt in these cells infection by KOS37 and RUL46 increases the level of phospho-S473 Akt relative to that of mock infected cells. Both samples also demonstrate a slight decrease in the electrophoretic mobility of total Akt. This change in the apparent molecular weight of Akt is consistent with activation-induced post-translational modifications including phosphorylation of S473. By contrast, samples infected by the VP11/12 null ΔUL46galK and ΔUL46 have lower levels of phospho-S473 Akt compared to KOS37, RUL46 and mock infected samples. Infection by ΔUL46galK and ΔUL46 also fails to increase the apparent molecular weight of Akt relative to mock infected cells. Importantly, normal levels of the late viral protein, VP16 accumulate during infection by ΔUL46galK and ΔUL46, suggesting that defects observed did not arise from reduced expression of viral proteins other than VP11/12 (Figure 27A). These findings indicate that HSV

infection requires VP11/12 to activate Akt. Furthermore, VP11/12 null virus infection seems to decrease phospho-S473 Akt relative to mock infection, suggesting that the absence of VP11/12 allows for infection-induced inhibition of Akt.

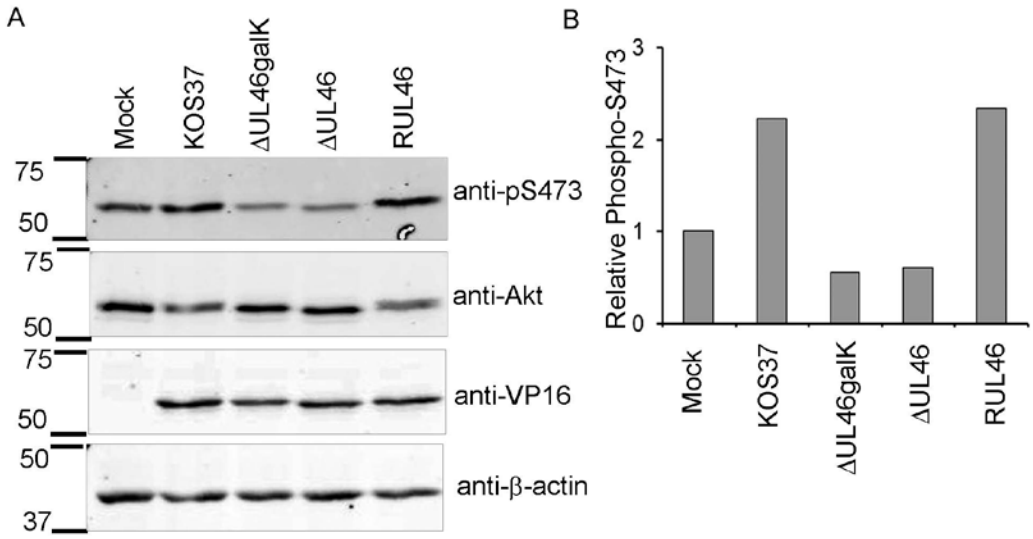

Figure 27. VP11/12 is necessary for Akt activation in HSV infected Jurkat cells.

Jurkat T cells were mock infected, and infected by HSV-1 KOS37, the VP11/12 null mutants ΔUL46galK and ΔUL46, and the VP11/12- repaired virus RUL46. Cell lysate was collected at 9 hours post-infection and analyzed by western blot. A) Western blot analysis of the activating phosphorylation of Akt at S473 (anti-pS473), total Akt, VP16 and β-Actin. B) The infrared signal of anti-pS473 was divided by the signal of total Akt and normalized to yield a value of one for the mock-infected sample. Similar results were obtained in three separate experiments.

5.2.2 VP11/12 is Required for Akt Activation Late in Primary HEL Fibroblast Infection.

A thorough investigation of VP11/12 dependent Akt signalling was not possible in Jurkat T cells due to the PTEN deficiency in these cells (192). We initially examined primary T cells. Others have shown that primary T cells cannot be infected by cell-free HSV (8, 101, 167). Therefore, those studies were complicated by the need to infect the T cells through exposure to infected fibroblasts. During the course of those studies it became apparent that infected primary human embryonic lung fibroblasts (HEL) display a high level of the active Akt. HEL fibroblasts infected by HSV-1 F and HSV-1 KOS were collected at various times post-infection (hours post-infection, hpi) and phospho-S473 Akt was detected by western blot (Figure 28A). Signals from anti-Akt demonstrated that Akt expression is constant throughout infection. Thus, increases in phospho-S473 Akt are indicative of enhanced Akt activation. As reported previously infection by HSV-1 F induced a transient increase in phospho-S473 Akt, 4 hpi (12). However, infection by both HSV-1 strains, F and KOS, increased the level of phospho-S473 Akt late in infection (past 12 hpi). Post translational modifications of Akt were also evident late in infection (16-20 hpi) through the reduced electrophoretic mobility of total cellular Akt.

The necessity of VP11/12 in Akt activation was also assessed in infected HEL fibroblasts (Figure 28 B,C). Activation of Akt was assessed using antibodies

specific for Akt phosphorylated at both activating residues, threonine 308 of the activation loop and serine 473 of the hydrophobic motif (anti-pT308, anti-pS473). Increased levels of Akt phosphorylated at T308 and S473 were apparent in fibroblasts infected by KOS37 and RUL46 compared to mock infected samples or samples infected by VP11/12 null viruses, ΔUL46galK and ΔUL46. This demonstrates that VP11/12 is required for activation of Akt in HSV-1 infected primary HEL fibroblasts, and provides us with a good model system for studying VP11/12-dependent activation of Akt.

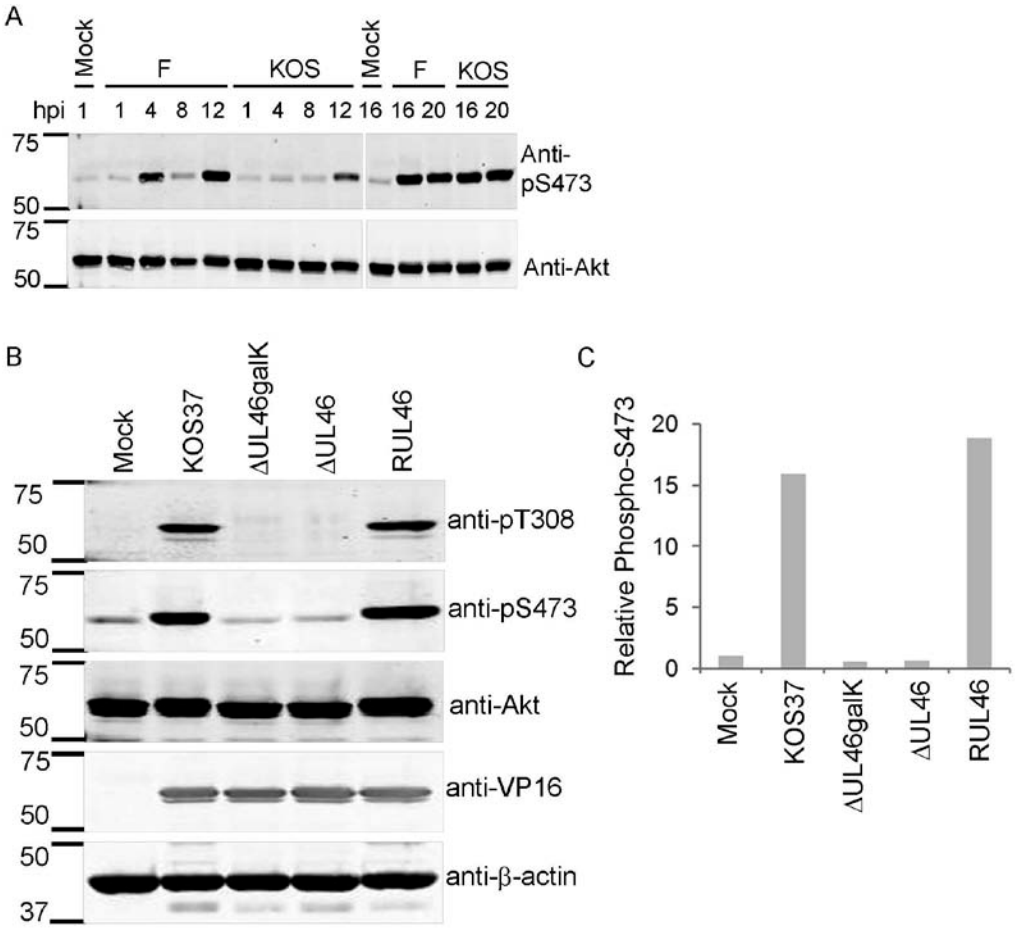

Figure 28. HSV requires VP11/12 to activate Akt late during HEL fibroblast infection.

A) HEL fibroblasts were mock infected, infected by HSV-1 F or infected by HSV-1 KOS. Cell lysate was collected at the indicated times post-infection (hours post-infection, hpi) and analyzed by western blot using anti-pS473 and anti-Akt. B) HEL fibroblasts were mock infected, and infected by HSV-1 KOS37, VP11/12 null viruses, ΔUL46galK and ΔUL46, and the VP11/12 repaired virus, RUL46. Cell lysate was collected 15 hours post-infection and analyzed by western blot using antibodies against the activating phosphorylation of Akt at T308 (anti-pT308) and S473 (anti-pS473), total Akt, VP16, and β-Actin. C) The infrared signal of anti-pS473 in B was divided by the signal of anti-Akt and normalized to yield a value of one for the mock-infected sample. This experiment was repeated once.

5.2.3 HSV Infection Activates Akt Through Cellular PI3K

In cellular Akt signalling PI3K is upstream of Akt. To assess whether PI3K activity contributes to Akt activation late in HEL fibroblast infection, we infected with HSV-1 KOS and treated infected samples with the PI3K specific inhibitors Wortmannin and Ly294002 (Figure 29). The LY294002 inactive analogue, LY303511 was also used to treat infected cells as a negative control. Phosphorylation of S473 of Akt was undetectable in samples treated with either Wortmannin or Ly294002. By contrast, untreated KOS infection and infection treated with the inactive Ly294002 analogue, LY303511 generated comparable levels of phospho-S473 Akt. This indicates that specific inhibition of PI3K prevents HSV infection-induced activation of Akt, and suggests that HSV infection initiates the cellular PI3K-Akt signalling pathway.

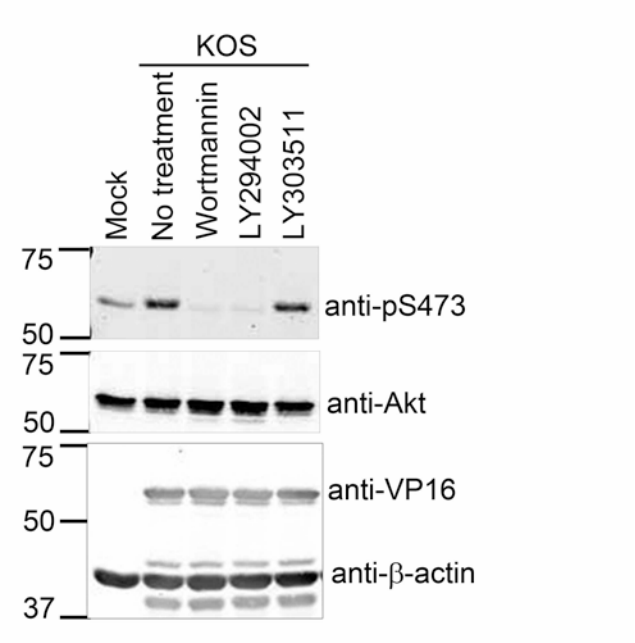

Figure 29. Infection-induced activation of Akt is PI3K-dependent.

HEL fibroblasts were mock infected and infected by HSV-1 KOS. Kinase inhibitors specific for PI3K, Wortmannin and LY294002, and the inactive analogue of LY294002, LY303511 were added to cultures at 11 hours post-infection as indicated. Cell lysate was collected at 19 hours post-infection and analyzed by western blot using the indicated antibodies. This experiment was repeated once.

5.2.4 VP11/12 Interacts With PI3K During Infection

Analysis of the VP11/12 sequence using the Scansite 2.0 algorithm identifies putative SH2 and SH3 binding motifs predicted to bind the regulatory subunit of PI3K, p85 (156). VP11/12 has been reported to associate with infected cell membranes (148). Therefore direct interactions between PI3K and VP11/12 could activate cellular PI3K-Akt signalling by recruiting PI3K to its substrate PIP2. To address this possibility we asked whether VP11/12 interacts with PI3K during infection by immunoprecipitation/western blot assays for p85 and a GFP tagged VP11/12 (Figure 30). Jurkat T cells were mock infected, infected by HSV-1 KOS or infected by the KOS derivatives, GHSV-UL46 and KOSG which encode a GFP tagged version of VP11/12 and a GFP expression cassette, respectively. Immunoprecipitation of GFP yielded detectable levels of p85 only in the GHSV-UL46 infected samples (Figure 30A). Conversely, the VP11/12-GFP fusion protein is pulled down by immunoprecipitation with anti-p85 and not by immunoprecipitation with non-specific rabbit IgG (Figure 30B). Analysis of phosphotyrosine in anti-p85 immunoprecipitate further suggests that p85 interacts with the tyrosine phosphorylated form of the native VP11/12 (ca. 90 kDa) and the VP11/12-GFP fusion protein (ca. 120 kDa). Collectively, these assays indicate that VP11/12 interacts with p85 either directly or indirectly during infection.

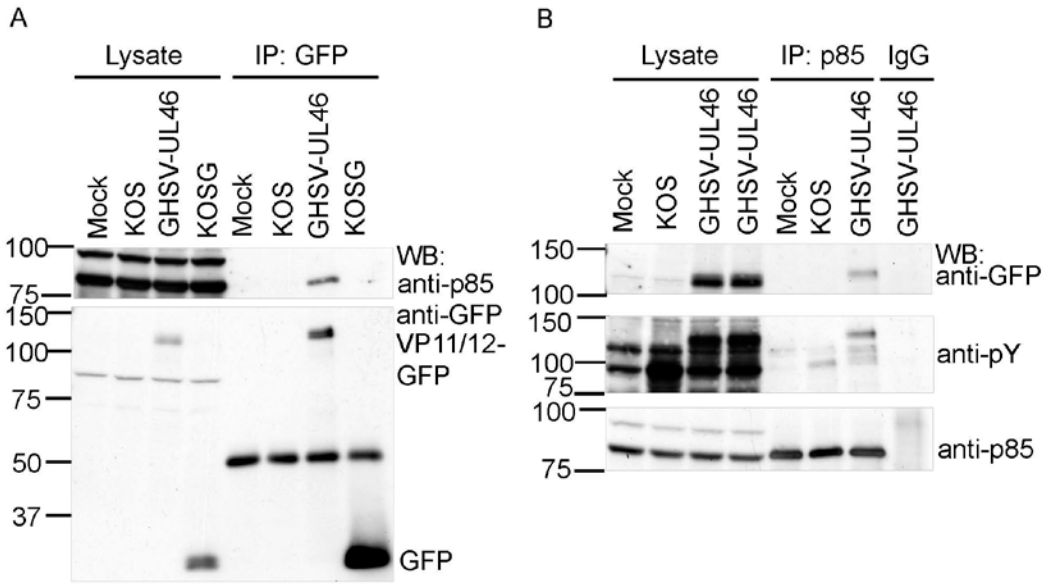

Figure 30. VP11/12 interacts with p85 during infection.

Jurkat T cells were mock-infected, infected by KOS, and infected by KOS derived viruses which express either GFP (KOS-G) or a VP11/12-GFP fusion protein (GHSV-UL46). A) Western blot analysis of p85 and GFP in anti-GFP immunoprecipitates. Lysate controls were analyzed by western blot directly. B) Western blot analysis of p85 and GFP in immunoprecipitates obtained with anti-p85 or rabbit IgG. Lysate controls were analyzed by western blot directly. Results are typical of three separate experiments.

5.2.5 SFK Activation Does Not Strictly Require VP11/12 in HEL Fibroblasts

Active SFKs are upstream of the PI3K-Akt pathway in cellular signalling through immune receptors (75, 203) and growth receptors (151), and in viral signalling through v-Src (131) and polyoma MTAg (26). In the previous chapters, we demonstrated that VP11/12 is required for HSV infection induced activation of Lck (Chapter 4), and that Lck activity contributed to tyrosine phosphorylation of VP11/12 (Chapter 3). VP11/12-dependent activation of Lck may be upstream of HSV-induced Akt signalling in T cells, and although HEL fibroblasts do not express Lck, other SFKs may compensate to activate Akt signalling in these cells. To address this hypothesis, we first examined whether VP11/12-dependent activation of SFKs was detectable in HEL fibroblasts, as it had been for Lck in Jurkat T cells (Chapter 4). To detect SFK activation we used antisera specific for SFKs phosphorylated at the activating tyrosine residue (the equivalent of Y416 of Src) (Figure 31A). SFKs phosphorylated at this residue increased relative to β-actin during HSV infection, and the relative level of active SFKs was decreased only slightly during infection by VP11/12 null viruses, ΔUL46galK and ΔUL46 (Figure 31B). Previously, ICP0, US3 and UL13 were implicated in activating SFKs, other than Lck, in Vero epithelial cells (113). Our results indicate that VP11/12 may be partially responsible for SFK activation in infected HEL fibroblasts; however ICP0, US3, UL13 or other factors specific to HSV infection make a greater contribution to SFK activation in these cells.

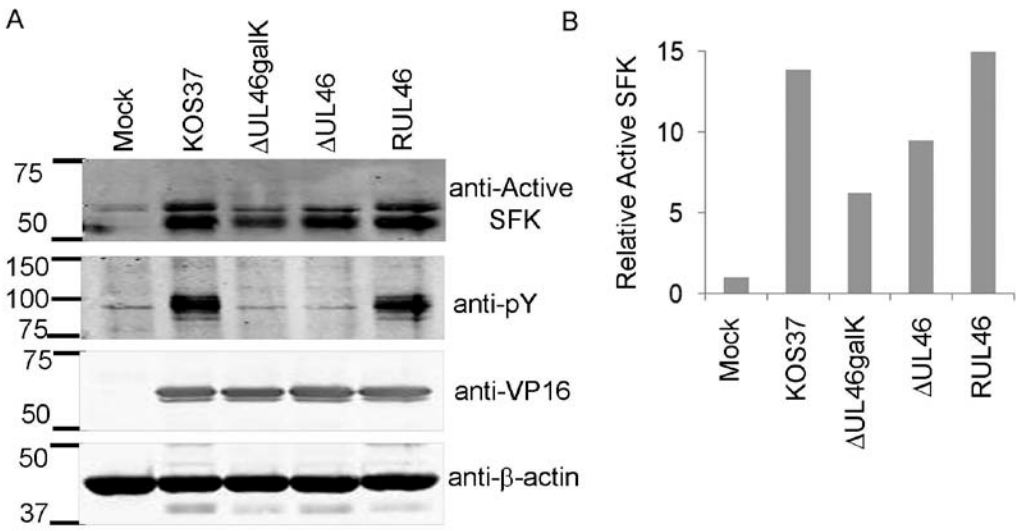

Figure 31. VP11/12 is tyrosine phosphorylated and promotes SFK activation in infected HEL fibroblasts.

HEL fibroblasts were infected by HSV-1 KOS37, ΔUL46galK, ΔUL46, and RUL46. Cell lysate was collected at 21 hours post-infection and analyzed by western blot. A) Western blot detection of SFKs phosphorylated at the activating tyrosine (the equivalent of phospho-Y416 of Src, anti-active SFK), phosphotyrosine, VP16 and β-actin. B) The infrared signal obtained with anti-active SFK was divided by the signal obtained with anti-β-actin and normalized to give a value of one in the mock-infected sample. Similar results were obtained in three separate experiments measuring active SFKs.

5.2.6 SFK Activity is Required for VP11/12-PI3K Interactions

As outlined in the introduction, some signalling pathways utilize SFK-dependent phosphorylation of a p85 SH2 binding motif in a membrane associated protein to recruit PI3K to its substrate. VP11/12 is membrane associated in infected cells (148), and we have shown that Lck activity enhances tyrosine phosphorylation of VP11/12 in T cells (Chapter 3). Thus, we reasoned that SFK-mediated phosphorylation of VP11/12 could facilitate SH2 mediated PI3K binding and allow for PI3K activity.

HEL fibroblasts do not express Lck, therefore we first assessed whether SFK activity contributes to tyrosine phosphorylation of VP11/12 in these cells. Although tyrosine phosphorylation of the ca. 90 kDa VP11/12 is weaker in HEL fibroblasts and Vero epithelial cells compared to lymphocytes (Chapter 3), it is still detectable by western blot analysis (Figure 31). To determine whether SFK activity mediates VP11/12 tyrosine phosphorylation in these cells, we gauged the effects of the SFK specific inhibitor PP2 on VP11/12 phosphorylation (Figure 32). At 11 hpi, HSV-1 KOS infected fibroblasts were treated with PP2 at a concentration of 10, 20 or 30 μM or treated with the inactive analogue of PP2, PP3 (30 μM). The anti-phosphotyrosine signal of VP11/12 was assessed at 20 hpi. Exposure to PP2 (10, 20, and 30 μM) greatly decreased the abundance of the tyrosine phosphorylated form of VP11/12, while exposure to PP3 (30 μM) had no

effect. Thus, SFKs endogenous to HEL cells are responsible for generating most, if not all, of the phosphotyrosine residues in VP11/12.

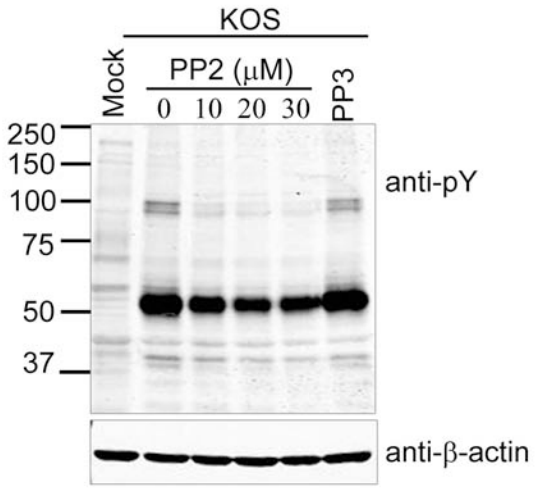

Figure 32. SFK activity is required for tyrosine phosphorylation of VP11/12.

HEL fibroblasts were mock infected and infected by HSV-1 KOS. At 11hours post-infection the specific SFK inhibitor PP2 was added at 10, 20 and 30 μM and the inactive analogue of PP2, PP3 was added at 30 μM as indicated. Cell lysate was collected at 16 hours post-infection and analyzed by western blot using anti-phosphotyrosine and anti-β-actin. Similar results were obtained in two separate experiments.

To examine whether SFK-dependent phosphorylation of VP11/12 allows for VP11/12-PI3K interactions we assessed whether these two proteins associate in the presence of SFK inhibition (Figure 33). HEL fibroblasts were infected by the HSV-1 KOS derivatives, GHSV-UL46 and KOSG. Cells infected by GHSV-UL46 were treated at 11 hpi with PP2 (10 μM) and PP3 (10 μM). Co-precipitation of p85 was detected in anti-GFP immunoprecipitates from untreated and PP3 treated GHSV-UL46 infected cells. However, p85 was not detected in anti-GFP precipitates from GHSV-UL46 infected cells treated with PP2. Similarly, anti-GFP precipitates from untreated GHSV-UL46 infected Jurkat cells yielded more p85 than precipitates from GHSV-UL46 infected JCAM1.6 cells or GHSV-UL46 infected Jurkat cells treated with 10 μM PP2 (Figure 34). This demonstrates that SFK activity promotes p85-VP11/12 interactions, and it suggests that SFK activation is one of the initial steps in VP11/12-dependent PI3K-Akt signalling.

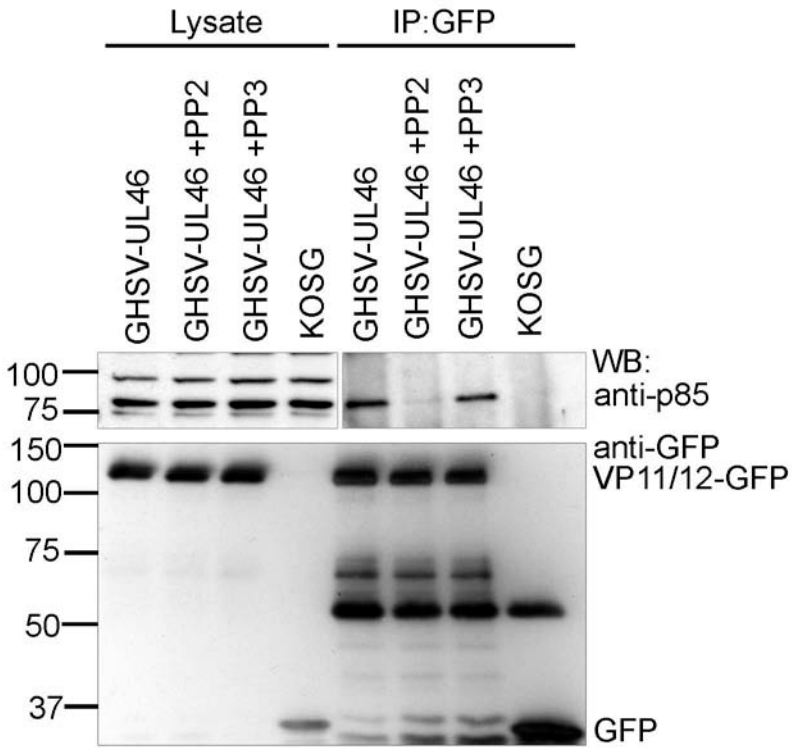

Figure 33. SFK activity is required for VP11/12-p85 interaction(s) in HEL cells.

HEL fibroblasts were infected by HSV-1 KOS derived viruses, KOSG and GHSV-UL46. At 11 hours post-infection, PP2 (10 μM) and PP3 (10μM) were added to cultures as indicated. Cell lysate was collected at 18 hours post-infection. Anti-GFP immunoprecipitates were analyzed by western blot using the indicated antibodies. Lysate controls were analyzed directly. This experiment was repeated once.

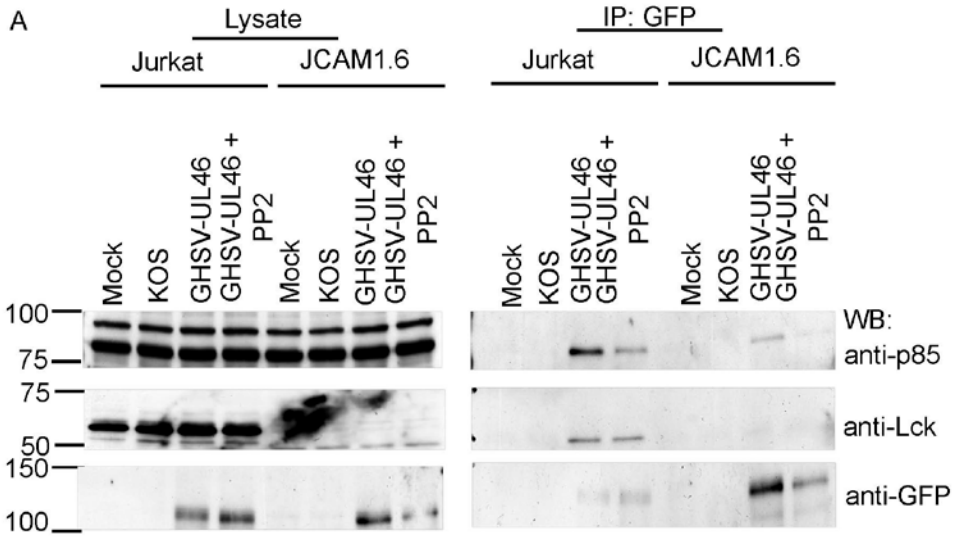

Figure 34. Lck enhances VP11/12-p85 interaction(s) in Jurkat T cells.

Jurkat cells and JCAM1.6 cells were infected by HSV-1 KOS and the KOS derivative GHSV-UL46. Some GHSV-UL46 infections were performed in the presence of PP2 (10 μM) for the 9 hour infection. Anti-GFP immunoprecipitates were analyzed by western blot using the indicated antibodies. Lysate controls were analyzed directly.

5.2.7 HSV-mediated Akt Activation Requires SFK Activity

To determine if SFK activity enhances downstream activation of Akt, phospho-S473Akt was measured in infected HEL fibroblasts treated with PP2 and PP3 (as described for Figure 32). Enhanced phosphorylation of S473 of Akt was detected in KOS infected cells that were left untreated or treated with the inactive PP2 analogue, PP3 (Figure 35). By contrast, treatment with PP2 inhibited S473 phosphorylation. This demonstrates that SFK activity contributes to infection induced activation of Akt.

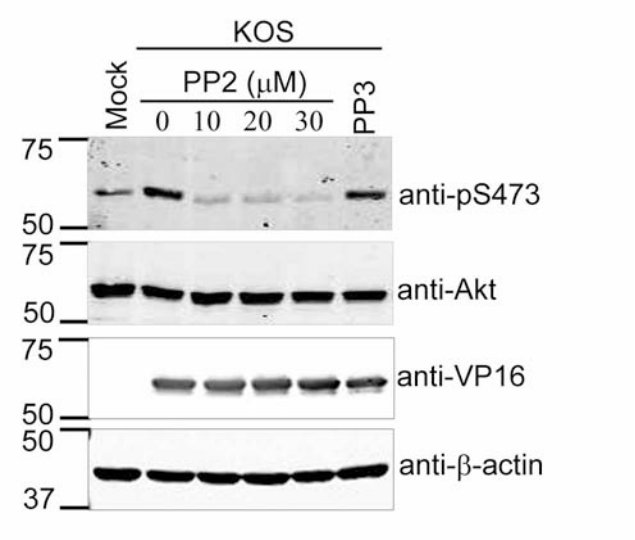

Figure 35. Inhibition of SFK activity prevents Akt activation during HSV infection.

HEL fibroblasts were mock infected or infected by HSV-1 KOS. At 11 hours post-infection the specific SFK inhibitor PP2 was added at a concentration of 10, 20 and 30 μM and the inactive analogue of PP2, PP3 was added at a 30 μM concentration. Cell lysate was collected at 16 hours post-infection and analyzed by western blot using the indicated antibodies. This experiment was repeated twice.

5.2.8 VP11/12 is Not Required for HSV Induced Activation of mTORC1

A previous report by Walsh and Mohr demonstrated that HSV infection induces signalling through mTORC1 to hyperphosphorylate and inactivate 4EBP1 (237). Akt is a well known activator of mTORC1 signalling. Therefore, we asked if VP11/12 mediated activation of Akt contributes to mTORC1 signalling by examining the downstream consequences of mTORC-1 activation, 4EBP1 hyperphosphorylation and inhibition and S6K phosphorylation and activation (Figure 36). As seen previously (237), infection by wild type virus, KOS37 induced the hyperphosphorylation of 4EBP1, evident by an increase in the apparent molecular weight of the protein. KOS37 infection also enhanced the fraction of S6K phosphorylated at the activating residue, T389 (anti-pT389) compared to mock infection. These changes are consistent with HSV-1 infection induced activation of mTORC-1. However, hyperphosphorylation of 4EBP1 and an increase in the amount of phospho-T389 S6K was also evident during infection by VP11/12 null viruses, indicating that VP11/12 is not required for infection induced activation of mTORC-1.

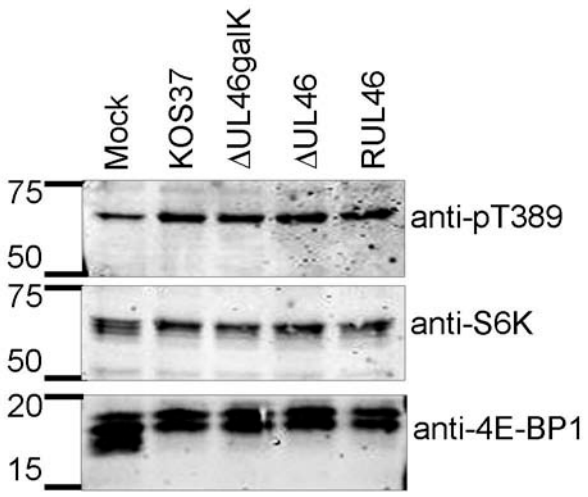

Figure 36. VP11/12 is not required for infection induced phosphorylation of mTORC1 substrates, S6K and 4E-BP1.

HEL fibroblasts were infected by HSV-1 KOS37, ΔUL46galK, ΔUL46, and RUL46. At 20 hours post-infection lysate was collected. The activating phosphorylation of S6K at T389 (anti-pT389), total S6K and total 4E-BP1 were detected by western blot. This experiment was repeated once.

Phosphorylation/inhibition of the Akt substrate, GSK3β was also examined. The inhibitory phosphorylation of GSK-3β at S9 was induced during infection by wild type HSV KOS37, ΔUL46galK, ΔUL46 and RUL46 (Figure 39). Thus, HSV infection enhances GSK-3β phosphorylation and is likely to inhibit GSK-3β activity, but it does not require VP11/12 to do so.

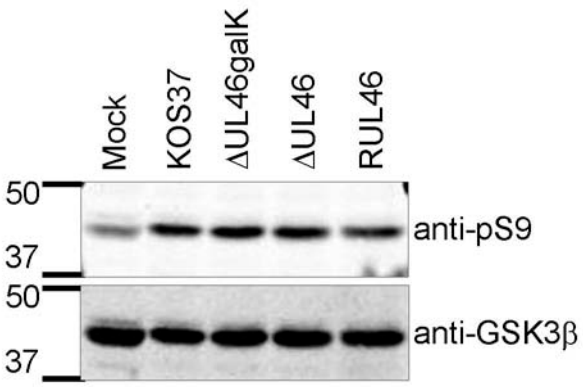

Figure 37. VP11/12 is not required for HSV-mediated deactivation of GSK-3β.

HEL fibroblasts were mock infected, and infected by HSV-1 KOS37, ΔUL46galK, ΔUL46, and RUL46. Cell lysate was analyzed by western blot for inhibitory phosphorylation GSK-3β at S9 (anti-pS9) and total GSK-3β. This experiment was repeated once.

5.3 SUMMARY OF RESULTS

This chapter establishes VP11/12 as a signalling modulator that activates SFK-PI3K-Akt signalling in Jurkat T cells, HEL fibroblasts, and potentially in other cell types. We have demonstrated that VP11/12 activates Akt through the PI3K-Akt signalling axis (Figure 29) in Jurkat T cells (Figure 27) and HEL fibroblasts (Figure 28). We observed interactions between VP11/12 and PI3K or PI3K signalling complexes, which suggests that VP11/12 activates PI3K directly during infection (Figure 30). SFK-dependent tyrosine phosphorylation of a membrane associated protein, like VP11/12, can mediate activation of the PI3K-Akt pathway (discussed in detail in section 6.9). In accordance with such a mechanism, SFK activity is required for VP11/12 tyrosine phosphorylation (Figure 32), PI3K-VP11/12 interactions (Figure 33 and 34) and Akt activation (Figure 35) in HEL fibroblasts.

In this chapter, VP11/12 demonstrated interactions with SFKs in fibroblasts analogous to those observed previously with Lck, in T cells (Chapter 3 and 4). In Chapter 4, VP11/12 was shown to be strictly required for Lck activation during T cell infection (Figure 22 and 24) and VP11/12 is now observed to contribute, albeit to a lesser degree, to the increased level of active SFKs induced during HEL fibroblast infection (Figure 31). As seen in Jurkat T cells (Chapter 3, Figure 13), HEL fibroblasts also induce SFK-dependent tyrosine

phosphorylation of VP11/12 (Figure 32). Collectively, this data suggests that VP11/12 activates Lck in T cells and other SFKs in HEL cells to induce its own tyrosine phosphorylation.

Inhibition of SFKs by PP2 was used in this chapter to indicate that SFKs are upstream of the PI3K-Akt signalling axis during HSV-1 infection. The effects observed on PI3K-Akt interactions (Figure 33 and 34) and the effects observed on Akt activation (Figure 35) were due to specific inhibition of SFKs since the inactive analogue PP3 did not alter these events even at the highest concentration tested (30 μM). Furthermore, inhibition of Akt activation by PP2 does not appear to be caused by an adverse effect on viral replication since this drug did not alter expression of the late viral protein, VP16 (Figure 35). It is interesting that VP11/12-PI3K interactions in Jurkat T cells were not completely blocked by 10 μM PP2 (Figure 34). As well, in some experiments Akt activation was not completely abrogated by 10 μM PP2, and instead it exhibited a dose-dependent response at 10-30 μM PP2 (data not shown). This data is intriguing, since 10 μM PP2 is known to be sufficient to block TCR signalling (confirmed in our laboratory; data not shown). This may indicate that, compared to TCR signalling, HSV infection holds a greater number of SFK molecules in a constitutively active state. In support of this theory, we were unable to detect an increase in phospho-Y394Lck upon TCR ligation using western blot analysis and the anti-active SFK antibody, whereas, an increase in phospho-Y394Lck was readily detected during HSV-1 infection (Chapter 4).

In HSV infection, the target substrate(s) of active Akt and the corresponding cellular function have yet to be identified. Although HSV-1 infection was previously shown to activate the Akt target, mTORC1 (237), VP11/12 is not required for this activity (Figure 36) suggesting that VP11/12 mediated Akt activation is not upstream of mTORC1 signalling. Likewise, HSV-1 infection enhances phosphorylation of the known Akt substrate GSK-3β independent of VP11/12 (Figure 37) suggesting that Akt activity induced by VP11/12 does not deactivate GSK-3β. Activation of mTORC1 and deactivation of GSK-3β may occur through the activity of another HSV-1 encoded signalling modulator(s) or through an alternative cellular signalling pathway(s) activated in response to HSV-1 infection. Further experimentation is required to determine how mTORC1 and GSK-3β are activated and deactivated, respectively during HSV-1 infection. Further investigation is also needed to identify the substrate(s) of active Akt, the associated cellular function(s), and the importance of this function in terms of HSV survival.

CHAPTER 6: Discussion

6.1 Thesis Summary

The results in this communication support a model where the HSV-1 tegument protein VP11/12 activates Lck and other SFKs to initiate signalling through the PI3K-Akt signalling axis (Figure 38). The sequence of VP11/12 contains a putative high affinity SFK SH2 binding motif, YEEI. From our observation that VP11/12 interacts with Lck and is necessary to activate Lck during T cell infection (Chapter 4), we predict that this motif binds and activates Lck and other SFKs. These active SFKs or downstream kinase(s) are likely to phosphorylate tyrosine residues in VP11/12, since Lck is necessary and sufficient to induce robust phosphorylation of VP11/12 in T cells (Chapter 3) and the activity of other SFKs are necessary for this phosphorylation in HEL cells (Chapter 5). One of these phosphotyrosine residues may be within VP11/12's putative p85 SH2 binding motif, YTHM. In theory, phosphorylation at this site could facilitate interactions between PI3K and the membrane-associated VP11/12 to activate PI3K-Akt signalling (Chapter 5).

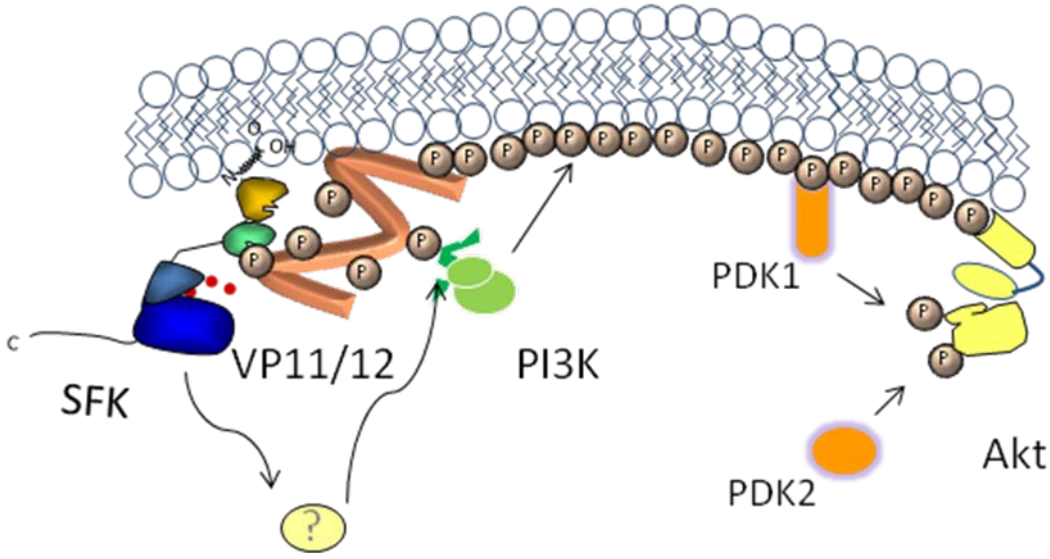

Figure 38. Model of VP11/12 Signalling.

Our results and the sequence of VP11/12 suggest a model where VP11/12 activates SFKs through interactions at the putative SH2 binding motif, YEEI. The active SFK or a downstream kinase(s) then phosphorylates VP11/12 at tyrosine residues, including that of the putative p85 SH2 binding motif, YTHM. Phosphorylation at this site facilitates interactions between PI3K and the membrane-associated VP11/12 to activate PI3K-Akt signalling.

6.2 Tyrosine Phosphorylation of VP11/12

The data in chapter 3 provides the first description of VP11/12 tyrosine phosphorylation. One earlier report examined tyrosine phosphorylation during HSV-1 and HSV-2 infection of HeLa epithelial and NIH 3T3 fibroblast cell lines. It was shown that the α gene product ICP22 and at least four other infected cell proteins are tyrosine phosphorylated during infection of these non-lymphoid cells (13). Our data adds to this initial observation by identifying VP11/12 as a tyrosine phosphorylated protein in HSV-1 and HSV-2 infected lymphoid cells (Chapter 3) and in HSV-1 infected fibroblasts (Figure 31A).

VP11/12 has been known for some time to be a phosphoprotein, however phosphorylation of VP11/12 was previously thought to be restricted to serine and threonine residues. It was shown some time ago that a number of tegument proteins, including VP11/12 are phosphorylated by virion associated kinases (110). These kinases were later identified as the virus encoded serine/threonine kinases US3 and UL13 (38, 58). One report suggested that these phosphorylation events may contribute to the dispersal of tegument proteins upon viral entry (143). For VP11/12 specifically, it has been suggested that serine/threonine phosphorylation is necessary for VP11/12 expression since US3 phosphorylates VP11/12 *in vitro* and VP11/12 is absent during infection by US3 null HSV-2 (132).

VP11/12 phosphorylation may facilitate interactions with multiple signalling effectors to activate various cellular signalling pathways. Analysis of the VP11/12 sequence using the Scansite 2.0 algorithm (156) suggests that phosphotyrosine residues recruit many SH2 domain containing proteins and at least one protein containing a phosphotyrosine binding group (PTB) domain. In addition, VP11/12 is also predicted to be the target of serine/threonine kinases and to facilitate at least one phosphoserine-specific interaction. These predictions call for a thorough investigation of VP11/12 phosphorylation and the signalling events resulting from this phosphorylation. Phosphorylation sites could be determined through two dimensional phosphopeptide mapping. Along with the Scansite 2.0 analysis, the identified phosphorylation sites could be used to predict novel interactions with signalling effectors, and aid in fully elucidating the signalling capabilities of VP11/12.

Phosphorylation of VP11/12 may also facilitate interactions with signal transducing proteins downstream of active Akt. One residue in VP11/12 (S478 in VP11/12 of HSV-1 strain 17) is predicted to be a substrate of Akt, and upon phosphorylation it is predicted to bind the adaptor protein and downstream effector of Akt, 14-3-3. Potential phosphorylation of this motif could be detected using an antibody designed to detect the phosphorylated form of any Akt substrate or through two dimensional phosphopeptide mapping. If phosphorylation is detectable then PI3K inhibitors could be used to determine whether Akt phosphorylates this site. In parallel, immunoprecipitation/western blot

experiments could determine whether 14-3-3 interacts with VP11/12, and if an interaction is detected then VP11/12 mutagenesis could be used to address whether interaction requires phosphorylation of S478.

6.3 Tyrosine Phosphorylated Infected Lymphocyte Proteins

Tyrosine phosphorylated proteins with an apparent molecular weight lower than that of VP11/12 are induced by HSV lymphocyte infection and co-transfection (Figure 8, 11, 12) of VP11/12 and Lck (Figure 14). Most notable were the phosphoproteins of ca. 35-40 kDa; ca. 50-60kDa; and ca. 60-65 kDa as they are prominent during infection by wild type KOS37 and RUL46, but are much less apparent during infection by VP11/12 null virus (Figure 11).

Based on the data in this document, it is difficult to speculate on the identity of any of these tyrosine phosphorylation proteins. Immunoprecipitation of VP11/12-GFP during GHSV-UL46 infection produced tyrosine phosphorylated proteins of a lower molecular weight (ca. 50, 65, and 90 kDa) (Figure 9). Therefore, these proteins may associate with VP11/12 and their tyrosine phosphorylation may be dependent on the formation of a VP11/12 signalling complex. These proteins may also be products of VP11/12 degradation or cleavage. Western blot analysis using anti-GFP demonstrated that VP11/12-GFP is capable of generating low molecular weight products in cells transfected to express VP11/12-GFP (Figure 14).

The ca. 50-60 kDa phosphoprotein(s) are likely to represent Lck and other SFKs, since VP11/12 is required for infection induced tyrosine phosphorylation of the SFK Lck in infected T cells (Chapter 4). Consistent with this hypothesis, a tyrosine phosphorylated protein of ca. 50-60 kDa was observed in Vero cells transfected with active Lck alone (Figure 14).

6.4 A Putative Mechanism for Binding and Activation of Lck

As noted in the introduction, SFK substrates can induce kinase activation through high-affinity binding to the SH2 and/or SH3 domain (reviewed in (17, 54)). All SFKs are held in an inactive conformation by low affinity intramolecular interactions of the SH2 and SH3 domains. In theory, high affinity binding of the SH2 domain by a motif like YEEI interrupts inhibitory intramolecular interactions and locks the enzyme in an open and active conformation. In this context, it is interesting to note that the scansite algorithm (156) identifies two putative Lck SH2 binding motifs in VP11/12 of HSV-1 and HSV-2 (located at Y613 and Y624 in VP11/12 of HSV-1 strain17). The motif at Y624 has the sequence YEEI, which corresponds to the peptide sequence preferred by the SH2 domains of Lck, Fgr, Fyn, and Src (204). Indeed the affinity of the Lck SH2 domain for YEEI is so high that a mutant Lck with YEEI in the place of the native C-terminal inhibitory motif cannot be activated by the TCR (153). Thus, an attractive model is that the YEEI motif of VP11/12 binds the SH2 domain of Lck with high affinity, displacing the C-terminal inhibitory motif.

Interestingly, the YEEI motif of MTAg is also thought to facilitate Src activation (17, 188), suggesting that these two viral proteins use analogous mechanisms to activate Src-PI3K-Akt signalling (discussed in detail in section 6.9).

Further experimentation is required to determine whether VP11/12 interacts with Lck directly and if so, which motifs in VP11/12 facilitate that interaction.

6.5 VP11/12 Activates Multiple SFKs in Multiple Cell Lineages

The prominent tyrosine phosphorylation of VP11/12 by Lck in lymphocytes (Chapter 3) initially led us to speculate that VP11/12 functions only in lymphocytes to initiate lymphocyte specific signalling. Collectively our data now suggest that VP11/12 activates lymphoid and non-lymphoid SFKs, and SFKs from multiple cell lineages phosphorylate VP11/12. Tyrosine phosphorylation of VP11/12 can be detected in the 721.221 B cell line (Figure 8), and at a much lower level in HEL fibroblasts (Figure 12 and 31) and the Vero epithelial cell line (Figure 12). None of these cell lines are likely to express Lck suggesting that VP11/12 is phosphorylated by multiple SFKs. During HEL fibroblast infection, we have also observed that a small fraction of active SFKs are induced by infection in a VP11/12 dependent manner (Figure 31) and that the subtle tyrosine phosphorylation of VP11/12 is dependent on SFK activity (Figure 32). This suggests that other SFKs are activated by VP11/12 in the absence of Lck.

6.6 Studies of VP11/12 May Provide Insight Into Cellular PI3K-Akt Signalling

VP11/12 interacts with PI3K, suggesting that it directly activates PI3K. However, it is unclear whether VP11/12-PI3K interactions are sufficient to induce PI3K-Akt signalling. For this to be the case, VP11/12 may need to modulate multiple signalling components in the PI3K-Akt signalling axis. For example, one might predict that VP11/12 would need to simultaneously activate PI3K and suppress its antagonist, PTEN to allow for activation of Akt. Our evidence suggests that VP11/12 mediates the activation of multiple Src family members and one report previously demonstrated that active SFKs interfere with PTEN activity (124). Thus, VP11/12 mediated SFK activity may also disrupt the activity of PTEN. Our results suggest PTEN involvement since the PTEN deficient Jurkat cells demonstrate weak HSV mediated activation of Akt compared to HEL cells (Figure 27 and 28). However, we have yet to directly investigate the involvement of PTEN. PTEN null mouse embryonic fibroblasts (MEFs) have been described. Assuming that HSV infection is able to activate Akt in MEFs then PTEN null MEFs could be used to specifically address whether PTEN suppression contributes to infection induced activation of Akt. Furthermore, it was previously suggested that PTEN impairment was due to SFK-mediated phosphorylation and subsequent degradation of PTEN (124). These events could also be examined during HSV infection. Determining whether SFKs inhibit PTEN during HSV infection may be a first step in determining whether this mechanism activates PI3K-Akt signalling in multiple biological systems.

VP11/12 also needs to modulate phosphorylation of S473 of the hydrophobic motif for full activation of Akt (186). The kinase(s) responsible for S473 phosphorylation is known as PDK-2; the identity of this kinase(s) and the trigger for its activation has yet to be conclusively determined. In a potentially pivotal study, mTORC2 was shown to be necessary and sufficient for the phosphorylation of the hydrophobic loop serine (S473) of Akt (186). This suggests that VP11/12 mediated activation of Akt requires activation of mTORC2; a possibility that can be easily investigated by examining HSV induced S473 phosphorylation in the presence of inhibitors torin1 and rapamycin. Torin1 inhibits the mTOR kinase of mTORC1 and mTORC2. Since rapamycin specifically inhibits mTORC1, the effects of mTORC2 can be elucidated from any effect seen in torin1 treated samples that is absent in rapamycin treated samples. Kevin Quach from our lab has already used these experiments to determine that mTORC2 is necessary to phosphorylate S473 of Akt during HSV infection. HSV-1 infection can now be used as a model to study the mechanism(s) of mTORC2 activation, therefore providing insight into the cellular mechanisms governing Akt phosphorylation and activation.

6.7 HSV May Encode Multiple Regulators of Akt Signalling

It remains possible that HSV-1 may encode other signalling modulators that contribute to VP11/12-dependent activation of Akt. One report demonstrated that expression of HSV-1 LAT in neuroblastoma cells was sufficient to increase

levels of total Akt and phosphorylated Akt (112). Another report suggested that the serine/threonine kinase activity of HSV-2 ICP10 is required to activate Akt during infection (68). HSV-1 null mutants lacking LAT (163), and ICP6 (the homologue of HSV-2 ICP10; (15)) have been previously described. These mutants could be examined to determine if either LAT or ICP6 is necessary for activation of Akt in infected HEL fibroblasts.

Previously, the HSV-1 serine threonine kinase US3 was shown to mediate a decrease in the amount of active/phosphorylated Akt during HSV-1 infection of the epithelial cervical carcinoma cell line, HEp-2 (12). Combined with our findings, this suggests that HSV encodes both positive (VP11/12) and negative (US3) regulators of Akt. In support of this theory, infection of Jurkat T cells by VP11/12 null HSV-1 induces a decrease in the level of phospho-S473 Akt compared to mock infected samples (Figure 27). If HSV does utilize these two viral proteins to activate and inhibit Akt, then the activation status of Akt during infection may depend on the availability of the targeted cellular signalling effectors, which may be dictated by cell line specific basal signalling events. The use of such an intricate system to control Akt activity would imply that Akt signalling is a critical variable in determining the course of HSV infection.

US3 has also been implicated in the phosphorylation of several Akt substrates. Prevention of apoptosis by US3 occurs in part through phosphorylation of the Akt substrate BAD (23). Infection induced mTORC1

signalling requires US3-dependent phosphorylation of the upstream effector TSC2 (140). Furthermore, our lab has now shown that US3 is necessary for phosphorylation of the canonical Akt substrates GSK-3β, and FOXO1 during infection (Kevin Quach unpublished data). Collectively, these studies suggest that US3 suppresses cellular Akt and acts as a viral mimic of Akt to phosphorylate targets that benefit the virus.

Future experiments in our lab will specifically address the role of US3 in VP11/12 mediated Akt activation. As outlined above, US3 null HSV-1 demonstrates enhanced Akt activation compared to wild type HSV-1 infection of HEp-2 cells (12). However, a study of US3 null HSV-2 demonstrated that this virus fails to express VP11/12 (132). We hypothesize that viral or cellular mechanisms control the relative expression of US3 and VP11/12 in order to control Akt activation. This leads us to ask whether VP11/12 is present and responsible for Akt activation during US3 null HSV-1 infection of HEp-2 cells (12), and whether US3 is responsible for Akt inhibition during VP11/12 null HSV-1 infection of Jurkat T cells (Figure 27). Presently, our experiments have confirmed that US3 null HSV-1 demonstrates enhanced infection induced activation of Akt in HEp-2 cells (Fred Wu unpublished data). Expression of VP11/12 in these samples will now be assessed. If VP11/12 is expressed, an HSV-1 mutant null for both VP11/12 and US3 will be constructed, and used to determine whether VP11/12 is responsible for Akt activation in the absence of US3. Similarly, in Jurkat cells, infection by this null mutant will determine

whether US3 is responsible for inhibition of Akt in the absence of VP11/12. Others have speculated that VP11/12 expression may be stabilized by US3-mediated phosphorylation since US3 phosphorylates VP11/12 *in vitro* (132). Therefore, we will also investigate whether US3 phosphorylates VP11/12 *in vivo*, and whether this phosphorylation stabilizes VP11/12 expression. Since US3 is known to phosphorylate Akt substrates this may include an examination of VP11/12's putative Akt phosphorylation site (located at S478 in VP11/12 of HSV-1 strain17).

6.8 A Putative Mechanism for VP11/12-dependent PI3K Activation

As reviewed in the introduction, membrane associated growth receptors and MTAg use a p85 SH2 binding motif (YXXM) to recruit PI3K to the membrane and allow for subsequent PI3K activity. VP11/12 may use an analogous mechanism to activate the canonical PI3K-Akt signalling pathway. In support of this theory, VP11/12 is found in a complex with PI3K during infection, and the sequence of VP11/12 contains a putative p85 SH2 binding motif (YTHM; located at Y519 in VP11/12 of HSV-1 strain17). VP11/12 is also known to associate with membrane during infection (148), therefore binding of PI3K by VP11/12 simultaneously recruits PI3K to its substrate PIP2. Finally, tyrosine phosphorylation is a prerequisite for SH2 binding, thus, the fact that SFK activity is required for VP11/12 tyrosine phosphorylation, VP11/12-PI3K binding, and activation of Akt provides additional support for this hypothesis. Further

experimentation is needed to determine which motifs on VP11/12 are required for PI3K binding and PI3K-Akt signalling.

6.9 A Putative Mechanism for VP11/12-dependent SFK-PI3K-Akt Signalling

The sequence of VP11/12 suggests it may activate multiple Src family members upstream of PI3K in a mechanism analogous to that of the growth receptor, PDGFR-β and the polyoma MTAg. As described in section 6.4 and 6.8, we theorize that the predicted SFK SH2 binding motif, YEEI and the putative p85 SH2 binding motif YTHM in VP11/12 activate SFKs and PI3K, respectively. Similar motifs coordinate Src-PI3K-Akt signalling downstream of PDGFR-β. High affinity binding of the SH2 domain of Src by PDGFR-β activates Src (142), allows for massive tyrosine phosphorylation of PDGFR-β, and subsequently allows for SH2-mediated binding of PI3K (10). Similarly, MTAg is thought to bind and activate Src at a YEEI motif, leading to phosphorylation of a p85 SH2 binding motif within MTAg; MTAg-PI3K binding; and subsequent PI3K activity (221). It therefore seems likely that VP11/12 also uses its putative SH2 binding motifs (YEEI for SFKs and YTHM for p85) to propagate SFK-PI3K-Akt signalling.

Future studies will address whether VP11/12 mediates signalling through SH2 binding. SH2-mediated interactions can be detected through *in vitro* binding assays. *In vivo*, a tyrosine to phenylalanine mutation of the putative SFK and p85 SH2 binding motifs in VP11/12 can also be used to determine whether SH2

binding is required for signalling. Of note, the two motifs predicted to bind the SFK SH2 domain (Y613 and Y624 in VP11/12 of HSV-1 strain 17) may function redundantly and mutagenesis of both may be required to accurately gauge the effect of these motifs on SFK binding and activation.

6.10 Conservation of the Putative SFK and PI3K SH2 Binding Motifs

The biological function of VP11/12 remains elusive, but conservation of amino acids within the putative SH2 binding motifs for SFKs and PI3K suggests that signalling propagated through these motifs is critical for virus survival. As discussed in sections 6.4 and 6.8, VP11/12 contains one putative p85 and two putative SFK SH2 binding motifs, which can, in theory promote kinase activity. We compared the known amino acid sequences of VP11/12 and VP11/12 homologues within the alphaherpesvirinae. We found conservation of these putative SH2 binding motifs within the Simplexviruses, which includes sequences reported for HSV-1, HSV-2, cercopithecine herpesvirus (CHV)-1, CHV-2, and CHV-16 (Figure 39). Each sequence demonstrates conservation of the consensus sequence of p85 SH2 binding (YXXM) at the equivalent of Y519 for HSV-1 strain 17, and perfect sequence identity at the high affinity SFK SH2 binding motif (YEEI), the equivalent of Y624 for HSV-1 strain 17. Conservation of these sequences suggests that evolutionary pressure has forced all Simplexviruses to retain the ability to activate SFK-PI3K-Akt signalling through VP11/12.

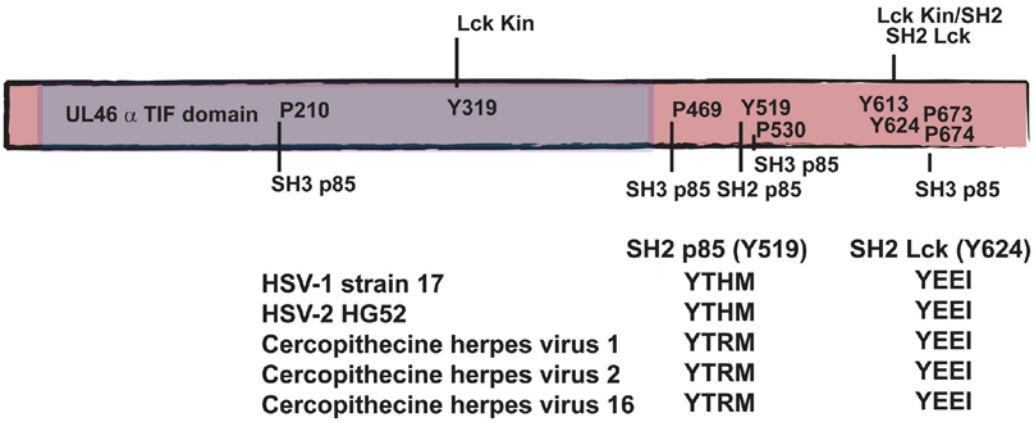

Figure 39. Simplexviruses conserve the SFK and p85 SH2 binding motifs of VP11/12.

Known VP11/12 sequences of the alphaherpesvirinae were examined for the presence or absence of the SFK SH2 high affinity binding sequence (YEEI) and the consensus sequence for p85 SH2 domain binding (YXXM). SFK and p85 SH2 binding motifs demonstrated conservation of sequence within the simplex genus of the alphaherpesvirinae.

6.11 VP11/12 May Serve Multiple and/or Cell-type Specific Functions

Like many viral proteins, VP11/12 is likely to have more than one function and some of these functions may be cell type specific. One indication that VP11/12 has multiple functions is found in the amino acid sequence. The SFK and p85 SH2 binding motifs that are conserved in simplexviruses lie outside of an N-terminal region which is conserved among all alphaherpesvirinae (Figure 39; α-TIF domain; the equivalent of amino acid 27-443 for HSV-1 strain 17). We predict that the SH2 binding motifs of VP11/12 facilitate SFK-PI3K-Akt signalling in simplexvirus infection, while the conserved N-terminal region serves a function that is critical to the survival of all alphaherpesviruses.

We have shown that Lck generates intense tyrosine phosphorylation of VP11/12 compared to the SFKs in fibroblasts or epithelial cells (Chapter 3). Since Lck expression is largely restricted to lymphocytes (130), this data suggests that VP11/12 may serve one or more lymphocyte-specific functions. The prominent phosphorylation of VP11/12 may indicate phosphorylation of additional tyrosines residues in lymphocytes, and these phosphotyrosines could allow for the recruitment of additional signal transducing proteins capable of initiating multiple lymphocyte specific signalling pathways. Thus, the enhanced tyrosine phosphorylation of VP11/12 may generate unique signals in infected lymphocytes for the manipulation of signalling related to lymphocyte function. Alternatively, the intense phosphorylation of VP11/12 in lymphocytes may

indicate that a greater proportion of VP11/12 molecules are phosphorylated in these cells. While this would not necessarily result in lymphocyte specific signalling, it may indicate an importance of VP11/12 mediated signalling and viral manipulation of the associated cellular function(s) in these cells.

HSV infects T lymphocytes and is known to alter their function as an immune evasion strategy (reviewed in (89)). HSV is able to infect primary T cells *in vitro* (8, 101, 166, 167, 173, 199), and also infects peripheral T cells in the intact human host (8). Infected T cells and NK cells display diminished target cell killing, and infected T cells also display enhanced fratricide, and altered cytokine profiles (167, 168, 173, 199, 200, 251), at least in part due to HSV-induced modifications to the TCR signalling pathway (199, 200). We have shown that VP11/12 is not required for inhibition of TCR signalling (Figure 15) or NK cell killing (Figure 16). However, this data cannot exclude VP11/12 as a potential modulator of immune related signalling. The laboratory of K.R. Jerome has accumulated data which demonstrates that overexpression of VP11/12 is sufficient to inhibit TCR signals (K.R. Jerome, personal communication). Thus, VP11/12 along with other virus proteins may contribute to viral manipulation of immune receptor signalling including signalling emanating from the TCR.

VP11/12-Lck interactions may also exert cell-type specific effects in neurons rather than lymphocytes. Although Lck is conventionally considered a lymphocyte specific kinase, immunocytochemistry has been used to suggest that

Lck is present in mouse and rat neurons (157, 230). Neural expression of Lck has not been widely confirmed in the literature. One report indicates that Lck cannot be detected in a normal human brain (211). Moreover, the expression level of Lck reported for the mouse brain is tenfold lower than that of the murine thymus (157). If Lck is present in neurons then VP11/12-Lck interactions could conceivably play a role in signalling that promotes the establishment of latency, reactivation of the virus, or neural spread of HSV.

6.12 VP11/12 Null Virus as a Vector in Genetic-based Cancer Therapeutics

HSV-1 has been investigated for its ability to act as a vector carrying "suicide" genes for expression in cancerous cells. Our data showing VP11/12 dependent activation of PI3K-Akt signalling (Chapter 5) suggests that a VP11/12 null version of this vector may be more efficient at killing tumour cells. VP11/12 null HSV-1 should be capable of efficiently expressing a "suicide" gene, since removal of VP11/12 from an HSV-1 vector was previously shown to enhance long-term expression of a reporter gene (118). In addition, removal of VP11/12 may enhance the vector's ability to induce cell death, since VP11/12 activates the PI3K-Akt signalling axis and this pathway is known to enhance tumour cell survival (reviewed in (119)). Further investigation is required to determine whether removal of VP11/12 makes the HSV-1 vector into a more efficient killer.

6.13 Future Directions

The work in this thesis provides the first description of a function for VP11/12 during infection. Our data indicating that VP11/12 activates SFK-PI3K-Akt signalling has defined VP11/12 as a cell signalling modulator. This adds to our knowledge of HSV infection induced changes to cell signalling and is a first step toward understanding the signalling capabilities of VP11/12. Work in our lab presently aims to define other cellular signalling pathways targeted by VP11/12; other virus encoded signalling modulators; and the molecular mechanisms governing VP11/12 mediated activation of the SFK-PI3K-Akt pathway.

Regrettably, we have yet to determine the biological function(s) of VP11/12. Working in cell culture, VP11/12 null virus produced progeny at levels comparable to that of wild type virus, and the absence of VP11/12 during infection produced no major changes in infected cell morphology. Therefore, future studies should aim to determine the impact of VP11/12 on viral pathogenesis. Such studies would involve the examination of HSV-1 null for VP11/12, and HSV-1 with targeted mutations of VP11/12 including removal of the α-TIF domain and a tyrosine to phenylalanine mutation of any SH2 binding motifs that function in infection induced signalling. Infection by these viruses in a mouse or rabbit model will demonstrate how VP11/12, VP11/12 mediated signalling, and the conserved α-TIF domain function to promote virus survival. One would expect VP11/12 to influence processes that cannot be easily measured

in cell culture systems, such as the establishment of latency, virus reactivation, viral spread, innate immunity or adaptive immunity.

CHAPTER 7: Bibliography

1. ICTVdB Management (2006). 00.031 Herpesviridae., ICTVdB-The Universal Virus Database, version 3. Buchen-Osmond, C. (Ed), Columbia University, New York, USA.

2. **Abraham, N., and A. Veillette.** 1990. Activation of p56lck through mutation of a regulatory carboxy-terminal tyrosine residue requires intact sites of autophosphorylation and myristylation. Mol Cell Biol **10:**5197-206.

3. **Alwine, J. C., W. L. Steinhart, and C. W. Hill.** 1974. Transcription of herpes simplex type 1 DNA in nuclei isolated from infected HEp-2 and KB cells. Virology **60:**302-7.

4. **Ambagala, A. P., J. C. Solheim, and S. Srikumaran.** 2005. Viral interference with MHC class I antigen presentation pathway: the battle continues. Vet Immunol Immunopathol **107:**1-15.

5. **Amrein, K. E., and B. M. Sefton.** 1988. Mutation of a site of tyrosine phosphorylation in the lymphocyte-specific tyrosine protein kinase, p56lck, reveals its oncogenic potential in fibroblasts. Proc Natl Acad Sci U S A **85:**4247-51.

6. **Atkinson, E. A., M. Barry, A. J. Darmon, I. Shostak, P. C. Turner, R. W. Moyer, and R. C. Bleackley.** 1998. Cytotoxic T lymphocyte-assisted suicide. Caspase 3 activation is primarily the result of the direct action of granzyme B. J Biol Chem **273:**21261-6.

7. **Aubert, M., Z. Chen, R. Lang, C. H. Dang, C. Fowler, D. D. Sloan, and K. R. Jerome.** 2008. The antiapoptotic herpes simplex virus glycoprotein J localizes to multiple cellular organelles and induces reactive oxygen species formation. J Virol **82:**617-29.

8. **Aubert, M., M. Yoon, D. D. Sloan, P. G. Spear, and K. R. Jerome.** 2009. The virological synapse facilitates herpes simplex virus entry into T cells. J Virol **83:**6171-83.

9. **Baucke, R. B., and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. V. Identification of an Fc-binding glycoprotein. J Virol **32:**779-89.

10. **Baxter, R. M., J. P. Secrist, R. R. Vaillancourt, and A. Kazlauskas.** 1998. Full activation of the platelet-derived growth factor beta-receptor kinase involves multiple events. J Biol Chem **273:**17050-5.

11. **Benati, D., and C. T. Baldari.** 2008. SRC family kinases as potential therapeutic targets for malignancies and immunological disorders. Curr Med Chem **15:**1154-65.

12. **Benetti, L., and B. Roizman.** 2006. Protein kinase B/Akt is present in activated form throughout the entire replicative cycle of deltaU(S)3 mutant virus but only at early times after infection with wild-type herpes simplex virus 1. J Virol **80:**3341-8.

13. **Blaho, J. A., C. S. Zong, and K. A. Mortimer.** 1997. Tyrosine phosphorylation of the herpes simplex virus type 1 regulatory protein ICP22 and a cellular protein which shares antigenic determinants with ICP22. J Virol **71:**9828-32.

14. **Bolen, J. B., C. J. Thiele, M. A. Israel, W. Yonemoto, L. A. Lipsich, and J. S. Brugge.** 1984. Enhancement of cellular src gene product associated tyrosyl kinase activity following polyoma virus infection and transformation. Cell **38:**767-77.

15. **Brandt, C. R., R. L. Kintner, A. M. Pumfery, R. J. Visalli, and D. R. Grau.** 1991. The herpes simplex virus ribonucleotide reductase is required for ocular virulence. J Gen Virol **72 (Pt 9):**2043-9.

16. **Brinkmann, M. M., and T. F. Schulz.** 2006. Regulation of intracellular signalling by the terminal membrane proteins of members of the Gammaherpesvirinae. J Gen Virol **87:**1047-74.

17. **Brown, M. T., and J. A. Cooper.** 1996. Regulation, substrates and functions of src. Biochim Biophys Acta **1287:**121-49.

18. **Brunet, A., A. Bonni, M. J. Zigmond, M. Z. Lin, P. Juo, L. S. Hu, M. J. Anderson, K. C. Arden, J. Blenis, and M. E. Greenberg.** 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell **96:**857-68.

19. **Bubeck Wardenburg, J., C. Fu, J. K. Jackman, H. Flotow, S. E. Wilkinson, D. H. Williams, R. Johnson, G. Kong, A. C. Chan, and P. R. Findell.** 1996. Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. J Biol Chem **271:**19641-4.

20. **Buchkovich, N. J., Y. Yu, C. A. Zampieri, and J. C. Alwine.** 2008. The TORrid affairs of viruses: effects of mammalian DNA viruses on the PI3K-Akt-mTOR signalling pathway. Nat Rev Microbiol **6:**266-75.

21. **Bucks, M. A., K. J. O'Regan, M. A. Murphy, J. W. Wills, and R. J. Courtney.** 2007. Herpes simplex virus type 1 tegument proteins VP1/2 and UL37 are associated with intranuclear capsids. Virology **361:**316-24.

22. **Cartier, A., E. Broberg, T. Komai, M. Henriksson, and M. G. Masucci.** 2003. The herpes simplex virus-1 Us3 protein kinase blocks CD8T cell lysis by preventing the cleavage of Bid by granzyme B. Cell Death Differ **10:**1320-8.

23. **Cartier, A., T. Komai, and M. G. Masucci.** 2003. The Us3 protein kinase of herpes simplex virus 1 blocks apoptosis and induces phosporylation of the Bcl-2 family member Bad. Exp Cell Res **291:**242-50.

24. **Chan, A. C., M. Iwashima, C. W. Turck, and A. Weiss.** 1992. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. Cell **71:**649-62.

25. **Chen, S. H., M. F. Kramer, P. A. Schaffer, and D. M. Coen.** 1997. A viral function represses accumulation of transcripts from productive-cycle genes in mouse ganglia latently infected with herpes simplex virus. J Virol **71:**5878-84.

26. **Cheng, J., J. A. DeCaprio, M. M. Fluck, and B. S. Schaffhausen.** 2009. Cellular transformation by Simian Virus 40 and Murine Polyoma Virus T antigens. Semin Cancer Biol **19:**218-28.

27. **Cho, N. H., P. Feng, S. H. Lee, B. S. Lee, X. Liang, H. Chang, and J. U. Jung.** 2004. Inhibition of T cell receptor signal transduction by tyrosine kinase-interacting protein of Herpesvirus saimiri. J Exp Med **200:**681-7.

28. **Confer, D. L., G. M. Vercellotti, D. Kotasek, J. L. Goodman, A. Ochoa, and H. S. Jacob.** 1990. Herpes simplex virus-infected cells disarm killer lymphocytes. Proc Natl Acad Sci U S A **87:**3609-13.

29. **Cooper, J. A., K. L. Gould, C. A. Cartwright, and T. Hunter.** 1986. Tyr527 is phosphorylated in pp60c-src: implications for regulation. Science **231:**1431-4.

30. **Copeland, A. M., W. W. Newcomb, and J. C. Brown.** 2009. Herpes simplex virus replication: roles of viral proteins and nucleoporins in capsid-nucleus attachment. J Virol **83:**1660-8.

31. **Corcoran, J. A., W. L. Hsu, and J. R. Smiley.** 2006. Herpes simplex virus ICP27 is required for virus-induced stabilization of the ARE-

containing IEX-1 mRNA encoded by the human IER3 gene. J Virol **80:**9720-9.

32. **Court, D. L., J. A. Sawitzke, and L. C. Thomason.** 2002. Genetic engineering using homologous recombination. Annu Rev Genet **36:**361-88.

33. **Courtneidge, S. A.** 1985. Activation of the pp60c-src kinase by middle T antigen binding or by dephosphorylation. EMBO J **4:**1471-7.

34. **Courtneidge, S. A., and A. E. Smith.** 1983. Polyoma virus transforming protein associates with the product of the c-src cellular gene. Nature **303:**435-9.

35. **Cross, D. A., D. R. Alessi, P. Cohen, M. Andjelkovich, and B. A. Hemmings.** 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature **378:**785-9.

36. **Cunningham, A. L., R. R. Turner, A. C. Miller, M. F. Para, and T. C. Merigan.** 1985. Evolution of recurrent herpes simplex lesions. An immunohistologic study. J Clin Invest **75:**226-33.

37. **D'Oro, U., K. Sakaguchi, E. Appella, and J. D. Ashwell.** 1996. Mutational analysis of Lck in CD45-negative T cells: dominant role of tyrosine 394 phosphorylation in kinase activity. Mol Cell Biol **16:**4996-5003.

38. **Daikoku, T., S. Shibata, F. Goshima, S. Oshima, T. Tsurumi, H. Yamada, Y. Yamashita, and Y. Nishiyama.** 1997. Purification and characterization of the protein kinase encoded by the UL13 gene of herpes simplex virus type 2. Virology **235:**82-93.

39. **Darr, C. D., A. Mauser, and S. Kenney.** 2001. Epstein-Barr virus immediate-early protein BRLF1 induces the lytic form of viral replication through a mechanism involving phosphatidylinositol-3 kinase activation. J Virol **75:**6135-42.

40. **Datta, S. R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, and M. E. Greenberg.** 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell **91:**231-41.

41. **Davison, A. J., R. Eberle, B. Ehlers, G. S. Hayward, D. J. McGeoch, A. C. Minson, P. E. Pellett, B. Roizman, M. J. Studdert, and E. Thiry.** 2009. The order Herpesvirales. Arch Virol **154:**171-7.

42. **Dawson, C. W., G. Tramountanis, A. G. Eliopoulos, and L. S. Young.** 2003. Epstein-Barr virus latent membrane protein 1 (LMP1) activates the phosphatidylinositol 3-kinase/Akt pathway to promote cell survival and induce actin filament remodeling. J Biol Chem **278:**3694-704.

43. **del Peso, L., M. Gonzalez-Garcia, C. Page, R. Herrera, and G. Nunez.** 1997. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science **278:**687-9.

44. **DeLuca, N. A., A. M. McCarthy, and P. A. Schaffer.** 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J Virol **56:**558-70.

45. **Desai, P., G. L. Sexton, J. M. McCaffery, and S. Person.** 2001. A null mutation in the gene encoding the herpes simplex virus type 1 UL37 polypeptide abrogates virus maturation. J Virol **75:**10259-71.

46. **Desai, P. J.** 2000. A null mutation in the UL36 gene of herpes simplex virus type 1 results in accumulation of unenveloped DNA-filled capsids in the cytoplasm of infected cells. J Virol **74:**11608-18.

47. **Deshmane, S. L., and N. W. Fraser.** 1989. During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. J Virol **63:**943-7.

48. **Diefenbach, R. J., M. Miranda-Saksena, M. W. Douglas, and A. L. Cunningham.** 2008. Transport and egress of herpes simplex virus in neurons. Rev Med Virol **18:**35-51.

49. **Divito, S., T. L. Cherpes, and R. L. Hendricks.** 2006. A triple entente: virus, neurons, and CD8+ T cells maintain HSV-1 latency. Immunol Res **36:**119-26.

50. **Dohner, K., A. Wolfstein, U. Prank, C. Echeverri, D. Dujardin, R. Vallee, and B. Sodeik.** 2002. Function of dynein and dynactin in herpes simplex virus capsid transport. Mol Biol Cell **13:**2795-809.

51. **Dubin, G., E. Socolof, I. Frank, and H. M. Friedman.** 1991. Herpes simplex virus type 1 Fc receptor protects infected cells from antibody-dependent cellular cytotoxicity. J Virol **65:**7046-50.

52. **Efeyan, A., and D. M. Sabatini.** 2010. mTOR and cancer: many loops in one pathway. Curr Opin Cell Biol **22:**169-76.

53. **Ellery, J. M., and P. J. Nicholls.** 2002. Alternate signalling pathways from the interleukin-2 receptor. Cytokine Growth Factor Rev **13:**27-40.

54. **Engen, J. R., T. E. Wales, J. M. Hochrein, M. A. Meyn, 3rd, S. Banu Ozkan, I. Bahar, and T. E. Smithgall.** 2008. Structure and dynamic regulation of Src-family kinases. Cell Mol Life Sci **65:**3058-73.

55. **Farnsworth, A., T. W. Wisner, M. Webb, R. Roller, G. Cohen, R. Eisenberg, and D. C. Johnson.** 2007. Herpes simplex virus glycoproteins gB and gH function in fusion between the virion envelope and the outer nuclear membrane. Proc Natl Acad Sci U S A **104:**10187-92.

56. **Feldman, L. T., A. R. Ellison, C. C. Voytek, L. Yang, P. Krause, and T. P. Margolis.** 2002. Spontaneous molecular reactivation of herpes simplex virus type 1 latency in mice. Proc Natl Acad Sci U S A **99:**978-83.

57. **Flint, J., and T. Shenk.** 1997. Viral transactivating proteins. Annu Rev Genet **31:**177-212.

58. **Frame, M. C., F. C. Purves, D. J. McGeoch, H. S. Marsden, and D. P. Leader.** 1987. Identification of the herpes simplex virus protein kinase as the product of viral gene US3. J Gen Virol **68 (Pt 10):**2699-704.

59. **Francois, F., and M. E. Klotman.** 2003. Phosphatidylinositol 3-kinase regulates human immunodeficiency virus type 1 replication following viral entry in primary CD4+ T lymphocytes and macrophages. J Virol **77:**2539-49.

60. **Frank, I., and H. M. Friedman.** 1989. A novel function of the herpes simplex virus type 1 Fc receptor: participation in bipolar bridging of antiviral immunoglobulin G. J Virol **63:**4479-88.

61. **Friedman, H. M., G. H. Cohen, R. J. Eisenberg, C. A. Seidel, and D. B. Cines.** 1984. Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. Nature **309:**633-5.

62. **Fries, L. F., H. M. Friedman, G. H. Cohen, R. J. Eisenberg, C. H. Hammer, and M. M. Frank.** 1986. Glycoprotein C of herpes simplex virus 1 is an inhibitor of the complement cascade. J Immunol **137:**1636-41.

63. **Fruehling, S., R. Swart, K. M. Dolwick, E. Kremmer, and R. Longnecker.** 1998. Tyrosine 112 of latent membrane protein 2A is

essential for protein tyrosine kinase loading and regulation of Epstein-Barr virus latency. J Virol **72:**7796-806.

64. **Fukuda, M., and R. Longnecker.** 2004. Latent membrane protein 2A inhibits transforming growth factor-beta 1-induced apoptosis through the phosphatidylinositol 3-kinase/Akt pathway. J Virol **78:**1697-705.

65. **Fukui, Y., and H. Hanafusa.** 1989. Phosphatidylinositol kinase activity associates with viral p60src protein. Mol Cell Biol **9:**1651-8.

66. **Garber, D. A., P. A. Schaffer, and D. M. Knipe.** 1997. A LAT-associated function reduces productive-cycle gene expression during acute infection of murine sensory neurons with herpes simplex virus type 1. J Virol **71:**5885-93.

67. **Gierasch, W. W., D. L. Zimmerman, S. L. Ward, T. K. Vanheyningen, J. D. Romine, and D. A. Leib.** 2006. Construction and characterization of bacterial artificial chromosomes containing HSV-1 strains 17 and KOS. J Virol Methods **135:**197-206.

68. **Gober, M. D., J. M. Laing, S. M. Thompson, and L. Aurelian.** 2006. The growth compromised HSV-2 mutant DeltaRR prevents kainic acid-induced apoptosis and loss of function in organotypic hippocampal cultures. Brain Res **1119:**26-39.

69. **Goldsmith, M. A., and A. Weiss.** 1987. Isolation and characterization of a T-lymphocyte somatic mutant with altered signal transduction by the antigen receptor. Proc Natl Acad Sci U S A **84:**6879-83.

70. **Granzow, H., B. G. Klupp, and T. C. Mettenleiter.** 2005. Entry of pseudorabies virus: an immunogold-labeling study. J Virol **79:**3200-5.

71. **Green, M. T., R. J. Courtney, and E. C. Dunkel.** 1981. Detection of an immediate early herpes simplex virus type 1 polypeptide in trigeminal ganglia from latently infected animals. Infect Immun **34:**987-92.

72. **Hagmann, M., O. Georgiev, W. Schaffner, and P. Douville.** 1995. Transcription factors interacting with herpes simplex virus alpha gene promoters in sensory neurons. Nucleic Acids Res **23:**4978-85.

73. **Hardwick, J. S., and B. M. Sefton.** 1997. The activated form of the Lck tyrosine protein kinase in cells exposed to hydrogen peroxide is phosphorylated at both Tyr-394 and Tyr-505. J Biol Chem **272:**25429-32.

74. **Hargett, D., T. McLean, and S. L. Bachenheimer.** 2005. Herpes simplex virus ICP27 activation of stress kinases JNK and p38. J Virol **79:**8348-60.

75. **Harwood, N. E., and F. D. Batista.** 2008. New insights into the early molecular events underlying B cell activation. Immunity **28:**609-19.

76. **Heldwein, E. E., and C. Krummenacher.** 2008. Entry of herpesviruses into mammalian cells. Cell Mol Life Sci **65:**1653-68.

77. **Herold, B. C., R. J. Visalli, N. Susmarski, C. R. Brandt, and P. G. Spear.** 1994. Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. J Gen Virol **75 (Pt 6):**1211-22.

78. **Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear.** 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J Virol **65:**1090-8.

79. **Hjorthaug, H. S., and H. C. Aasheim.** 2007. Ephrin-A1 stimulates migration of CD8+CCR7+ T lymphocytes. Eur J Immunol **37:**2326-36.

80. **Holen, H. L., M. Shadidi, K. Narvhus, O. Kjosnes, A. Tierens, and H. C. Aasheim.** 2008. Signaling through ephrin-A ligand leads to activation of Src-family kinases, Akt phosphorylation, and inhibition of antigen receptor-induced apoptosis. J Leukoc Biol **84:**1183-91.

81. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J Virol **14:**8-19.

82. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc Natl Acad Sci U S A **72:**1276-80.

83. **Horak, I. D., R. E. Gress, P. J. Lucas, E. M. Horak, T. A. Waldmann, and J. B. Bolen.** 1991. T-lymphocyte interleukin 2-dependent tyrosine protein kinase signal transduction involves the activation of p56lck. Proc Natl Acad Sci U S A **88:**1996-2000.

84. **Imperiale, M. J., Major, E.O.** 2007. Polyomaviruses, p. 2272-2273. *In* D. M. Knipe, Howley, P.M. (ed.), Fields Virology, 5 ed, vol. 2. Lippincott, Williams, and Wikins, Philadelphia.

85. **Ingham, R. J., J. Raaijmakers, C. S. Lim, G. Mbamalu, G. Gish, F. Chen, L. Matskova, I. Ernberg, G. Winberg, and T. Pawson.** 2005. The Epstein-Barr virus protein, latent membrane protein 2A, co-opts tyrosine kinases used by the T cell receptor. J Biol Chem **280:**34133-42.

86. **Inoki, K., Y. Li, T. Zhu, J. Wu, and K. L. Guan.** 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol **4:**648-57.

87. **Inoue, H., H. Motani-Saitoh, K. Sakurada, H. Ikegaya, D. Yajima, M. Hayakawa, Y. Sato, K. Otsuka, K. Kobayashi, S. Nagasawa, and H. Iwase.** Detection of varicella-zoster virus DNA in 414 human trigeminal ganglia from cadavers by the polymerase chain reaction: a comparison of the detection rate of varicella-zoster virus and herpes simplex virus type 1. J Med Virol **82:**345-9.

88. **Iwashima, M., B. A. Irving, N. S. van Oers, A. C. Chan, and A. Weiss.** 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. Science **263:**1136-9.

89. **Jerome, K. R.** 2008. Viral modulation of T-cell receptor signaling. J Virol **82:**4194-204.

90. **Jerome, K. R., J. F. Tait, D. M. Koelle, and L. Corey.** 1998. Herpes simplex virus type 1 renders infected cells resistant to cytotoxic T-lymphocyte-induced apoptosis. J Virol **72:**436-41.

91. **Johnson, D. C., and V. Feenstra.** 1987. Identification of a novel herpes simplex virus type 1-induced glycoprotein which complexes with gE and binds immunoglobulin. J Virol **61:**2208-16.

92. **Johnson, D. C., M. C. Frame, M. W. Ligas, A. M. Cross, and N. D. Stow.** 1988. Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J Virol **62:**1347-54.

93. **Johnson, R. A., X. Wang, X. L. Ma, S. M. Huong, and E. S. Huang.** 2001. Human cytomegalovirus up-regulates the phosphatidylinositol 3-kinase (PI3-K) pathway: inhibition of PI3-K activity inhibits viral replication and virus-induced signaling. J Virol **75:**6022-32.

94. **Jung, J. U., S. M. Lang, T. Jun, T. M. Roberts, A. Veillette, and R. C. Desrosiers.** 1995. Downregulation of Lck-mediated signal transduction by tip of herpesvirus saimiri. J Virol **69:**7814-22.

95. **Karre, K., H. G. Ljunggren, G. Piontek, and R. Kiessling.** 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature **319:**675-8.

96. **Kato, K., T. Daikoku, F. Goshima, H. Kume, K. Yamaki, and Y. Nishiyama.** 2000. Synthesis, subcellular localization and VP16 interaction of the herpes simplex virus type 2 UL46 gene product. Arch Virol **145:**2149-62.

97. **Kaufman, H. E., A. M. Azcuy, E. D. Varnell, G. D. Sloop, H. W. Thompson, and J. M. Hill.** 2005. HSV-1 DNA in tears and saliva of normal adults. Invest Ophthalmol Vis Sci **46:**241-7.

98. **Kavathas, P., F. H. Bach, and R. DeMars.** 1980. Gamma ray-induced loss of expression of HLA and glyoxalase I alleles in lymphoblastoid cells. Proc Natl Acad Sci U S A **77:**4251-5.

99. **Kelly, B. J., C. Fraefel, A. L. Cunningham, and R. J. Diefenbach.** 2009. Functional roles of the tegument proteins of herpes simplex virus type 1. Virus Res **145:**173-86.

100. **Kemp, L. M., C. L. Dent, and D. S. Latchman.** 1990. Octamer motif mediates transcriptional repression of HSV immediate-early genes and octamer-containing cellular promoters in neuronal cells. Neuron **4:**215-22.

101. **Kirchner, H., Kleinicke, C., Northoff, H.** 1977. Replication of Herpes Simplex Virus in Human Peripheral T Lymphocytes. J Gen Virol **37:**647-649.

102. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of pp60c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell **49:**65-73.

103. **Koegl, M., P. Zlatkine, S. C. Ley, S. A. Courtneidge, and A. I. Magee.** 1994. Palmitoylation of multiple Src-family kinases at a homologous N-terminal motif. Biochem J **303 (Pt 3):**749-53.

104. **Koretzky, G. A., J. Picus, M. L. Thomas, and A. Weiss.** 1990. Tyrosine phosphatase CD45 is essential for coupling T-cell antigen receptor to the phosphatidyl inositol pathway. Nature **346:**66-8.

105. **Kramer, M. F., and D. M. Coen.** 1995. Quantification of transcripts from the ICP4 and thymidine kinase genes in mouse ganglia latently infected with herpes simplex virus. J Virol **69:**1389-99.

106. **Krause, P. R., K. D. Croen, S. E. Straus, and J. M. Ostrove.** 1988. Detection and preliminary characterization of herpes simplex virus type 1 transcripts in latently infected human trigeminal ganglia. J Virol **62:**4819-23.

107. **Kristensson, K., E. Lycke, M. Roytta, B. Svennerholm, and A. Vahlne.** 1986. Neuritic transport of herpes simplex virus in rat sensory neurons in vitro. Effects of substances interacting with microtubular function and axonal flow [nocodazole, taxol and erythro-9-3-(2-hydroxynonyl)adenine]. J Gen Virol **67 (Pt 9):**2023-8.

108. **Kuhns, M. S., M. M. Davis, and K. C. Garcia.** 2006. Deconstructing the form and function of the TCR/CD3 complex. Immunity **24:**133-9.

109. **Kummer, M., A. T. Prechtel, P. Muhl-Zurbes, N. M. Turza, and A. Steinkasserer.** 2009. HSV-1 upregulates the ARE-binding protein tristetraprolin in a STAT1- and p38-dependent manner in mature dendritic cells. Immunobiology **214:**852-60.

110. **Lemaster, S., and B. Roizman.** 1980. Herpes simplex virus phosphoproteins. II. Characterization of the virion protein kinase and of the polypeptides phosphorylated in the virion. J Virol **35:**798-811.

111. **Lerner, E. C., and T. E. Smithgall.** 2002. SH3-dependent stimulation of Src-family kinase autophosphorylation without tail release from the SH2 domain in vivo. Nat Struct Biol **9:**365-9.

112. **Li, S., D. Carpenter, C. Hsiang, S. L. Wechsler, and C. Jones.** 2010. Herpes simplex virus type 1 latency-associated transcript inhibits apoptosis and promotes neurite sprouting in neuroblastoma cells following serum starvation by maintaining protein kinase B (AKT) levels. J Gen Virol **91:**858-66.

113. **Liang, Y., and B. Roizman.** 2006. State and role of SRC family kinases in replication of herpes simplex virus 1. J Virol **80:**3349-59.

114. **Liedtke, W., B. Opalka, C. W. Zimmermann, and E. Lignitz.** 1993. Age distribution of latent herpes simplex virus 1 and varicella-zoster virus genome in human nervous tissue. J Neurol Sci **116:**6-11.

115. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate into cells. J Virol **62:**1486-94.

116. **Lillycrop, K. A., C. L. Dent, S. C. Wheatley, M. N. Beech, N. N. Ninkina, J. N. Wood, and D. S. Latchman.** 1991. The octamer-binding protein Oct-2 represses HSV immediate-early genes in cell lines derived from latently infectable sensory neurons. Neuron **7:**381-90.

117. **Linnemann, T., Y. H. Zheng, R. Mandic, and B. M. Peterlin.** 2002. Interaction between Nef and phosphatidylinositol-3-kinase leads to activation of p21-activated kinase and increased production of HIV. Virology **294:**246-55.

118. **Liu, M., J. Tang, X. Wang, T. Yang, and A. I. Geller.** 2005. Enhanced long-term expression from helper virus-free HSV-1 vectors packaged in the presence of deletions in genes that modulate the function of VP16, U L 46 and U L 47. J Neurosci Methods **145:**1-9.

119. **Liu, P., H. Cheng, T. M. Roberts, and J. J. Zhao.** 2009. Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov **8:**627-44.

120. **Liu, T., K. M. Khanna, X. Chen, D. J. Fink, and R. L. Hendricks.** 2000. CD8(+) T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. J Exp Med **191:**1459-66.

121. **Liu, X., S. R. Brodeur, G. Gish, Z. Songyang, L. C. Cantley, A. P. Laudano, and T. Pawson.** 1993. Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. Oncogene **8:**1119-26.

122. **Liu, X., L. E. Marengere, C. A. Koch, and T. Pawson.** 1993. The v-Src SH3 domain binds phosphatidylinositol 3'-kinase. Mol Cell Biol **13:**5225-32.

123. **Lowell, C. A.** 2004. Src-family kinases: rheostats of immune cell signaling. Mol Immunol **41:**631-43.

124. **Lu, Y., Q. Yu, J. H. Liu, J. Zhang, H. Wang, D. Koul, J. S. McMurray, X. Fang, W. K. Yung, K. A. Siminovitch, and G. B. Mills.** 2003. Src family protein-tyrosine kinases alter the function of PTEN to regulate phosphatidylinositol 3-kinase/AKT cascades. J Biol Chem **278:**40057-66.

125. **Luo, K. X., and B. M. Sefton.** 1990. Analysis of the sites in p56lck whose phosphorylation is induced by tetradecanoyl phorbol acetate. Oncogene **5:**803-8.

126. **Manning, B. D., and L. C. Cantley.** 2007. AKT/PKB signaling: navigating downstream. Cell **129:**1261-74.

127. **Manning, B. D., A. R. Tee, M. N. Logsdon, J. Blenis, and L. C. Cantley.** 2002. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol Cell **10:**151-62.

128. **Marth, J. D., J. A. Cooper, C. S. King, S. F. Ziegler, D. A. Tinker, R. W. Overell, E. G. Krebs, and R. M. Perlmutter.** 1988. Neoplastic transformation induced by an activated lymphocyte-specific protein tyrosine kinase (pp56lck). Mol Cell Biol **8:**540-50.

129. **Marth, J. D., D. B. Lewis, M. P. Cooke, E. D. Mellins, M. E. Gearn, L. E. Samelson, C. B. Wilson, A. D. Miller, and R. M. Perlmutter.** 1989. Lymphocyte activation provokes modification of a lymphocyte-specific protein tyrosine kinase (p56lck). J Immunol **142:**2430-7.

130. **Marth, J. D., R. Peet, E. G. Krebs, and R. M. Perlmutter.** 1985. A lymphocyte-specific protein-tyrosine kinase gene is rearranged and overexpressed in the murine T cell lymphoma LSTRA. Cell **43:**393-404.

131. **Martin, G. S.** 2001. The hunting of the Src. Nat Rev Mol Cell Biol **2:**467-75.

132. **Matsuzaki, A., Y. Yamauchi, A. Kato, F. Goshima, Y. Kawaguchi, T. Yoshikawa, and Y. Nishiyama.** 2005. US3 protein kinase of herpes simplex virus type 2 is required for the stability of the UL46-encoded tegument protein and its association with virus particles. J Gen Virol **86:**1979-85.

133. **McKnight, J. L., P. E. Pellett, F. J. Jenkins, and B. Roizman.** 1987. Characterization and nucleotide sequence of two herpes simplex virus 1 genes whose products modulate alpha-trans-inducing factor-dependent activation of alpha genes. J Virol **61:**992-1001.

134. **McLauchlan, J., and F. J. Rixon.** 1992. Characterization of enveloped tegument structures (L particles) produced by alphaherpesviruses: integrity of the tegument does not depend on the presence of capsid or envelope. J Gen Virol **73 (Pt 2):**269-76.

135. **McLean, T. I., and S. L. Bachenheimer.** 1999. Activation of cJUN N-terminal kinase by herpes simplex virus type 1 enhances viral replication. J Virol **73:**8415-26.

136. **Mettenleiter, T. C., B. G. Klupp, and H. Granzow.** 2009. Herpesvirus assembly: an update. Virus Res **143:**222-34.

137. **Miller, C. L., A. L. Burkhardt, J. H. Lee, B. Stealey, R. Longnecker, J. B. Bolen, and E. Kieff.** 1995. Integral membrane protein 2 of Epstein-Barr virus regulates reactivation from latency through dominant negative effects on protein-tyrosine kinases. Immunity **2:**155-66.

138. **Miller, C. L., R. Longnecker, and E. Kieff.** 1993. Epstein-Barr virus latent membrane protein 2A blocks calcium mobilization in B lymphocytes. J Virol **67:**3087-94.

139. **Minaker, R. L., K. L. Mossman, and J. R. Smiley.** 2005. Functional inaccessibility of quiescent herpes simplex virus genomes. Virol J **2:**85.

140. **Mohr, I.** 2009. Presented at the The 34th International Herpesvirus Workshop, Ithaca, New York, USA.

141. **Monroe, J. G.** 2006. ITAM-mediated tonic signalling through pre-BCR and BCR complexes. Nat Rev Immunol **6:**283-94.

142. **Mori, S., L. Ronnstrand, K. Yokote, A. Engstrom, S. A. Courtneidge, L. Claesson-Welsh, and C. H. Heldin.** 1993. Identification of two juxtamembrane autophosphorylation sites in the PDGF beta-receptor; involvement in the interaction with Src family tyrosine kinases. EMBO J **12:**2257-64.

143. **Morrison, E. E., Y. F. Wang, and D. M. Meredith.** 1998. Phosphorylation of structural components promotes dissociation of the herpes simplex virus type 1 tegument. J Virol **72:**7108-14.

144. **Motani, H., K. Sakurada, H. Ikegaya, T. Akutsu, M. Hayakawa, Y. Sato, D. Yajima, K. Sato, K. Kobayashi, and H. Iwase.** 2006. Detection of herpes simplex virus type 1 DNA in bilateral human trigeminal ganglia and optic nerves by polymerase chain reaction. J Med Virol **78:**1584-7.

145. **Mou, F., E. Wills, and J. D. Baines.** 2009. Phosphorylation of the U(L)31 protein of herpes simplex virus 1 by the U(S)3-encoded kinase regulates localization of the nuclear envelopment complex and egress of nucleocapsids. J Virol **83:**5181-91.

146. **Mou, F., E. G. Wills, R. Park, and J. D. Baines.** 2008. Effects of lamin A/C, lamin B1, and viral US3 kinase activity on viral infectivity, virion egress, and the targeting of herpes simplex virus U(L)34-encoded protein to the inner nuclear membrane. J Virol **82:**8094-104.

147. **Munter, S., M. Way, and F. Frischknecht.** 2006. Signaling during pathogen infection. Sci STKE **2006:**re5.

148. **Murphy, M. A., M. A. Bucks, K. J. O'Regan, and R. J. Courtney.** 2008. The HSV-1 tegument protein pUL46 associates with cellular membranes and viral capsids. Virology **376:**279-89.

149. **Myers, M. G., Jr., J. M. Backer, X. J. Sun, S. Shoelson, P. Hu, J. Schlessinger, M. Yoakim, B. Schaffhausen, and M. F. White.** 1992. IRS-1 activates phosphatidylinositol 3'-kinase by associating with src homology 2 domains of p85. Proc Natl Acad Sci U S A **89:**10350-4.

150. **Naldinho-Souto, R., H. Browne, and T. Minson.** 2006. Herpes simplex virus tegument protein VP16 is a component of primary enveloped virions. J Virol **80:**2582-4.

151. **Nelson, J.** 2008. Structure and Function in Cell Signalling. John Wiley & Sons Canada Ltd, Mississauga, ON.

152. **Newton, A. C.** 2009. Lipid activation of protein kinases. J Lipid Res **50 Suppl:**S266-71.

153. **Nika, K., L. Tautz, Y. Arimura, T. Vang, S. Williams, and T. Mustelin.** 2007. A weak Lck tail bite is necessary for Lck function in T cell antigen receptor signaling. J Biol Chem **282:**36000-9.

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

155. **O'Shea, C., K. Klupsch, S. Choi, B. Bagus, C. Soria, J. Shen, F. McCormick, and D. Stokoe.** 2005. Adenoviral proteins mimic nutrient/growth signals to activate the mTOR pathway for viral replication. EMBO J **24:**1211-21.

156. **Obenauer, J. C., L. C. Cantley, and M. B. Yaffe.** 2003. Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res **31:**3635-41.

157. **Omri, B., P. Crisanti, M. C. Marty, F. Alliot, R. Fagard, T. Molina, and B. Pessac.** 1996. The Lck tyrosine kinase is expressed in brain neurons. J Neurochem **67:**1360-4.

158. **Orange, J. S.** 2002. Human natural killer cell deficiencies and susceptibility to infection. Microbes Infect **4:**1545-58.

159. **Paladino, P., and K. L. Mossman.** 2009. Mechanisms employed by herpes simplex virus 1 to inhibit the interferon response. J Interferon Cytokine Res **29:**599-607.

160. **Pang, M. F., K. W. Lin, and S. C. Peh.** 2009. The signaling pathways of Epstein-Barr virus-encoded latent membrane protein 2A (LMP2A) in latency and cancer. Cell Mol Biol Lett **14:**222-47.

161. **Park, R., and J. D. Baines.** 2006. Herpes simplex virus type 1 infection induces activation and recruitment of protein kinase C to the nuclear membrane and increased phosphorylation of lamin B. J Virol **80:**494-504.

162. **Pellett, P. E., Roizman, B.** 2007. The Family Herpesviridae: A Brief Introduction. *In* D. M. Knipe, Howley, P.M. (ed.), Fields Virology, 5th ed. Lippincott Williams & Wilkins.

163. **Perng, G. C., E. C. Dunkel, P. A. Geary, S. M. Slanina, H. Ghiasi, R. Kaiwar, A. B. Nesburn, and S. L. Wechsler.** 1994. The latency-associated transcript gene of herpes simplex virus type 1 (HSV-1) is required for efficient in vivo spontaneous reactivation of HSV-1 from latency. J Virol **68:**8045-55.

164. **Pingel, J. T., and M. L. Thomas.** 1989. Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. Cell **58:**1055-65.

165. **Polak, P., and M. N. Hall.** 2009. mTOR and the control of whole body metabolism. Curr Opin Cell Biol **21:**209-18.

166. **Posavad, C. M., J. J. Newton, and K. L. Rosenthal.** 1994. Infection and inhibition of human cytotoxic T lymphocytes by herpes simplex virus. J Virol **68:**4072-4.

167. **Posavad, C. M., J. J. Newton, and K. L. Rosenthal.** 1993. Inhibition of human CTL-mediated lysis by fibroblasts infected with herpes simplex virus. J Immunol **151:**4865-73.

168. **Posavad, C. M., and K. L. Rosenthal.** 1992. Herpes simplex virus-infected human fibroblasts are resistant to and inhibit cytotoxic T-lymphocyte activity. J Virol **66:**6264-72.

169. **Potter, C. J., L. G. Pedraza, and T. Xu.** 2002. Akt regulates growth by directly phosphorylating Tsc2. Nat Cell Biol **4:**658-65.

170. **Prasad, K. V., O. Janssen, R. Kapeller, M. Raab, L. C. Cantley, and C. E. Rudd.** 1993. Src-homology 3 domain of protein kinase p59fyn mediates binding to phosphatidylinositol 3-kinase in T cells. Proc Natl Acad Sci U S A **90:**7366-70.

171. **Preston, C. M.** 1979. Abnormal properties of an immediate early polypeptide in cells infected with the herpes simplex virus type 1 mutant tsK. J Virol **32:**357-69.

172. **Purves, F. C., W. O. Ogle, and B. Roizman.** 1993. Processing of the herpes simplex virus regulatory protein alpha 22 mediated by the UL13 protein kinase determines the accumulation of a subset of alpha and gamma mRNAs and proteins in infected cells. Proc Natl Acad Sci U S A **90:**6701-5.

173. **Raftery, M. J., C. K. Behrens, A. Muller, P. H. Krammer, H. Walczak, and G. Schonrich.** 1999. Herpes simplex virus type 1 infection of activated cytotoxic T cells: Induction of fratricide as a mechanism of viral immune evasion. J Exp Med **190:**1103-14.

174. **Rajcani, J., V. Andrea, and R. Ingeborg.** 2004. Peculiarities of herpes simplex virus (HSV) transcription: an overview. Virus Genes **28:**293-310.

175. **Read, G. S., and M. Patterson.** 2007. Packaging of the virion host shutoff (Vhs) protein of herpes simplex virus: two forms of the Vhs polypeptide are associated with intranuclear B and C capsids, but only one is associated with enveloped virions. J Virol **81:**1148-61.

176. **Resh, M. D.** 1993. Interaction of tyrosine kinase oncoproteins with cellular membranes. Biochim Biophys Acta **1155:**307-22.

177. **Reth, M.** 1989. Antigen receptor tail clue. Nature **338:**383-4.

178. **Reynolds, A. E., L. Liang, and J. D. Baines.** 2004. Conformational changes in the nuclear lamina induced by herpes simplex virus type 1 require genes U(L)31 and U(L)34. J Virol **78:**5564-75.

179. **Reynolds, A. E., E. G. Wills, R. J. Roller, B. J. Ryckman, and J. D. Baines.** 2002. Ultrastructural localization of the herpes simplex virus type 1 UL31, UL34, and US3 proteins suggests specific roles in primary envelopment and egress of nucleocapsids. J Virol **76:**8939-52.

180. **Rock, D. L., A. B. Nesburn, H. Ghiasi, J. Ong, T. L. Lewis, J. R. Lokensgard, and S. L. Wechsler.** 1987. Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. J Virol **61:**3820-6.

181. **Roizman, B., Knipe, D.M., Whitley, R.J.** 2007. Herpes Simplex Virus. *In* D. M. Knipe, Howley, P.M. (ed.), Fields Virology, 5th ed. Lippincott Williams & Wilkins.

182. **Roskoski, R., Jr.** 2005. Src kinase regulation by phosphorylation and dephosphorylation. Biochem Biophys Res Commun **331:**1-14.

183. **Roskoski, R., Jr.** 2004. Src protein-tyrosine kinase structure and regulation. Biochem Biophys Res Commun **324:**1155-64.

184. **Sacks, W. R., and P. A. Schaffer.** 1987. Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. J Virol **61:**829-39.

185. **Saeed, M. F., A. A. Kolokoltsov, A. N. Freiberg, M. R. Holbrook, and R. A. Davey.** 2008. Phosphoinositide-3 kinase-Akt pathway controls cellular entry of Ebola virus. PLoS Pathog **4:**e1000141.

186. **Sarbassov, D. D., D. A. Guertin, S. M. Ali, and D. M. Sabatini.** 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science **307:**1098-101.

187. **Schaffhausen, B. S., H. Dorai, G. Arakere, and T. L. Benjamin.** 1982. Polyoma virus middle T antigen: relationship to cell membranes and apparent lack of ATP-binding activity. Mol Cell Biol **2:**1187-98.

188. **Schaffhausen, B. S., and T. M. Roberts.** 2009. Lessons from polyoma middle T antigen on signaling and transformation: A DNA tumor virus contribution to the war on cancer. Virology **384:**304-16.

189. **Schneider, U., H. U. Schwenk, and G. Bornkamm.** 1977. Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. Int J Cancer **19:**621-6.

190. **Scholle, F., K. M. Bendt, and N. Raab-Traub.** 2000. Epstein-Barr virus LMP2A transforms epithelial cells, inhibits cell differentiation, and activates Akt. J Virol **74:**10681-9.

191. **Seavitt, J. R., L. S. White, K. M. Murphy, D. Y. Loh, R. M. Perlmutter, and M. L. Thomas.** 1999. Expression of the p56(Lck) Y505F mutation in CD45-deficient mice rescues thymocyte development. Mol Cell Biol **19:**4200-8.

192. **Shan, X., M. J. Czar, S. C. Bunnell, P. Liu, Y. Liu, P. L. Schwartzberg, and R. L. Wange.** 2000. Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. Mol Cell Biol **20:**6945-57.

193. **Shieh, M. T., D. WuDunn, R. I. Montgomery, J. D. Esko, and P. G. Spear.** 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J Cell Biol **116:**1273-81.

194. **Shih, W. L., M. L. Kuo, S. E. Chuang, A. L. Cheng, and S. L. Doong.** 2000. Hepatitis B virus X protein inhibits transforming growth factor-beta -induced apoptosis through the activation of phosphatidylinositol 3-kinase pathway. J Biol Chem **275:**25858-64.

195. **Sicheri, F., I. Moarefi, and J. Kuriyan.** 1997. Crystal structure of the Src family tyrosine kinase Hck. Nature **385:**602-9.

196. **Simmons, A., and D. C. Tscharke.** 1992. Anti-CD8 impairs clearance of herpes simplex virus from the nervous system: implications for the fate of virally infected neurons. J Exp Med **175:**1337-44.

197. **Simpson-Holley, M., R. C. Colgrove, G. Nalepa, J. W. Harper, and D. M. Knipe.** 2005. Identification and functional evaluation of cellular and viral factors involved in the alteration of nuclear architecture during herpes simplex virus 1 infection. J Virol **79:**12840-51.

198. **Skepper, J. N., A. Whiteley, H. Browne, and A. Minson.** 2001. Herpes simplex virus nucleocapsids mature to progeny virions by an envelopment --> deenvelopment --> reenvelopment pathway. J Virol **75:**5697-702.

199. **Sloan, D. D., J. Y. Han, T. K. Sandifer, M. Stewart, A. J. Hinz, M. Yoon, D. C. Johnson, P. G. Spear, and K. R. Jerome.** 2006. Inhibition of TCR signaling by herpes simplex virus. J Immunol **176:**1825-33.

200. **Sloan, D. D., and K. R. Jerome.** 2007. Herpes simplex virus remodels T-cell receptor signaling, resulting in p38-dependent selective synthesis of interleukin-10. J Virol **81:**12504-14.

201. **Sloan, D. D., G. Zahariadis, C. M. Posavad, N. T. Pate, S. J. Kussick, and K. R. Jerome.** 2003. CTL are inactivated by herpes simplex virus-infected cells expressing a viral protein kinase. J Immunol **171:**6733-41.

202. **Smiley, J. R.** 2004. Herpes simplex virus virion host shutoff protein: immune evasion mediated by a viral RNase? J Virol **78:**1063-8.

203. **Smith-Garvin, J. E., G. A. Koretzky, and M. S. Jordan.** 2009. T cell activation. Annu Rev Immunol **27:**591-619.

204. **Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnofsky, R. J. Lechleider, and et al.** 1993. SH2 domains recognize specific phosphopeptide sequences. Cell **72:**767-78.

205. **Soula, M., B. Rothhut, L. Camoin, J. L. Guillaume, D. Strosberg, T. Vorherr, P. Burn, F. Meggio, S. Fischer, and R. Fagard.** 1993. Anti-CD3 and phorbol ester induce distinct phosphorylated sites in the SH2 domain of p56lck. J Biol Chem **268:**27420-7.

206. **Spear, P. G., and B. Roizman.** 1967. Buoyant density of herpes simplex virus in solutions of caesium chloride. Nature **214:**713-4.

207. **Spear, P. G., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J Virol **9:**143-59.

208. **Staal, S. P.** 1987. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci U S A **84:**5034-7.

209. **Stackpole, C. W.** 1969. Herpes-type virus of the frog renal adenocarcinoma. I. Virus development in tumor transplants maintained at low temperature. J Virol **4:**75-93.

210. **Stephens, L., K. Anderson, D. Stokoe, H. Erdjument-Bromage, G. F. Painter, A. B. Holmes, P. R. Gaffney, C. B. Reese, F. McCormick, P. Tempst, J. Coadwell, and P. T. Hawkins.** 1998. Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science **279:**710-4.

211. **Stettner, M. R., W. Wang, L. B. Nabors, S. Bharara, D. C. Flynn, J. R. Grammer, G. Y. Gillespie, and C. L. Gladson.** 2005. Lyn kinase activity is the predominant cellular SRC kinase activity in glioblastoma tumor cells. Cancer Res **65:**5535-43.

212. **Stevens, J. G., L. Haarr, D. D. Porter, M. L. Cook, and E. K. Wagner.** 1988. Prominence of the herpes simplex virus latency-associated transcript in trigeminal ganglia from seropositive humans. J Infect Dis **158:**117-23.

213. **Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science **235:**1056-9.

214. **Stokoe, D., L. R. Stephens, T. Copeland, P. R. Gaffney, C. B. Reese, G. F. Painter, A. B. Holmes, F. McCormick, and P. T. Hawkins.** 1997. Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science **277:**567-70.

215. **Stow, N. D., and E. C. Stow.** 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. J Gen Virol **67 (Pt 12):**2571-85.

216. **Straus, D. B., and A. Weiss.** 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. Cell **70:**585-93.

217. **Summers, S. A., L. Lipfert, and M. J. Birnbaum.** 1998. Polyoma middle T antigen activates the Ser/Thr kinase Akt in a PI3-kinase-dependent manner. Biochem Biophys Res Commun **246:**76-81.

218. **Sun, G., A. K. Sharma, and R. J. Budde.** 1998. Autophosphorylation of Src and Yes blocks their inactivation by Csk phosphorylation. Oncogene **17:**1587-95.

219. **Swart, R., I. K. Ruf, J. Sample, and R. Longnecker.** 2000. Latent membrane protein 2A-mediated effects on the phosphatidylinositol 3-Kinase/Akt pathway. J Virol **74:**10838-45.

220. **Szilagyi, J. F., and C. Cunningham.** 1991. Identification and characterization of a novel non-infectious herpes simplex virus-related particle. J Gen Virol **72 (Pt 3):**661-8.

221. **Talmage, D. A., R. Freund, A. T. Young, J. Dahl, C. J. Dawe, and T. L. Benjamin.** 1989. Phosphorylation of middle T by pp60c-src: a switch for binding of phosphatidylinositol 3-kinase and optimal tumorigenesis. Cell **59:**55-65.

222. **Theil, D., T. Derfuss, I. Paripovic, S. Herberger, E. Meinl, O. Schueler, M. Strupp, V. Arbusow, and T. Brandt.** 2003. Latent

herpesvirus infection in human trigeminal ganglia causes chronic immune response. Am J Pathol **163:**2179-84.

223. **Thoulouze, M. I., N. Sol-Foulon, F. Blanchet, A. Dautry-Varsat, O. Schwartz, and A. Alcover.** 2006. Human immunodeficiency virus type-1 infection impairs the formation of the immunological synapse. Immunity **24:**547-61.

224. **Tigges, M. A., S. Leng, D. C. Johnson, and R. L. Burke.** 1996. Human herpes simplex virus (HSV)-specific CD8+ CTL clones recognize HSV-2-infected fibroblasts after treatment with IFN-gamma or when virion host shutoff functions are disabled. J Immunol **156:**3901-10.

225. **Topp, K. S., K. Bisla, N. D. Saks, and J. H. Lavail.** 1996. Centripetal transport of herpes simplex virus in human retinal pigment epithelial cells in vitro. Neuroscience **71:**1133-44.

226. **Turner, A., B. Bruun, T. Minson, and H. Browne.** 1998. Glycoproteins gB, gD, and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system. J Virol **72:**873-5.

227. **Vaillancourt, R. R., L. E. Heasley, J. Zamarripa, B. Storey, M. Valius, A. Kazlauskas, and G. L. Johnson.** 1995. Mitogen-activated protein kinase activation is insufficient for growth factor receptor-mediated PC12 cell differentiation. Mol Cell Biol **15:**3644-53.

228. **van Genderen, I. L., R. Brandimarti, M. R. Torrisi, G. Campadelli, and G. van Meer.** 1994. The phospholipid composition of extracellular herpes simplex virions differs from that of host cell nuclei. Virology **200:**831-6.

229. **van Oers, N. S., N. Killeen, and A. Weiss.** 1996. Lck regulates the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 in murine thymocytes. J Exp Med **183:**1053-62.

230. **Van Tan, H., G. Allee, C. Benes, J. V. Barnier, J. D. Vincent, and R. Fagard.** 1996. Expression of a novel form of the p56lck protooncogene in rat cerebellar granular neurons. J Neurochem **67:**2306-15.

231. **Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen.** 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. Cell **55:**301-8.

232. **Veillette, A., I. D. Horak, and J. B. Bolen.** 1988. Post-translational alterations of the tyrosine kinase p56lck in response to activators of protein kinase C. Oncogene Res **2:**385-401.

233. **Veillette, A., I. D. Horak, E. M. Horak, M. A. Bookman, and J. B. Bolen.** 1988. Alterations of the lymphocyte-specific protein tyrosine kinase (p56lck) during T-cell activation. Mol Cell Biol **8:**4353-61.

234. **Vittone, V., E. Diefenbach, D. Triffett, M. W. Douglas, A. L. Cunningham, and R. J. Diefenbach.** 2005. Determination of interactions between tegument proteins of herpes simplex virus type 1. J Virol **79:**9566-71.

235. **Vogel, L. B., and D. J. Fujita.** 1993. The SH3 domain of p56lck is involved in binding to phosphatidylinositol 3'-kinase from T lymphocytes. Mol Cell Biol **13:**7408-17.

236. **Wagner, M. J., and J. R. Smiley.** 2009. Herpes simplex virus requires VP11/12 to induce phosphorylation of the activation loop tyrosine (Y394) of the Src family kinase Lck in T lymphocytes. J Virol **83:**12452-61.

237. **Walsh, D., and I. Mohr.** 2004. Phosphorylation of eIF4E by Mnk-1 enhances HSV-1 translation and replication in quiescent cells. Genes Dev **18:**660-72.

238. **Wange, R. L., R. Guitian, N. Isakov, J. D. Watts, R. Aebersold, and L. E. Samelson.** 1995. Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70. J Biol Chem **270:**18730-3.

239. **Warming, S., N. Costantino, D. L. Court, N. A. Jenkins, and N. G. Copeland.** 2005. Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res **33:**e36.

240. **Watson, R. J., and J. B. Clements.** 1978. Characterization of transcription-deficient temperature-sensitive mutants of herpes simplex virus type 1. Virology **91:**364-79.

241. **Watts, J. D., J. S. Sanghera, S. L. Pelech, and R. Aebersold.** 1993. Phosphorylation of serine 59 of p56lck in activated T cells. J Biol Chem **268:**23275-82.

242. **Wild, P., M. Engels, C. Senn, K. Tobler, U. Ziegler, E. M. Schraner, E. Loepfe, M. Ackermann, M. Mueller, and P. Walther.** 2005. Impairment of nuclear pores in bovine herpesvirus 1-infected MDBK cells. J Virol **79:**1071-83.

243. **Willard, M.** 2002. Rapid directional translocations in virus replication. J Virol **76:**5220-32.

244. **Winkler, D. G., I. Park, T. Kim, N. S. Payne, C. T. Walsh, J. L. Strominger, and J. Shin.** 1993. Phosphorylation of Ser-42 and Ser-59 in the N-terminal region of the tyrosine kinase p56lck. Proc Natl Acad Sci U S A **90:**5176-80.

245. **Wisner, T. W., C. C. Wright, A. Kato, Y. Kawaguchi, F. Mou, J. D. Baines, R. J. Roller, and D. C. Johnson.** 2009. Herpesvirus gB-induced fusion between the virion envelope and outer nuclear membrane during virus egress is regulated by the viral US3 kinase. J Virol **83:**3115-26.

246. **WuDunn, D., and P. G. Spear.** 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J Virol **63:**52-8.

247. **Wysocka, J., and W. Herr.** 2003. The herpes simplex virus VP16-induced complex: the makings of a regulatory switch. Trends Biochem Sci **28:**294-304.

248. **Xu, W., A. Doshi, M. Lei, M. J. Eck, and S. C. Harrison.** 1999. Crystal structures of c-Src reveal features of its autoinhibitory mechanism. Mol Cell **3:**629-38.

249. **Xu, W., S. C. Harrison, and M. J. Eck.** 1997. Three-dimensional structure of the tyrosine kinase c-Src. Nature **385:**595-602.

250. **Yamaguchi, H., and W. A. Hendrickson.** 1996. Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation. Nature **384:**484-9.

251. **York, I. A., and D. C. Johnson.** 1993. Direct contact with herpes simplex virus-infected cells results in inhibition of lymphokine-activated killer cells because of cell-to-cell spread of virus. J Infect Dis **168:**1127-32.

252. **York, I. A., C. Roop, D. W. Andrews, S. R. Riddell, F. L. Graham, and D. C. Johnson.** 1994. A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. Cell **77:**525-35.

253. **Yu, J. C., M. A. Heidaran, J. H. Pierce, J. S. Gutkind, D. Lombardi, M. Ruggiero, and S. A. Aaronson.** 1991. Tyrosine mutations within the alpha platelet-derived growth factor receptor kinase insert domain abrogate receptor-associated phosphatidylinositol-3 kinase activity without affecting mitogenic or chemotactic signal transduction. Mol Cell Biol **11:**3780-5.

254. **Yu, K. T., D. K. Werth, I. H. Pastan, and M. P. Czech.** 1985. src kinase catalyzes the phosphorylation and activation of the insulin receptor kinase. J Biol Chem **260:**5838-46.

255. **Zachos, G., B. Clements, and J. Conner.** 1999. Herpes simplex virus type 1 infection stimulates p38/c-Jun N-terminal mitogen-activated protein kinase pathways and activates transcription factor AP-1. J Biol Chem **274:**5097-103.

256. **Zahariadis, G., M. J. Wagner, R. C. Doepker, J. M. Maciejko, C. M. Crider, K. R. Jerome, and J. R. Smiley.** 2008. Cell-type-specific tyrosine phosphorylation of the herpes simplex virus tegument protein VP11/12 encoded by gene UL46. J Virol **82:**6098-108.

257. **Zhang, B., D. F. Spandau, and A. Roman.** 2002. E5 protein of human papillomavirus type 16 protects human foreskin keratinocytes from UV B-irradiation-induced apoptosis. J Virol **76:**220-31.

258. **Zhang, W., and L. E. Samelson.** 2000. The role of membrane-associated adaptors in T cell receptor signalling. Semin Immunol **12:**35-41.

259. **Zhang, W., J. Sloan-Lancaster, J. Kitchen, R. P. Trible, and L. E. Samelson.** 1998. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell **92:**83-92.

260. **Zhang, Y., and J. L. McKnight.** 1993. Herpes simplex virus type 1 UL46 and UL47 deletion mutants lack VP11 and VP12 or VP13 and VP14, respectively, and exhibit altered viral thymidine kinase expression. J Virol **67:**1482-92.

261. **Zhang, Y., D. A. Sirko, and J. L. McKnight.** 1991. Role of herpes simplex virus type 1 UL46 and UL47 in alpha TIF-mediated transcriptional induction: characterization of three viral deletion mutants. J Virol **65:**829-41.

262. **Zhou, Z. H., J. He, J. Jakana, J. D. Tatman, F. J. Rixon, and W. Chiu.** 1995. Assembly of VP26 in herpes simplex virus-1 inferred from structures of wild-type and recombinant capsids. Nat Struct Biol **2:**1026-30.