

UNIVERSITY OF ALBERTA

**HSV-1 REMODELS PI3-KINASE/AKT
SIGNALING**

by

KEVIN QUACH

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

MASTER OF SCIENCE

in

VIROLOGY

Department of Medical Microbiology and Immunology

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Spring 2013

Edmonton, Alberta

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ABSTRACT

AKT inhibits apoptosis and stimulates cap-dependent translation by phosphorylating key downstream cellular proteins. Many viruses therefore activate the PI3-kinase-AKT signaling pathway to promote cell survival and viral protein synthesis. HSV-1 activates AKT during lytic infection and the abundant tegument protein VP11/12 is required for this effect. Although VP11/12 is essential for AKT activation, deleting VP11/12 has no detectable effect on HSV-1 induced phosphorylation of the examined downstream target proteins. Rather, the protein kinase U_S3 is required for the phosphorylation of AKT targets and thus serves as an AKT mimic. Our data indicate that VP11/12 and U_S3 do not collaborate to provide redundant coverage of key AKT targets during infection. Interestingly, HSV-1 attenuates signals from activated AKT induced by external growth factors. Remarkably, neither VP11/12 nor U_S3 is required for this effect.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. James R. Smiley for providing an exceptional learning environment for the development of scientific writing and critical thinking skills. Thank you to past and current lab members for sharing your scientific ideas with me and for making graduate school an exceptionally enjoyable time. I would like to specifically thank Holly Saffran for making me feel welcome and providing support in the lab and personal life. Thank you to my committee members Dr. David Evans, Dr. Rob Ingham, and Dr. Richard Lamb for your assistance with science and academics. Additionally, many thanks to Anne Giles, Debbie Doudiet, Tabitha Vasquez, and the rest of the office staff for their support. Thank you to all my friends in the Department of Medical Microbiology and Immunology for your support and friendly conversations. I cannot begin to thank enough those I've developed close relationships with: Nancy Hu, Andre Deslauriers, Kinola Williams and Bettina Bareiss. Thank you to Nancy Hu with whom I have shared some of my best memories and who has made the last few years special. Furthermore, your scientific contribution to this thesis was invaluable. Thank you to Kinola Williams for providing the support that I needed in multiple situations. Thanks to all who have helped make Edmonton my home. In closing, I would like to dedicate this thesis to my sister Tara; you have taught me everything I know and have never left my side.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION.....	1
1.1 Overview of Herpes Simplex Virus-1 (HSV-1).....	2
1.1.1 A Brief Introduction.....	2
1.1.2 Taxonomy.....	3
1.1.3 The HSV Virion.....	4
1.1.4 HSV-1 Lytic Cycle.....	5
1.1.5 HSV-1 Latency.....	8
1.2 Cellular initiation of PI3K-AKT signaling.....	9
1.2.1 A Brief Introduction on PI3-Kinase.....	9
1.2.2 Activation of PI3-Kinase.....	11
1.2.3 Propagating PI3-Kinase Signals.....	13
1.2.4 AKT.....	15
1.2.5 AKT Signaling.....	18
1.3 Viruses modulate PI3K-AKT signaling.....	21
1.3.1 HSV-1 U _L 46.....	21
1.3.2 UL46 activates SFKs and PI3-Kinase to induce AKT activation.....	21
1.3.3 HSV-1 U _S 3.....	25
1.3.4 US3 promotes cell survival by circumventing cell signaling pathway.....	25
1.3.5 U _S 3 modulates AKT signaling.....	25
1.4 Thesis rationale.....	26
CHAPTER 2: MATERIALS AND METHODS.....	27
2.1 Mammalian cell cultures.....	28
2.2 Virus strains and growth conditions.....	28
2.3 Preparation of viral stocks.....	29
2.4 Virus titrations.....	30
2.5 Infecting cells studies of HSV-induced host signaling modifications.....	30
2.6 Addition of drugs and external growth ligands.....	31
2.7 Preparation of cell extracts.....	31
2.8 SDS-polyacrylamide gel.....	32

2.9 Western blot.....	33
2.10 Antibodies.....	33
CHAPTER 3: RESULTS.....	35
3.1 HSV-1 Infection Causes Maximal AKT Activation at late times during infection.....	36
3.2 HSV-1 Requires mTORC2 for AKT phosphorylation.....	38
3.3 U _S 3 Mediates the Phosphorylation of AKT Targets.....	44
3.4 HSV-1 also activates AKT in Human Foreskin Fibroblasts.....	47
3.5 U _S 3 Mediates the phosphorylation of AKT targets despite activation of AKT by U _L 46.....	51
3.6 Serum induces AKT activation, but there is no significant activation signal transduced to AKT targets at late times of infection.....	62
3.7 PDGF BB induces AKT activation, but the signal is not transduced to AKT targets.....	72
3.8 Titration of PDGF AA, AB and BB.....	80
3.9 PDGF ligands do not perform equally at activating AKT during infection.....	85
CHAPTER 4: DISCUSSION.....	94
4.1 Thesis summary.....	95
4.2 The HSV-1 kinase U _S 3 masquerades as an AKT mimic to regulate cellular processes.....	98
4.3 HSV-1 promotes cap-dependent translation.....	98
4.4 A potential role for U _L 46 in promoting cell survivalals.....	99
4.5 HSV-1 encodes proteins other than U _L 46 and U _S 3 to modulate AKT signaling.....	100
4.6 HSV-1 permits signaling to AKT from specific receptors.....	101
4.7 Other aspects of AKT activation that are modulated by HSV-1.....	102
4.6.1 A mechanism for the phosphorylation of AKT by HSV-1.....	102
4.6.2 U _S 3 negatively regulates the phosphorylation of the T308 residue of AKT.....	102

4.8 Future directions.....	103
BIBLIOGRAPHY.....	104
APPENDICES.....	128

LIST OF FIGURES

Figure 1.1: Structure of PI3K.....	10
Figure 1.2: PDGF-receptor activates PI3K.....	12
Figure 1.3: Transduction of an activated PI3K signal.....	14
Figure 1.4: Activation of AKT and propagation of an activated AKT signal...17	
Figure 1.5: AKT acts on multiple proteins.....	20
Figure 1.6: HSV-1 U _L 46 binds SFK and PI3K to activate AKT.....	24
Figure 3.1: HSV-1 activates AKT in a U _L 46-dependent manner.....	37
Figure 3.2: mTORC2 is required for AKT activation during HSV-1 infection....41	
Figure 3.3: DMSO does not dampen AKT activation during HSV-1 infection....43	
Figure 3.4: The AKT targets, GSK3 β and FoxO1, are phosphorylated in a U _L 46-independent fashion, but require U _S 3.....	46
Figure 3.5: HSV-1 activates AKT in a U _L 46-dependent manner at early and late times post infection.....	49
Figure 3.6: Despite U _L 46-activated AKT, GSK3 β is mainly phosphorylated in a U _S 3-dependent fashion at early times post infection.....	55
Figure 3.7: Multiple AKT targets are phosphorylated in a U _S 3-dependent fashion at late times post infection.....	58
Figure 3.8: 10% serum induces maximal AKT activation after 20 minutes.....	65
Figure 3.9: Serum induces AKT activation and downstream signaling in infected cells at early infection.....	68
Figure 3.10: Serum induces AKT activation and, but not downstream signaling in infected cells at late times post infection.....	70
Figure 3.11: PDGF BB activates AKT.....	75
Figure 3.12: PDGF BB induces AKT activation, but the signal is not propagated to AKT targets during late times post infection.....	77
Figure 3.13: Differing concentrations of PDGF AA, AB and BB induce comparable levels of AKT activation.....	81
Figure 3.14: PDGF ligands do not perform equally at phosphorylating S473 AKT during infection.....	88

Figure 3.15: PDGF ligands do not perform equally at phosphorylating T308 AKT during infection.....91

Figure 4.1: U_L46 promotes AKT activation, while U_S3 negatively regulates AKT signaling.....96

Figure 4.2: HSV-1 blocks AKT activation downstream of specific receptors.....97

ABBREVIATIONS

4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
α	Immediate-early
β	Early
γ	Late
AGC	Protein kinase A, protein kinase G, protein kinase C
AKT/PKB	Protein kinase B
BAC	Bacterial artificial chromosome
BCR	B cell receptor
BH3	Bcl-2 homology domain 3
CTL	Cytotoxic lymphocytes
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
EGFR	Epidermal growth factor receptor
g-	Glycoprotein
FBS	Fetal bovine serum
FoxO1	Forkhead box O1
GSK3 β	Glycogen synthase kinase 3 β
HCF	Host cell factor
HCMV/HHV-5	Human cytomegalovirus/human herpes virus-5
HEL	Human embryonic lung fibroblast
HFF	Human foreskin fibroblasts
HSV-1	Herpes simplex virus-1
HSV-2	Herpes simplex virus-2
IGFR	Insulin growth factor receptor
INPP5B	Type II phosphatidylinositol 5' phosphate
MHC	Major histocompatibility complex
mTORC1, mTORC2	Mammalian target of rapamycin complex 1, 2
NGFR	Nerve growth factor receptor
NK	Natural killer
OBB	Odyssey blocking buffer

Oct-1	Octamer transcription factor-1
ORF	Open reading frame
P	Penicillin
PDGFR	Platelet-derived growth factor receptor
PDK1	Phosphoinositide-dependent protein kinase-1
PH	Pleckstrin homology
PI3K	Phosphoinositide-3-Kinase
PIP2	Phosphatidylinositol-4,5-diphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKA	Protein kinase A
PLC	Phospholipase C
PP2A	Protein phosphatase 2A
PTEN	Phosphatase and tensin homolog on chromosome TEN
RTK	Receptor tyrosine kinase
S	Streptomycin
S-	Serine
S6K	S6 kinase
SFK	Src family kinase
SH-	Src homology-
T308	Threonine 308
TAP	Transporter associates with antigen presentation
TBST	TBS-Tween
TCR	T cell receptor
TSC2	Tuber sclerosis complex 2
U ₃ PK	U ₃ protein kinase
Vero	American monkey kidney cells
VHS	Virion host shut-off
VP11/12	Viral protein 11 and viral protein 12
VZV	Varicella zoster virus

CHAPTER 1: INTRODUCTION

This thesis focuses on the role of the Herpes Simplex Virus (HSV)-1 proteins VP11/12 and U_S3 in modulating the host cell PI3K-AKT signaling pathway. Below, I have provided the context for this research by outlining the HSV life cycle, reviewing the PI3K-AKT pathway, and summarizing previous knowledge about how HSV and other viruses manipulate this system.

1.1 Overview of Herpes Simplex Virus-1

1.1.1 A Brief Introduction

HSV-1 is a prevalent human pathogen found in 70-95% of the population depending on geographic location, and this percentage increases with age (255). Herpes simplex viruses are neurotropic, and infection causes the appearance of fluid-filled blisters or sores on the skin around the oral cavity (cold sores) or genitals that are caused by either HSV-1 or 2. Although clinical symptoms are mild in the majority of the population, HSV-1 can cause a variety of diseases such as infection of the mouth and gums (gingivostomatitis) and pharyngitis (80, 154). People with eczema have an increased risk of herpes infection outbreaks around eczema-prone areas of the body (eczema herpeticum) (213). Herpes simplex viruses are also known to cause other disorders such as encephalitis, herpetic whitlow, herpes gladiatorum, and herpes keratitis (198).

HSV is transmitted through direct skin contact or through the exchange of bodily fluids from an infected individual. HSV must be actively replicating to be transmitted. Active replication is phenotypically characterized by the appearance of blisters that contain infectious virus particles. However, herpes can be asymptomatic while viral shedding occurs (198). Most people who harbor latent HSV shed virus asymptotically. Viral shedding occurs in 2-9% of adults and 5-8% of children at any given time (255).

Following initial infection and lytic replication at the epithelia, HSV virions migrate to innervating sensory nerve endings and travel retrograde into nerve cell bodies (52, 197). It is here that the virus enters latency and can remain dormant throughout life. Alternatively, the virus can cycle between periods of lytic and latent replication. Recurrent active lytic infections have an unknown

etiology; however, immune system repression may be an initiating event. Repression of the immune system may occur during cancer, stress, infection, and certain drug therapies. During this time, the virus will infect epithelial cells linked to the site of latency by innervating neurons (reviewed in (197, 198)). Tingling, itching and pain at the site of cold sores are signs of an oncoming viral reactivation (71, 210, 211).

Unfortunately, herpes is a life-long infection and no cure has been developed. Effective antiviral treatments such as acyclovir can reduce viral shedding and alleviate the severity and duration of symptoms.

1.1.2 Taxonomy

Within the *Herpesvirales* order is a family of large, double-stranded, linear DNA viruses that are known to infect mammals, fish, birds, or molluscs (reviewed in (198)). Based on their sequence homology and the species they infect, the order can be divided into families, including *Alloherpesviridae*, *Herpesviridae*, and *Malacoherpesviridae*. The *Herpesviridae* family can be divided into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* (198). *Alphaherpesvirinae* have a broad host range, short replication cycle, and can establish latency in sensory neurons. *Betaherpesvirinae* generally have a more restricted host range, long reproductive cycle, grow slowly in culture and cause infected cells to enlarge. They often establish latency in tissues such as secretory glands and hematopoietic progenitor cells (198). *Gammaherpesvirinae* have a narrow host range, replicate in epithelial cells, and establish latency in T or B lymphocytes, which can lead to cellular immortalization. There are more than 200 different Herpesviruses, but only eight distinct types are known to cause disease in humans (253). The *Alphaherpesvirinae* subfamily includes Herpes Simplex Virus types 1 and 2 (HSV-1 and HSV-2), which are also called Human Herpes Viruses 1 and 2 (HHV-1 and HHV-2); and varicella zoster virus (VZV/HHV-3). HSV-1 and HSV-2 cause oral and genital herpes respectively, and severe ocular damage leading to blindness (Herpes keratitis) (15), while VZV is the causative agent of chicken pox and shingles. The *Betaherpesvirinae* subfamily includes

human cytomegalovirus (HCMV/HHV-5), herpes lymphotropic virus (HHV-6), and roseolovirus (HHV-7). HCMV can be associated with birth defects (223), and fatal diseases in immune-compromised individuals, including organ transplant recipients and individuals with AIDS (101). Lastly, the *Gammaherpesvirinae* subfamily is comprised of Epstein-Barr virus (EBV/HHV-4), which causes infectious mononucleosis, and Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8), which is more often associated with Castleman's disease (199).

1.1.3 The HSV Virion

The herpes virion consists of four essential parts: the genome, capsid, tegument and envelope. The size of herpes virions ranges from 120 to 300 nm depending on the variability in the thickness of the tegument layer (198). Herpesvirus virions have their DNA enclosed within an icosahedral capsid, which is wrapped by a proteinaceous layer called the tegument, and contained within a host-cell-derived lipid envelope (197, 256).

HSV-1 is a large DNA virus that contains approximately 90 open reading frames (ORFs). The genome is divided into two covalently linked regions known as the unique long (U_L) and unique short (U_S) regions, which are both flanked by inverted repeats. (150, 151) Of the 90 proteins, there are a reported 30 known and 10 suspected structural proteins distributed throughout the capsid, tegument and envelope (97, 198, 254).

The icosahedral capsid packages the viral DNA and is composed of 162 capsomers that can be broken down into seven proteins. There are only six genes to encode all seven proteins: U_L18 (VP23), U_L19 (VP5), U_L26 (VP21 and VP24), $U_L26.5$ (VP22a), U_L35 (VP26), and U_L38 (VP19C) (231). VP5, VP19C, VP23, and VP26 are structural components of the outer capsid while VP21, VP22a, and VP24 form the inner scaffold. A protein encoded by U_L26 has proteolytic activity to auto-cleave creating the byproducts VP21 and VP24. The U_L26 proteolytic enzyme also cleaves pre-VP22a into VP22a (97, 168). Additionally, there are four more gene products that form a minor component of the HSV capsid (U_L6 , U_L15 , U_L25) (25, 175, 233, 266).

Between the capsid and envelope lies an amorphous proteinaceous layer called the tegument (133). The tegument contains 14 identified proteins that aid the initial stages of infection, but the majority of tegument genes are not transcribed until the late stages (272). Some tegument proteins are VP16, virion host shut-off (VHS) protein, VP22, VP13/14, VP11/12, and VP1/2 (198). VP16 is an essential trans-activating factor of viral immediate-early promoters. VHS is important to viral replication since it has a role in shutting off host protein synthesis, degrading host mRNA, aiding viral replication and regulating viral gene expression (146). VP22 is used as a vehicle for intracellular trafficking of proteins, and reorganizes microtubules (59, 60). VP13/14 remains largely unexplored, but may influence modulation of the activity of VP16 (271). VP11/12 may have a role in regulation host cell signaling and T cell activation (247, 248, 269). VP1/2 is thought to influence release of HSV DNA into the nucleus (111).

The envelope is composed of a lipid bilayer that is acquired during egress from the cell membranes and is dotted with glycoprotein spikes. Glycoproteins are required for host cell recognition and binding, resulting in viral uptake into cells. There are 10 characterized glycoproteins found on the surface of the virion: gB, gC, gD, gE, gG, gH, gI, gK, gL, and gM (198). Additionally, the virion contains non-glycosylated membrane proteins that are encoded by U_L20, U_S9, U_L24, U_L43, and U_L34 (198). Several of these glycoproteins have essential roles in viral uptake by cells and the initial stages of infection.

1.1.4 HSV-1 Lytic Cycle

Lytic infection begins with viral attachment and entry into a cell that is mediated by host surface ligands and their cognate viral receptors. Once the virus enters the cell, gene transcription occurs in a sequential order. Immediate early (α) genes are the first to be transcribed and their products are required for the activation of other viral gene promoters. Expression of IE genes is necessary for the expression of early (β) genes and the onset of viral DNA replication. This is followed by the expression of leaky late (γ_1) and true late (γ_2) genes, and virion assembly and egress. Each step will be described in more detail below.

Preceding viral gene expression and viral DNA replication, HSV must first recognize and bind to cell surface receptors, and penetrate the cell membrane. Viral glycoproteins are required for recognition of cell receptors and entry of HSV into the cell (49, 205, 245). There are a total of five viral glycoproteins that are involved in viral entry: gB, gC, gD, gH, and gL (93, 219). Glycoproteins -B and -C mediate an initial weak binding interaction to cell surface heparan sulfate chains on cell surface proteoglycans like glycosaminoglycan (93, 209, 228, 261). Even though the initial binding is a weak interaction, virions lacking both gB and gC are severely impaired and unable to bind to cells (92).

The second step of viral attachment involves an association of viral gD to a plethora of entry receptors. On the cell surface, there are three main receptors to which HSV can bind: herpes virus entry mediator (HVEM) (159), nectin-1 and nectin-2 (74, 250), and specific sites in heparan sulfate. gD has the potential to bind to any one of the three cell surface molecules and mediate viral entry. However, the binding preference depends on the cell type and serotype of HSV.

The last step in viral entry is fusion of the viral envelope and host cell membrane. Glycoproteins -B, -D, -H, and -L act in concert to induce membrane fusion between the viral envelope and cell membrane (67, 136, 205, 212). Following fusion, the nucleocapsid and proteinaceous tegument are released into the cytosol. The tegument contains proteins that act immediately to aid viral gene expression and evade the immune response. The nucleocapsid interacts with dynein microtubular networks and the cofactor dynactin (56) for transportation to the nucleus (126, 215). The nucleocapsid interacts with importin- β and the nuclear pore complex (9, 215), triggering the release of viral DNA into the nucleus (107).

Upon lytic infection, α genes are transcribed by host RNA polymerase II at the periphery of nuclear domain (ND) 10 structures (37, 149), α gene expression does not require prior viral protein synthesis since proteins found in the tegument mediate the transcription of α genes (140). For example, a viral transcription activator, VP16, forms a complex with the host cell factor (HCF) (116, 130) and Octamer transcription factor 1 (Oct-1) (127, 128, 171, 182). This

complex binds upstream regulatory elements common to α genes, such as TAATGARATT sequences (139, 140, 237).

The VP16/HCF/Oct-1 aids in the transcriptional activation of five α gene products: infected cell protein (ICP) 0, ICP4, ICP22, ICP27, and ICP47 (98, 252, 257). Most α genes are required for subsequent gene expression (100). For example, ICP0 is an E3 ubiquitin ligase that induces proteasome-dependent degradation of components found in ND10 structures (PML, ATRx, SP100, and Daxx) that are thought to repress viral gene expression (63). ICP4 is a significant regulatory protein of HSV-1 transcription that mediates the transcriptional activation of β and γ genes (251). ICP27 is required for many aspects of viral translation and repressing host protein synthesis (88, 194, 216, 222, 238).

The majority of genes expressed early in an infection are required for viral DNA replication and nucleotide metabolism. The viral genome contains three origins of replication. Replication can begin at any one of the locations (232). Attached to the viral genome are several early proteins that promote DNA replication (129). U_L9 is a viral origin-binding protein that binds the replication origin to bend the DNA and cause a single-stranded (ss) stem loop structure (58). ICP8 binds the ss-DNA stem loop (36), stimulating the helicase activity of U_L9 (19). U_L5, U_L8, and U_L52 interact to make a helicase-primase complex that has 5'-3' activity (40). DNA polymerase is composed of U_L30/U_L42 and possesses 3' to 5' exonuclease activity for proofreading. U_L42 activity provides increased processivity (183). There are additional cellular enzymes such as DNA ligases and topoisomerases that aid in viral DNA replication (232).

Late genes are expressed shortly after viral DNA synthesis (99), so it makes sense their products are structural proteins, and are involved in the assembly of virions, or modification of cellular membranes. Late gene transcription occurs in replication compartments (178) and is promoted by the immediate early gene products ICP0 (30), ICP4, and ICP27 (201) as well as the early gene product, ICP8 (72). Late gene products can be divided into two classes (leaky-late and true-late), based on the kinetics of their expression. Leaky-late

gene expression occurs before DNA replication. Therefore, DNA replication is not required, but does enhance leaky-late gene expression (198).

Capsid assembly and DNA packaging are required to create infectious progeny. DNA packaging and capsid formation occurs in viral replication compartments found in the nucleus (48, 75). Cleavage and DNA packaging are tightly linked. These processes require six viral proteins: U_L6, U_L15, U_L25, U_L28, U_L32, and U_L33 (97). Viral DNA replication forms head-to-tail concatemeric complexes composed of tandem repeats of the viral genome. Concatemers are cleaved into unit-monomers and packaged into viral nucleocapsids (97).

Viral nucleocapsids bud through the nuclear membrane to become virions (162). The accepted model of virion assembly involves a multi-stage process of envelopment, de-envelopment and re-envelopment. It is thought that capsids with viral genomes egress through the nucleoplasm, and acquire a primary envelope at the inner nuclear membrane. The primary envelope fuses with the outer leaflet of the nuclear membrane or endoplasmic reticulum, forcing the loss of the primary envelope (reviewed in (156)). Non-enveloped nucleocapsids travel to Trans Golgi membranes, where they acquire mature glycoproteins and tegument (110). After maturation, virus particles bud into cytoplasmic vesicles derived from the trans-Golgi network or endosomes, and egress from the cell by exocytosis (156).

1.1.5 HSV-1 Latency

HSV-1 is a lifelong neurotropic virus of the sensory neural ganglia. Once the virus has replicated in epithelial cells, a progeny virus migrates and infects sensory neurons. In the sensory neural ganglia, the virus often enters a latent state (224). Latency occurs when the episomal viral genome is maintained in a circular conformation and non-replicating state in the neuronal nuclei (51, 155, 195, 196). The number of viral genomes found in a latent neuron can range from one to thousands (206). During latency, the expression of genes is highly restricted, with the exemption of latency-associated RNA transcripts (LATs) that accumulate to high levels in neuron nuclei (55). LATs are thought to influence chromatin

modulation of the viral episome, inhibit apoptosis and produce micro-RNAs that suppress lytic gene expression (18).

1.2 Cellular initiation of PI3K-AKT signaling

Viruses manipulate numerous host signaling pathways in order to enhance virus replication, suppress cellular death pathways and modulate the innate and adaptive immune responses. One cellular signaling pathway that is almost invariably targeted is the phosphoinositide 3-kinase (PI3K)-Protein Kinase B (AKT) axis, which as described below promotes cell survival and cap-dependent mRNA translation. Previous work has demonstrated that HSV-1 modulates this pathway, and identified two viral tegument proteins that contribute to this regulation.

Melany Wagner in our lab showed that the U_L46 gene product, VP11/12, is essential for AKT activation during infection (247), while Benetti and Roizman provided evidence that the serine-threonine protein kinase encoded by gene U_S3 dampens AKT activation (13). Below I review the PI3K-AKT pathway, and describe the properties of HSV U_S3 and VP11/12.

1.2.1 A Brief Introduction to PI3-Kinase

PI3Ks are a family of intracellular enzymes involved in critical signaling pathways. The PI3K family is divided into three classes (I, II, and III) (262) based on primary structure, regulation and *in vitro* lipid substrate specificity (132). Class I PI3Ks are subgrouped into class IA and class IB (260), but for the purpose of this thesis, PI3K class IA (henceforth referred to as PI3K) is thought to be the only relevant PI3K molecule for AKT signaling. PI3Ks are heterodimers that consist of a regulatory subunit, p85, and a catalytic subunit, p110 (32, 113). The p85 subunit consists of several domains, including two proline-rich fragments, a Src Homology (SH) 3 domain, and two SH2 domains (Fig. 1.1A). The SH3 and SH2 domains serve as docking sites for proteins that promote PI3K activation (114). The two SH2 domains are separated by the inter-SH2 sequence, which connects the p85 and p110 subunits (Fig. 1.1B) (113).

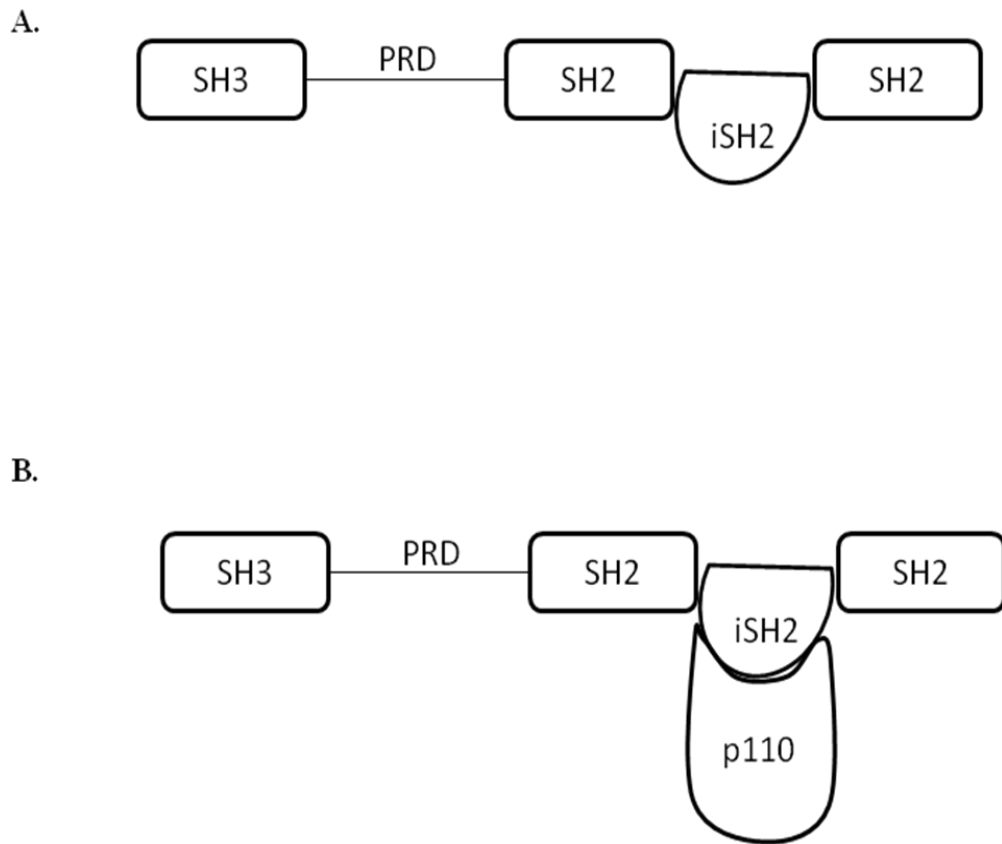


Figure 1.1: Structure of PI3K. A) The p85 subunit contains several domains, including proline rich domains, an SH3 domain and two SH2 binding domains. The SH2 domains are separated by the inter-SH2 sequence, B) and the inter-SH2 sequence mediates an interaction between the p85 and p110 subunits.

1.2.2 Activation of PI3-Kinase

PI3K activation is initiated downstream of several receptor tyrosine kinase (RTK) and cytoplasmic tyrosine kinase (non-RTK) signaling pathways. One example is signaling through platelet-derived growth factor receptor (PDGFR) (10, 163), a RTK that dimerizes and activates when a PDGF dimer-ligand is bound to the receptor (Fig. 1.2). This leads to auto-trans-phosphorylation on tyrosine residues in the cytoplasmic domain, including YXXM motifs, which serve as a binding site for the N-terminal SH2 domain of p85 (243). When the RTK is not activated, the p85 regulatory subunit renders the catalytic subunit inactive (268). Early studies demonstrated that the amino (N)-terminal SH2 domain is required to inhibit p110 activity (267). Binding of the SH2 domain of p85 induces a conformational change that leads to the activation of the p110 catalytic subunit. Src Family Kinases (SFKs) have also been implicated in the activation of PI3K. The SFK Lck phosphorylates Y688 on the C-terminal SH2 domain, which enhances p110 catalytic activity. Furthermore, Lck is a non-RTK that has been shown to phosphorylate YXXM motifs on CD28, resulting in recruitment and activation of PI3K downstream of T-cell receptor signaling. Binding of p85 to phosphorylated tyrosine motifs not only causes a substantial increase in phosphotransferase activity (ability to transfer phosphates) (270), but also increases the affinity of PI3K for phospholipids (28).

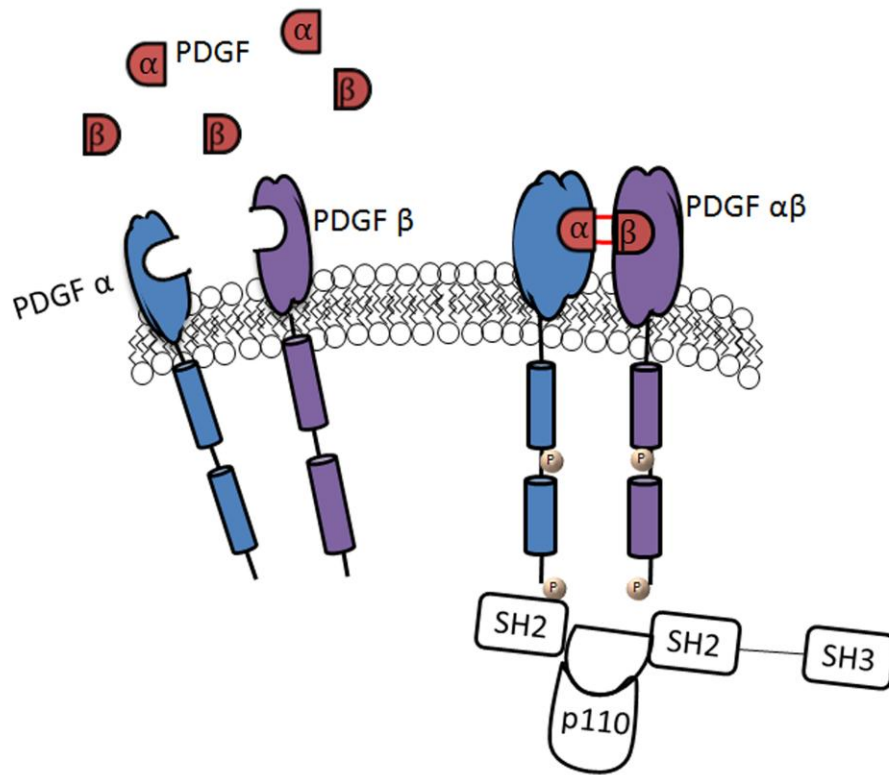


Figure 1.2: PDGF-receptor activates PI3K. PDGF receptors have two types of subunits, α and β , which both contain tyrosine kinase activity required for signal transduction (259, 265). When the growth factor binds to PDGF α and β receptors, it induces receptor homo- or heterodimerization and subsequent autophosphorylation of tyrosine residues on the receptor (120, 265). Translocating PI3K to these activated growth factor receptors stimulates p110 catalytic activity via binding of phospho-tyrosines to the SH2 domains of the PI3K p85 subunit (38, 96, 115, 118, 125).

1.2.3 Propagating PI3-Kinase Signals

In the active conformation, the p110 subunit of PI3K preferentially phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP₂) on the 3'-OH of its inositol ring, producing phosphatidylinositol-3,4,5-trisphosphate (PIP₃) at the plasma membrane (95, 239, 240). This allows some proteins containing pleckstrin homology (PH) domains, including AKT and phosphoinositide-dependent kinase 1 (PDK1), to be recruited to the plasma membrane (Fig. 1.3) (239). The PH domains of AKT and PDK1 preferentially bind to PIP₃ compared to other phosphoinositols (20, 106).

Phosphorylation of PIP₂ by PI3K is counteracted by the phosphatase PTEN, which catalyzes the dephosphorylation of PIP₃ to PIP₂ (Fig 1.3) (141). PTEN deficiency results in constitutive PI3K/AKT signaling, highlighting the critical role of PTEN in regulating the AKT pathway (112, 141). In addition to PI3K, PIP₂ is a substrate for phospholipase C (PLC) enzymes that hydrolyze inositol phospholipids into membrane-bound diacylglycerol and soluble inositol phosphates (IP₃) (50, 202) (reviewed in(124)). The production of IP₃ is often associated with subsequent cytosolic calcium release, and DAG remains bound to the membrane to trigger PKC activation (14, 54, 95, 192).

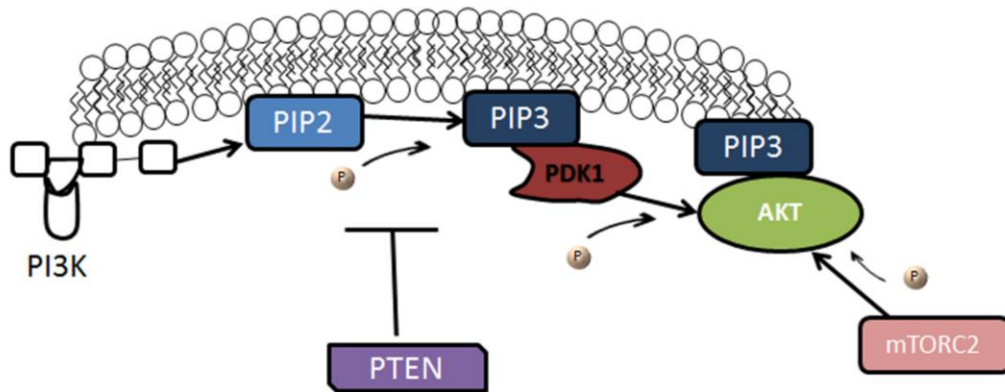


Figure 1.3: Transduction of an activated PI3K signal. Following activation, PI3K preferentially phosphorylates the 3'-OH inositol ring of phosphatidylinositol-4,5-diphosphate (PIP2) producing phosphatidylinositol-3,4,5-triphosphate (PIP3) on the cytoplasmic face of the plasma membrane. The generation of phosphorylated phosphoinositides by PI3K serves as docking sites for a plethora of effector molecules, including AKT and PDK1.

1.2.4 *AKT*

The AGC kinases are a family of protein kinases that regulate important cellular processes such as survival, translation, and cell-cycle progression. Members of the AGC kinase family include AKT, PDK1, S6 kinase (S6K), protein kinase C (PKC), and serum- and glucocorticoid-induced protein kinase (SGK) to name a few (6, 22, 131, 169). AGC kinases share sequence similarity in their catalytic domains (161) and are activated by phosphorylation in the activation loop (177).

As downstream effectors of PI3K, AKT and PDK1 are activated by a plethora of ligands including growth factors, hormones, and immune signals (81). AKT is a serine/threonine kinase that has been implicated in promoting cell survival, inhibiting apoptosis, regulating cellular proliferation and metabolism, and translation initiation (1, 44, 121, 217). The crucial role of AKT in regulating these important cellular processes is reflected in its phylogenetic conservation within invertebrate and vertebrate species (26, 241, 242). Dysregulation of AKT signaling has been linked to aberrant cell growth (208), diabetes, cardiovascular disease and neurological disorders (94).

AKT activation is characterized by phosphorylation on two residues: Serine 473 (S473) of the hydrophobic motif and Threonine 308 (T308) of the activation loop. The kinase that phosphorylates S473 is often referred to as “PDK2”. Several PDK2 candidates have been proposed including PDK1 (8), integrin-linked kinase (176), AKT itself (236), and DNA-PK (65). Mammalian target of rapamycin complex 2 (mTORC2) is the most widely accepted candidate responsible for the phosphorylation of S473, which can be blocked by RNA interference of mTORC2 (203, 204).

The phosphorylation of S473 is thought to precede and be required for the phosphorylation of T308 (2, 204, 207, 264). Phosphorylated S473 has been proposed to act as a docking site for the phosphate-binding pocket of PDK1, which helps to bring PDK1 in close proximity to the activation loop of AKT (264). More importantly, binding of the PH domain of PDK1 to PIP3 brings

PDK1 in close proximity to AKT (3, 16). The PH domain of AKT is thought to inhibit T308 phosphorylation by masking this residue, and an interaction between the PH domain and PIP3 releases this inhibition (17, 225), allowing PDK1 to phosphorylate T308 (3, 31, 157, 225) (Fig. 1.4).

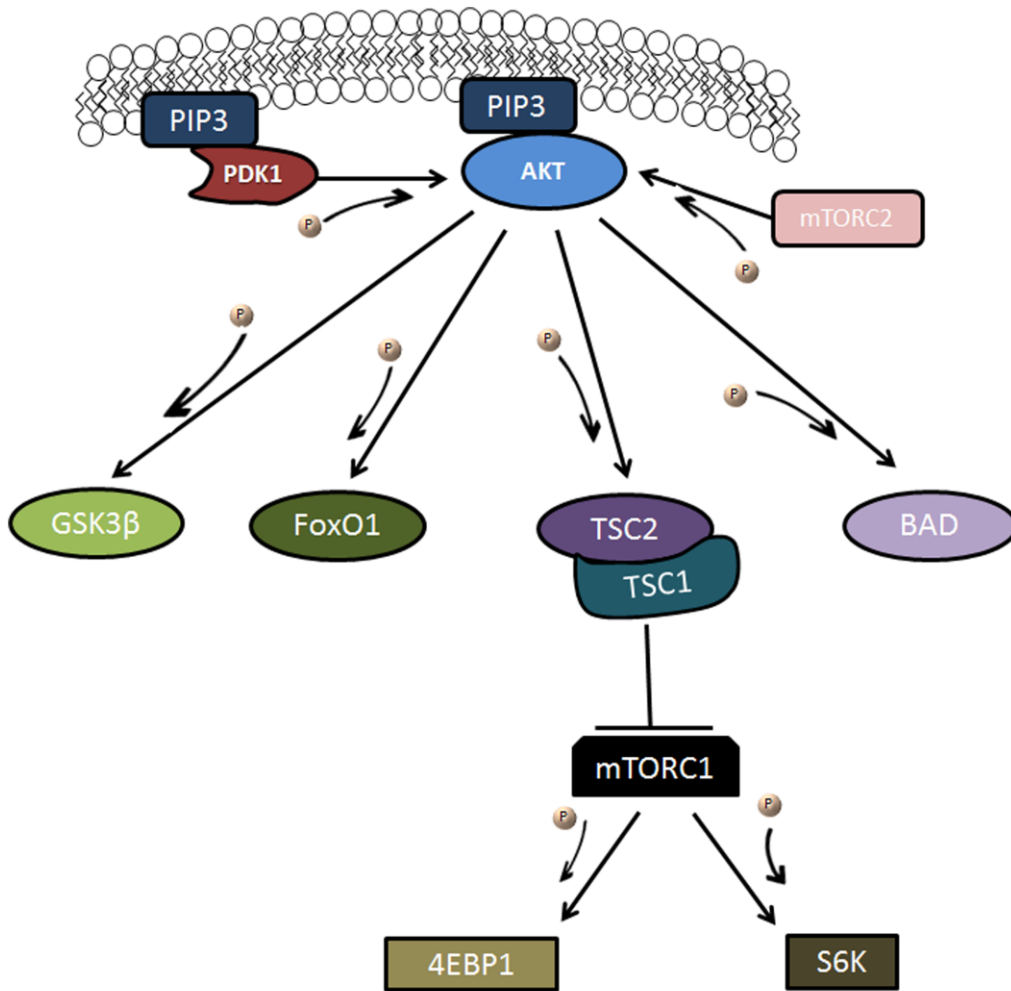


Figure 1.4: Activation and propagation of an activated AKT signal. AKT is activated by phosphorylation once bound to the plasma membrane. Phosphorylating AKT at its activating residues requires an interaction between PIP₃ and the PH domain on AKT. AKT activation is characterized by phosphorylation of two residues, S473 of the hydrophobic motif by mTORC2 and T308 of the activation loop by PDK1. Following activation, AKT phosphorylates the following substrate to inhibit their activity: GSK3 β , BAD, FoxO1, and TSC2. If not inhibited, TSC2 forms a heterodimeric complex with TSC1 and prevents activation of mTORC1. By inhibiting the TSC2/TSC1 complex, mTORC1 is able to phosphorylate downstream targets 4EBP1 and S6K.

1.2.5 *AKT signaling*

Following AKT activation, AKT acts on a multitude of effector molecules at the plasma membrane, cytosol, and nucleus (reviewed in (23)), until AKT is inactivated through dephosphorylation by protein phosphatase 2A (66). AKT is a major signaling arm of PI3K that acts on numerous proteins (Fig. 1.5, Cell Signaling), but only a small subset of these proteins will be discussed (Fig. 1.4). AKT targets are involved in important cellular processes such as cell survival, growth and translation (reviewed in (142)) (Fig. 1.4). For example, the AKT target glycogen synthase kinase 3 β (GSK3 β) is a serine/threonine kinase that regulates metabolism, cell survival, growth, differentiation, and migration (103, 104, 158, 214). GSK3 β , a negatively regulator of β -catenin, is phosphorylated by AKT on serine 9 (S9) (39). Phosphorylated GSK3 β inhibits the kinase, allowing β -catenin to drive gene transcription (50).

AKT also promotes cell survival by inhibiting the pro-apoptotic protein Bcl-2-associated death (BAD) (45) and the transcription factor forkhead box O1 (FoxO1). Through physical interaction, BAD inhibits pro-survival Bcl-2 proteins at the mitochondria to promote the release of cytochrome c and apoptosis (reviewed in (82)). AKT phosphorylates BAD on serine 136 (S136) (45), enabling 14-3-3 proteins to sequester BAD in the cytoplasm, inhibiting its activity (45, 46). BAD can also be inactivated by phosphorylation on Serine 112 and 155 by kinases other than AKT (244, 246). FoxO1 is a member of a family of transcription factors that induces genes involved in promoting apoptosis, cell cycle arrest, and metabolic processes (27). Activated AKT translocates to the nucleus and phosphorylates FoxO1 on serine 256 (S256), which promotes translocation of FoxO1 out of the nucleus (27).

Aside from cell survival, AKT is known for its function in promoting cell growth and translation. This can occur through AKT-mediated phosphorylation and inhibition of the tuberous sclerosis complex 2/1 (TSC2/TSC1) complex at serine 939 of TSC2 (105, 181). When TSC2 is inhibited, Ras-related small G protein, Rheb-GTP, acts as an activator for mTORC1 (73, 143, 230).

mTORC1 and mTORC2 are protein complexes that contain the kinase mTOR. mTORC2 contains the subunits mTOR, mLST8/GβL, Sin1 and rictor (70, 83, 84, 138, 203), while mTORC1 contains mTOR, mLST8/GβL, PRAS40, deptor and raptor (87, 122, 123, 138). Both mTORC1 and mTORC2 are inhibited by torin1, while selective inhibition of mTORC1 is achieved with rapamycin. mTORC1 inhibition is reflected in reduced phosphorylation of eukaryotic translation initiation factor (eIF) 4E binding protein 1 (4EBP1) and the hydrophobic motif of S6K (29) (77, 234) (Fig. 1.4).

The phosphorylation of S6K by mTORC1 results in its activation, which allows S6K to phosphorylate the 40S ribosomal protein S6, leading to enhanced translation (108). 4EBP1 inhibits the incorporation of the cap-binding protein eIF4E (79) into the translation initiation complex eIF4F, which is also composed of the scaffolding protein (eIF4G) and an RNA helicase (eIF4A) (78). The eIF4F complex binds and promotes the translation of methylated-5'-capped mRNA, including most HSV-1 and cellular mRNAs. mTORC1 phosphorylation of 4EBP1 on multiple sites dissociates 4EBP1 from eIF4E, permitting the formation of the translation initiation complex (77).

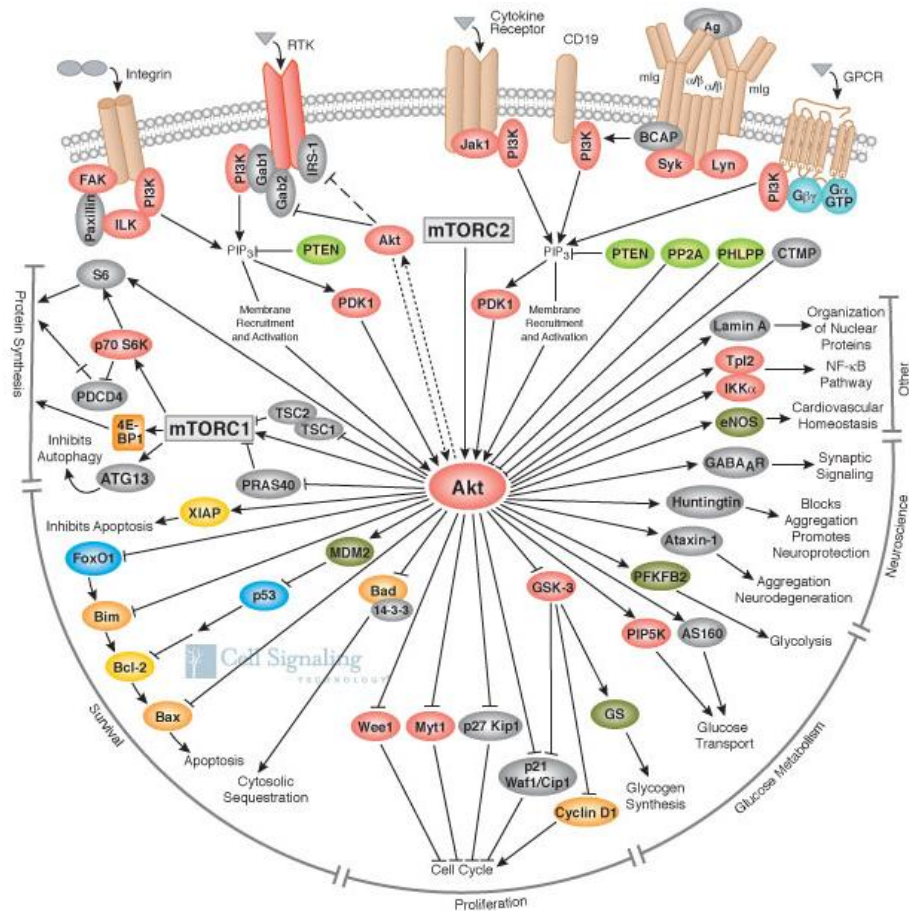


Figure 1.5: AKT acts on numerous proteins. AKT is a major signaling arm of PI3K. AKT acts on multiple proteins at the plasma membrane, and in the cytosol and nucleus (Cell Signaling).

1.3 Viruses modulate PI3K-AKT signaling

Given the importance of PI3K-AKT signaling in regulating cellular processes, it is of no surprise that viruses manipulate this signaling pathway. For example, the EBV immediate-early protein BRLF1 and latent membrane protein LMP1 activate PI3K, which promotes viral gene transcription and survival of infected cells (43, 47). Similarly, the polyoma virus protein middle T antigen (mTAG) also activates PI3K. mTAG is a membrane-bound protein that becomes tyrosine phosphorylated and recruits c-Src (190). This interaction leads to a conformational change in the mTAG-c-Src complex that creates a binding site for PI3K (229), leading to activation of PI3K-AKT signaling and cellular transformation (102). Similar to mTag, our lab has shown the HSV-1 protein VP11/12 recruits SFK and PI3K, leading to activation of AKT (247).

1.3.1 HSV-1 U_L46

VP11/12 was originally thought to be two proteins, VP11 and VP12 (220). However, in-depth analysis suggested that VP11/12 is one protein encoded by the ORF UL46 (133, 167, 271). VP11/12 is one of the most abundant structural proteins found in the virion tegument (258). As a structural protein of the virion, VP11/12 is produced in the late stages of infection and its synthesis is highly dependent on viral DNA replication (117). During infection, it is located throughout the cytoplasm, along membranous structures (167), and the periphery of the nucleus (117, 258). VP11/12 was originally thought to enhance α -gene transcription in collaboration with VP16, while suppressing α -gene transcription in the absence of VP16 (117, 152). However, this was brought into question when no detectable effect on immediate early gene expression was seen in a ΔU_L46 -virus infection (269).

1.3.2 U_L46 activates SFKs and PI3-Kinase to induce AKT activation

The first evidence that VP11/12 might influence cell signaling pathways emerged from studies designed to examine HSV-inactivated lymphocytes (NK, T and B cells), which were found to contain a 90 kDa tyrosine-phosphorylated protein identified as VP11/12. Tyrosine phosphorylation of VP11/12 is cell-type

specific, with weaker phosphorylation observed in fibroblasts and epithelial cells compared to lymphocytes (269). Accordingly, the lymphocyte-specific SFK Lck was found to be required for tyrosine phosphorylation of VP11/12 in Jurkat cells (269). In reciprocation, VP11/12 was required for tyrosine phosphorylation of Lck on the activation residue (Y394) (248).

Subsequent work by Dr. Wagner suggested another purpose, other than T-cell signaling, for VP11/12-Lck interactions. First, HSV mutants lacking VP11/12 retained the ability to block TCR signaling and inhibit cytolytic activity of an NK cell line. This indicated that VP11/12 is not essential for immune cell inactivation (269). Second, Lck expression is largely restricted to T and NK cells (145), yet a Western blot using anti-SFK phosphotyrosine antibody detected signals in B cells and weaker signals in epithelial cells and fibroblasts during HSV-1 infection (269). B cells express Lck-related SFKs, including Lyn, which is analogous to Lck in signaling from the BCR (reviewed in (263)). However, HSV does not appear to alter signaling from the BCR (86). Furthermore, epithelial cells and fibroblasts do not express Lck. These data suggest that VP11/12 serves as a substrate for other cellular tyrosine kinases (269).

Because SFKs have been reported to activate PI3K, Dr. Wagner examined the role of VP11/12 in regulating PI3K-AKT signaling during HSV-1 infection (247). Using a selective inhibitor of SFKs, PP2, Dr. Wagner discovered that SFK-induced tyrosine phosphorylation of VP11/12 is essential for an interaction between VP11/12 and the p85 regulatory subunit of PI3K (247). Using a scansite algorithm, Dr. Wagner's model predicts that VP11/12 is tyrosine phosphorylated on a predicted SH2 binding motif (YEEI) at position Y624, allowing for interaction with SFKs (248). Subsequent activation of SFK induces tyrosine phosphorylation of VP11/12 on predicted p85 SH2 (Y519, YTHM) binding motifs. Phosphorylation of the predicted p85 binding motifs on VP11/12 is thought to allow for binding of p85 and activation of PI3K (172, 247). By recruiting SFKs and PI3K to the plasma membrane, VP11/12 is hypothesized to

mimic an activated receptor, allowing HSV-1 to induce PI3K-AKT signaling (Fig 1.5) independently of activation of cellular receptors.

Indeed, AKT was found to be activated at late times post-infection in a VP11/12 and SFK-dependent manner. Furthermore, the phosphorylation of AKT is reduced in the presence of PI3K inhibitors, Ly294002 and Wortmannin, providing evidence for PI3K-dependent AKT activation (247). As expected, downstream AKT targets, S6K, 4EBP1 and GSK3 β , were also phosphorylated. Unexpectedly, although AKT was activated in a VP11/12-dependent fashion, AKT targets remained phosphorylated during infection in the absence of VP11/12. This suggests that another HSV-1 protein is involved in modulation of the AKT pathway.

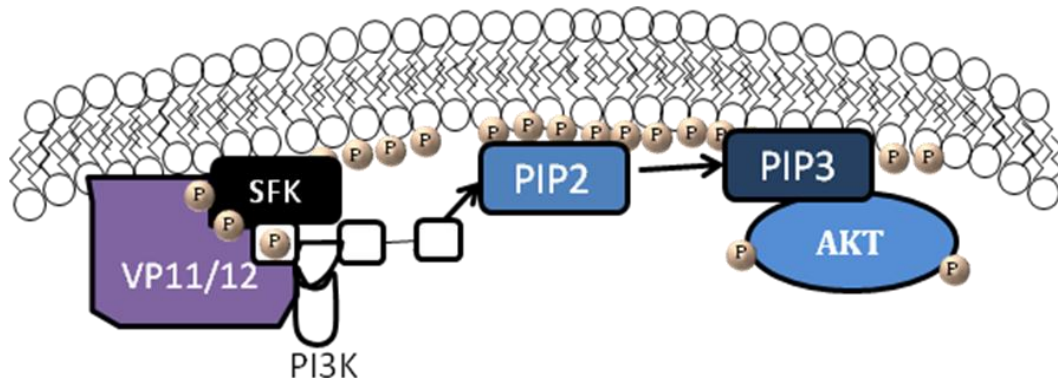


Figure 1.6: HSV-1 U_L46 binds SFK and PI3K to activate AKT. VP11/12 is thought to form a complex with Lck (or other SFKs) to initiate PI3K signaling. VP11/12 is tyrosine phosphorylated on a putative SH2-binding motif at residue Y624 (YEEL), serving as a docking site for Lck. Lck is tyrosine phosphorylated on an activating residue. Active Lck promotes tyrosine phosphorylation of VP11/12 on residue Y519 (YTHM), which is a putative p85 SH2 binding motif. Tyrosine phosphorylation of VP11/12 at residue YTHM serves as a docking site for the p85 subunit of PI3K, activating the lipid kinase. Activated PI3K signals are then propagated to phosphorylate AKT.

1.3.3 *HSV-1 U_S3*

U_S3 is a 68 kDa tegument protein encoded by the ORF U_S3 and expressed during early infection (68, 197). U_S3 is a protein kinase that phosphorylates several viral gene products including U_S9, U_L12, U_L31, U_L34, and U_L46 (41, 42, 147, 185, 187, 200). Although the effect of phosphorylation on most of these proteins has yet to be determined, the phosphorylation of U_L31 and U_L34 is thought to affect primary envelopment of the virus and egress of nucleocapsids (164, 191, 200). In addition to viral targets, U_S3 also phosphorylates the cellular proteins histone-deacetylating enzymes 1 and 2, resulting in enhanced gene expression (179, 180).

1.3.4 *U_S3 promotes cell survival through PKA-signaling*

While U_S3 is not required for viral spread in cell culture (184), U_S3-deficient viral mutants are severely restricted for growth in mice (4). The impairment in growth was attributed to increased apoptosis of infected cells (4), strongly suggesting that U_S3 functions to promote survival of host cells. Furthermore, U_S3 was shown to block apoptosis in cultured cells (11, 89, 173), and that its kinase activity is required for this function (166, 173, 200). One mechanism used by U_S3 to prevent apoptosis is the promotion of cyclic AMP-dependent protein kinase (PKA) signaling (12, 161), which is a pathway that regulates metabolism, survival, and proliferation of eukaryotic cells (174). U_S3 promotes PKA signaling by phosphorylating the regulatory subunit of PKA as well as PKA targets (12). Consequently, apoptosis is blocked by the concerted action of both activated PKA and U_S3. One protein inhibited by both PKA and U_S3 is BAD (12, 166). U_S3 inhibits apoptosis by promoting BAD phosphorylation at S112 and S136, which leads to sequestration of BAD by 14-3-3 proteins (166). Interestingly, phosphorylation of BAD at these two sites is not essential for the anti-apoptotic activity of U_S3 (11, 173), which indicates that U_S3 can inhibit apoptosis through PKA-independent mechanisms as well.

1.3.5 *U_S3 modulates AKT signaling*

While U_S3 promotes PKA signaling, it negatively regulates AKT activity (13). This was demonstrated by enhanced and prolonged AKT phosphorylation in cells infected with Δ U_S3-virus (13). It is peculiar that U_S3 negatively regulates AKT activation, given that both AKT and PKA have pro-survival roles. Although U_S3 negatively regulates AKT activation, it was found to act as an AKT mimic by phosphorylating AKT targets, during the course of this thesis (reviewed in (33)). This information, taken together with that of section 1.3.2, provides evidence that HSV-1 encodes both positive and negative regulators of AKT signaling.

1.4 Thesis Rationale

Regulation of the PI3K-AKT pathway by HSV-1 has only begun to be unraveled. Recent work by Dr. Wagner has raised two key questions. First, it is unclear whether VP11/12-PI3K interactions alone are sufficient to induce AKT activation. Activation of PI3K by VP11/12 is thought promote the recruitment of PDK1 to the plasma membrane, which subsequently phosphorylates the activation loop of AKT. Activation of AKT also involves phosphorylation of its hydrophobic motif by mTORC2. Phosphorylation of the hydrophobic motif of AKT has also been shown to be dependent on VP11/12 (247). However, it is currently unknown whether the VP11/12-dependent phosphorylation of the hydrophobic motif is mediated by mTORC2.

Second, it is unclear whether VP11/12-induced activation of AKT results in modification of downstream AKT targets in a VP11/12-dependent manner. Early evidence from Dr. Wagner suggests that HSV-1 infection induces S6K and GSK3 β phosphorylation on the same sites as AKT, but surprisingly, in a VP11/12-independent manner (247). Therefore, AKT targets may be phosphorylated by an AKT-independent mechanism during HSV-1 infection. We considered the idea that AKT targets are regulated by a viral protein kinase. HSV-1 encodes two protein kinases, U_S3 and U_L13. Of these, U_S3 is known to promote the phosphorylation of PKA targets such as BAD. Thus, I chose to examine whether U_S3 is involved in the phosphorylation of AKT targets.

MATERIALS AND METHODS

2.1 Mammalian cell cultures

Human Embryonic Lung (HEL) fibroblast, African monkey kidney epithelial (Vero) cells, and HeLa cervical carcinoma cells were obtained from the American Type Culture Collection. Telomerase immortalized human foreskin fibroblasts (HFF-Tel12, referred to as HFF below) were a gift from W. Bresnahan (24). Cells were cultured and passaged in 150 cm² tissue culture flasks (Corning) with 20 mL of Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin (P), 5 µg/mL streptomycin (S), 2mM L-glutamine and 1mM Sodium Pyruvate. Cultures were incubated at 37°C supplemented with 5% CO₂ in a humidified atmosphere. Cells were passaged when confluent (usually every 2-3 days dependent on the cell type) by trypsinizing (Trypsin, Gibco) until cells were no longer attached to the flask, re-suspending in DMEM/FBS and reseeding to approximately 25% confluence.

HEL cells are not immortal and were therefore passed up to a maximum of 15 times, while the immortal Vero, HeLa, and HFFs were passed indefinitely. Cells were trypsinized and their concentration was determined using a hemacytometer. Monolayers of confluent cells for infection were prepared by seeding 5x10⁵ cells (6-well plate) in tissue culture plates (Corning) that have a surface area of 9.5 cm² per well. The plates were incubated at 37°C supplemented with 5% CO₂ in a humidified atmosphere until confluence was observed under a light microscope, which was usually one day after seeding plates.

2.2 Virus strains and growth conditions

The following virus strains were used during the course of this investigation; all were grown and titered on Vero cells:

KOS37: is derived from the KOS37 BAC, an infectious bacterial artificial chromosome (BAC) that contains the entire HSV-1 strain KOS genome (76).

KOS37ΔU_L46-GalK(269): is a derivative of KOS37 in which the U_L46 open reading frame (ORF) has been precisely deleted and replaced with an *Escherichia coli* galK selective cassette.

KOS37 Δ U_L46(269): KOS37 Δ U_L46 is a derivative of KOS37 Δ U_L46-GalK in which the galK cassette has been precisely removed.

KOS37-RUL46(269): is an U_L46 mutant virus where the U_L46 ORF mutation present in KOS37 Δ U_L46-GalK has been restored to wild type. The mutant virus is a derivative of KOS37 Δ U_L46-GalK in which the galK cassette has been precisely removed and replaced with the KOS37 U_L46 ORF.

KOS37 Δ U_S3-GalK and KOS37 Δ U_S3 (Wu, F. unpublished data):

KOS37 Δ U_S3-GalK is a derivative of KOS37 in which the U_S3 ORF has been precisely removed and replaced by an E. coli galK selective cassette.

KOS37 Δ U_S3-GalK underwent further selection to remove galK and generate KOS37 Δ U_S3 constructs bearing a complete U_S3 deletion.

KOS37 Δ U_S3 Δ U_L46-GalK and KOS37 Δ U_S3 Δ U_L46 (Wu, F. unpublished data):

KOS37 Δ U_S3 Δ U_L46-GalK is a derivative of KOS37 Δ U_L46 in which the U_S3 ORF has been precisely removed and replaced by an E. coli galK selective cassette.

KOS37 Δ U_L46- Δ U_S3-GalK underwent further selection to remove galK and generate KOS37 Δ U_L46 Δ U_S3 constructs bearing a complete U_L46 and U_S3 deletion.

F(57): is a wild type HSV-1 virus isolated from individuals with skin lesions and characterized based on its adhesion and fusion properties to Hep-2 cells.

R7041 (F Δ U_S3)(185): is an F strain mutant in which the U_S3 ORF that has been removed.

R7306 (F-RU_S3)(187): is a derivative of R7041 in which the U_S3 ORF has been restored by co-transfection with a plasmid containing the entire U_S3 gene with flanking sequences.

R7356 (F Δ U_L13)(185, 186): is an F strain mutant in which the U_L13 ORF that has been removed.

2.3 Preparation of viral stocks

Confluent monolayers of Vero cells grown in 150 cm² flasks were infected with virus at an MOI of 0.05 PFU/cell in 5 mL serum-free DMEM for 1 hour at 37°C and rocked every 10-15 minutes. After an hour of incubation, medium was aspirated and replaced with 25 mL of medium 199 + P/S (Gibco) supplemented

with 10% FBS at 37°C in a humidified atmosphere supplemented with 5% CO₂. When the majority of cells exhibited total cytopathic effect (3-5 days post infection), they were harvested and pelleted by centrifugation (Beckman G5-R swinging bucket centrifuge) at 3000 revolutions per minute (rpm) for 10 minutes at 4°C. The medium was aspirated and samples were re-suspended in 1 mL serum free DMEM/flask, then subjected to a triplicate process of freezing at -80°C and thawing in a 37°C water bath in conjunction with vortexing in between freeze-thaws cycles. Cells were disrupted by three sonication cycles of 20 seconds (550 Sonic Dismembrator, Fisher Scientific) in conjunction with vortexing between cycles. Cell debris was separated by centrifugation at 3000 rpm for 10 minutes at 4°C. The supernatant was re-suspended in 100 µL DMEM/flask and aliquots of 100 µL were stored in cryovials (Nalgene) at -80°C.

2.4 Virus titrations

One day prior to infection, Vero cells were seeded in 6-well plates (Corning) to create a confluent monolayer of permissive cells. Virus stocks were titrated by plating ten-fold dilutions (ranging from 10⁻² to 10⁻⁸) in serum-free DMEM. Cells were infected with 0.5 mL of each dilution for 1 hour at 37°C and rocked every 15 minutes. The inoculum was replaced with 2 mL DMEM containing 10% human serum. Infections were incubated at 37°C in a humidified atmosphere supplemented with 5% CO₂ until plaques were visible (~2-5 days) by light microscopy. Medium was aspirated and cells were fixed with methanol for 10 minutes at room temperature. Samples were stained with 10% Geimsa stain (Sigma) for at least 1 hour. Geimsa stain was removed and plaques were counted for dilutions that produced 50-100 plaques.

2.5 Infecting Cells for studies of HSV-induced host signaling modifications

Once cells formed a confluent monolayer in a 6-well plate (Corning), they were serum-starved by incubation in 2 mL serum-free DMEM for 24 hours prior to infection. Immediately prior to infection, one well was sacrificed to determine the cell number. For the cell count, medium of the well was aspirated and the well was washed with 1 mL of 1xPBS. Cells were trypsinized and their concentration was determined using a hemacytometer. Virus was appropriately diluted in serum-

free DMEM and 0.5 mL was used to infect cells at an MOI of 10. The infection proceeded for 1 hour at 37°C with rocking every 15 minutes. The medium was aspirated and replaced with 2 mL serum-free DMEM. The plates were incubated for varying times at 37°C in a humidified atmosphere and supplemented with 5% CO₂.

2.6 Addition of drugs and growth factors

Inhibitory drugs were used to assess the requirement of specific kinases on AKT activation. Torin1 (NS Grey, Dana-Farber Cancer Institute) and rapamycin (Sigma-Aldrich) were stored in DMSO at -20°C at 250 nM and 1mM respectively. Rapamycin was used at a working concentration of 100nM in 1 mL serum-free DMEM for either the entire course of the infection or for the last 3 hours of post infection. Torin1 was used at a working concentration of 50nM in 1 mL serum-free DMEM for either the entire course of the infection or for the last 3 hours of post infection.

External growth factor ligands were used to assess the transduction of activated AKT signals. PDGF A-chain A-chain (AA), A-chain B-chain (AB) and B-chain B-chain (BB) (PDGF PeproTech) were reconstituted in water. Confluent monolayers of cells were serum-starved for 24 hours and treated with an increasing amount of serum (FBS), PDGF, or recombinant human PDGF AA, AB or BB that was diluted in DMEM. As described in the results, cells were treated with an amount of external ligand that induces detectable AKT phosphorylation at residues S473 and T308.

2.7 Preparation of cell extracts

Medium was removed into a conical centrifuge tube and the monolayers were washed with 0.5 mL 1xPBS, treated with 0.5 mL trypsin at 37°C and cells scraped off and the flask was washed with PBS once more. All of the washes were combined with the cells and original medium, and the cells were then pelleted at 1500 rpm for 5 minutes at 4°C. The supernatant was aspirated and the pellet resuspended in 0.5 mL 1xPBS. The resuspended pellet was placed under centrifugative force at 1681 rpm (Eppendorf Centrifuge 5417C) for 5 minutes. The supernatant was aspirated and the pellet was incubated in 200 uL lysis buffer

(1% Nonidet, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium fluoride (NaF), 1 mM sodium vanadate (Na₃VO₄), 1 mM β-glycerophosphate, 50 mM Tris-HCl pH 7.4) supplemented Complete protease inhibitor cocktail (Roche). After addition of 50 μL 6x SDS-Page lysis buffer (1M Tris-HCl pH 6.8, 30% glycerol, 10% SDS, 0.012% bromophenol blue, 6% 2-mercaptoethanol) for each sample, the samples were incubated at 4°C for 15-30 minutes and stored at -80°C.

2.8 SDS-polyacrylamide gel

A SDS-polyacrylamide gel consisted of a separating and stacking gel portion made from 5 and 1 mL solutions respectively. The separating gel portion was either made 8 or 15% from a 40% polyacrylamide (Fisher Bioagents) mix. An 8% acrylamide-SDS gel consists of 2.65 mL dH₂O, 1 mL 40% polyacrylamide mix, 1.3 mL 1.5 M Tris pH 8.8, 0.05 mL 10% SDS, 75 μL 10% ammonium persulfate (Bio-Rad), and 4.5 μL TEMED (EMD). A 15% acrylamide-SDS gel consists of 1.77 mL, 1.88 mL 40% polyacrylamide mix, 1.3 mL 1.5 M Tris pH 8.8, 0.05 mL 10% SDS, 75 μL 10% ammonium persulfate, and 4.5 μL TEMED. The separating gels were loaded into cleaned 1 mm glass gel plates (Bio-Rad) and an unmeasured small amount of water was pipetted on top to smoothly level the separating gel. The gels were left for 20-30 minutes to polymerize and the water was carefully removed. The stacking gel portion consists of 0.70 mL H₂O, 0.13 mL 40% polyacrylamide mix, 0.13 mL 1.0 M Tris pH 6.8, 0.01 mL 10% SDS, 30 μL 10% ammonium persulfate, and 3.0 μL TEMED. Immediately after loading the stacking solution onto the separating gel, a 10 or 15-well comb (Bio-Rad) was inserted and the gel was left to polymerize for 30 minutes. Upon polymerization, gels were loaded into a western blot apparatus (Bio-Rad) and the apparatus was filled with 1xSDS page buffer. 1xSDS page buffer is composed of 12.08 g Tris (Invitrogen), 57.6 g Glycine (Fisher Scientific), 4 g SDS (Bio-Rad), 1 L ddH₂O. The combs were carefully removed and a syringe (BD) filled with 1xSDS page buffer was used to remove any particulates left in the wells.

Samples were boiled at 95°C for 5 minutes and 15 μL was loaded into an 8% or 15% acrylamide-SDS gel along with 3 μL pre-stained SDS-Page standards,

Low Range (Fermentas). The gel was electrophoresed (Thermo) for 1-2 hours at 100 V in 1xSDS PAGE buffer.

2.9 Wet transfer

Nitrocellulose membranes (Hybond ECL, Amersham Pharmacia) were incubated in western transfer buffer (800 mL methanol (Fisher Scientific), 2.8 L ddH₂O, 400 mL 10x transfer buffer [121.2 g Tris, 575 g Glycine, 4 L ddH₂O]) for 10 minutes. Protein was transferred onto a nitrocellulose membrane using a wet protein transfer apparatus (Bio-Rad Trans-blot cell) at 75 V for 1.5 hours. After transfer, the membrane was incubated in Odyssey Blocking Buffer (OBB) (Aerius) and TBS-Tween (TBST) (25 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween-20) in a 1:1 ratio at room temperature for 1 hour.

2.10 Antibodies

The following dilutions of primary antibody were used for western blotting in this investigation: monoclonal anti-VP16 (LP1) (153), a kind gift of A. Minson, 1:15000; monoclonal anti-ICP27 1:7000 (Cat.# P1113, Virusys); polyclonal anti-actin 1:5000 (Cat.# A2066, Sigma-Aldrich); monoclonal GSK3 β 27C10 1:1000 (Cat.# 9315, Cell Signaling); polyclonal phospho-GSK3 β S9 1:1000 (Cat.# 9336, Cell Signaling); polyclonal AKT 1:1000 (Cat.# 9272, Cell Signaling); polyclonal phospho-AKT Thr308 1:1000 (Cat.# 9275, Cell Signaling); monoclonal phospho-AKT Ser473 1:1000 (Cat. #9271, Cell Signaling); monoclonal phospho-AKT Ser473 (D9E) 1:1000 (Cat. #4060, Cell Signaling); polyclonal BAD 1:1000 (Cat.# 5286, Cell Signaling); monoclonal phospho-BAD Ser136 1:1000 (Cat.# 9292, Cell Signaling); monoclonal FoxO1 C29H4 1:1000 (Cat.# 2880, Cell Signaling); polyclonal phospho-FoxO1 Ser256 1:1000 (Cat.# 9461, Cell Signaling); and polyclonal 4EBP1 1:1000 (Cat.# 9452, Cell Signaling). The membrane was incubated with primary antibody diluted in OBB:TBST in a 1:1 ratio overnight at 4°C. Membranes were washed in TBST 4-5 times for 10 minutes each at room temperature.

For infrared imaging, the membrane was incubated in secondary antibody in OBB:TBST in a 1:1 ratio for 1 hour at room temperature. The following dilutions of secondary antibody were used in this investigation: polyclonal donkey

anti-mouse IgG IRDye 800 diluted 1:10000 (Rockland); Alexa Fluor[®] 680 goat anti-rabbit IgG (H+L) diluted 1:15000 (Invitrogen). Following, washes in TBST 4-5 times for 10 minutes at room temperature, membranes were visualized using an infrared imager (Aerius).

FoxO1 and BAD antibody staining were visualized by enhanced chemiluminescence followed by detecting using X-ray film. Membranes that were incubated with primary FoxO1 or BAD antibody staining were washed in TBST 3-5 times for 10 minutes each. The membranes were incubated with the secondary antibody in TSBT/5% skim milk for 1 hour at room temperature: rabbit anti-mouse IgG-horseradish peroxidase (HRP) (Sigma) diluted 1:3000 and goat anti-rabbit IgG-HRP diluted 1:10000. After washing five times as before, the membrane was developed using ECL-plus system (Amersham Biosciences) according to the manufacturer's instructions. The signal was detected via exposure to Fuji X-Ray Medical Film (Fuji) on a M135A X-OMAT processor (Kodak).

CHAPTER 3: RESULTS

3.1 HSV-1 infection causes maximal AKT activation at late times during infection

As summarized in the rationale of Section 2.2, I sought to address two key questions raised by Dr. Wagner's previous research on HSV-1 induced phosphorylation of AKT. First, there was strong evidence for a role of PI3K (and by implication PDK1) in the VP11/12-dependent phosphorylation of AKT during HSV-infection, but her work did not address whether mTORC2 is also involved. Second, although HSV-1 strongly promotes phosphorylation of several AKT targets, such phosphorylation was independent of VP11/12. Therefore, one or more additional viral proteins influences phosphorylation of at least some AKT targets during infection. I examined if there are other viral proteins that regulate AKT signaling.

I first confirmed Dr. Wagner's findings that VP11/12 is required for HSV-1 induced AKT phosphorylation at late times post-infection in non-transformed human embryonic lung (HEL) fibroblasts (Fig. 3.1). To this end, confluent monolayers of cells were serum-starved 24 hours prior to infection and during the course of infection. They were mock-infected or infected with wild type HSV-1 KOS37, the VP11/12-null mutant ΔU_L46 , or the VP11/12-repaired virus RU_L46 at a MOI of 10 PFU. Cell lysates were collected at 18 hpi and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), and total AKT by Western blotting. Actin served as a loading control and HSV-1 VP16 was used as a control for virus infection. As expected, wild type virus enhanced phosphorylation on both AKT residues relative to the levels observed in mock-infected cells and this effect was abrogated in cells infected with the VP11/12-null mutant ΔU_L46 , and restored repairing the VP11/12 gene in RU_L46 (Fig. 3.1).

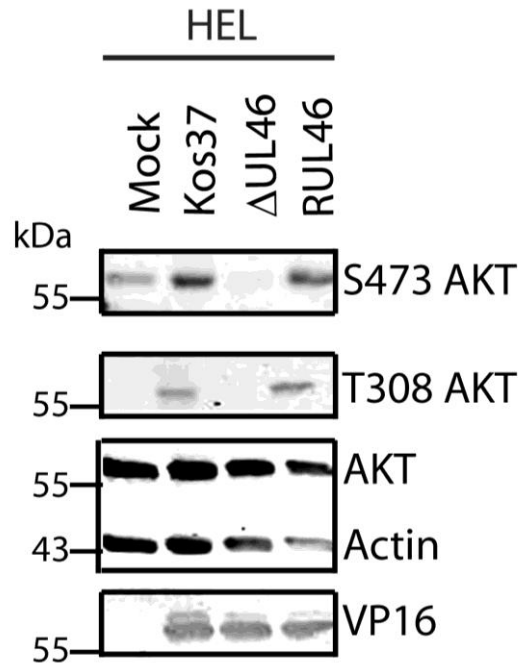


Figure 3.1: HSV-1 activates AKT in a U_L46-dependent manner. Confluent monolayers of HEL cells were serum-starved and mock-infected or infected with wild type HSV-1 KOS37, the VP11/12-null mutant Δ UL46, or the VP11/12-repaired virus RUL46. Cell lysates were collected at 18 hpi and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), total AKT, VP16, and β -actin by Western blotting.

3.2 HSV-1 requires mTORC2 for AKT phosphorylation

In uninfected cells, growth factor receptor signaling activates PI3K (sometimes with the assistance of SFKs), leading to activation of PDK1, which subsequently phosphorylates the activation loop of AKT. Activation of AKT also involves phosphorylation of its hydrophobic motif by PDK2, recently identified as mTORC2. The mechanisms that lead to the activation of mTORC2 remain largely unknown. HSV-induced activation of AKT involves VP11/12- and SFK-dependent activation of PI3K, strongly suggesting that in HSV-infected cells, PDK1 mediates phosphorylation of AKT as in uninfected cells. Phosphorylation of the hydrophobic motif of AKT has also been shown to be dependent on VP11/12. However, it is currently unknown whether the VP11/12-dependent phosphorylation of the hydrophobic motif is mediated by mTORC2. To address this question I examined the effects of inhibiting mTORC2 activity.

As described in the introduction, the mTOR kinase exists in two complexes, mTORC1 and mTORC2, that differ in their subunit composition and sensitivity to rapamycin. Both kinases contain the mTOR subunit that requires phosphatidic acid (PA) for mTOR activity. PA and rapamycin compete to interact with the FKBP12-rapamycin binding domain (FRB) of mTOR (64). Low concentrations of rapamycin are able to outcompete PA interactions with mTORC1. In contrast to mTORC1, mTORC2 is a more stable structure, so higher concentrations of rapamycin are required to dissociate the mTORC2 complex. Thus, rapamycin is an effective inhibitor of mTORC1. Another inhibitor of the mTOR complexes is torin1, which inhibits the mTOR catalytic subunit found in both mTORC1 and mTORC2 (235). Therefore, if mTORC2 is required for HSV-induced AKT activation, then one would predict that torin1 will block AKT activation, while rapamycin will have no effect.

To test this prediction, serum-starved confluent monolayers of HEL cells were mock-infected or infected with wild type HSV-1 KOS37, the VP11/12-null mutants ΔU_L46 and ΔU_L46 -GalK, or the VP11/12-repaired virus RU_L46 . Samples were treated with either 250 nM of torin1 or 100 nM of rapamycin for the entire course of infection (Fig. 3.2A) or the last three hours only (Fig. 3.2B). Cell lysates

were collected at 18 hpi and analyzed for the activating phosphorylation of AKT at residues S473 and T308, as well as for total AKT, 4EBP1, VP16, and β -actin by Western blotting. An equivalent concentration of the solvent DMSO (1 μ L/mL) was also used to discount any non-specific effects. DMSO had no detectable effect (either positive or negative) on AKT signaling or downstream phosphorylation of 4EBP1 in either mock-infected or infected cells (Fig. 3.3).

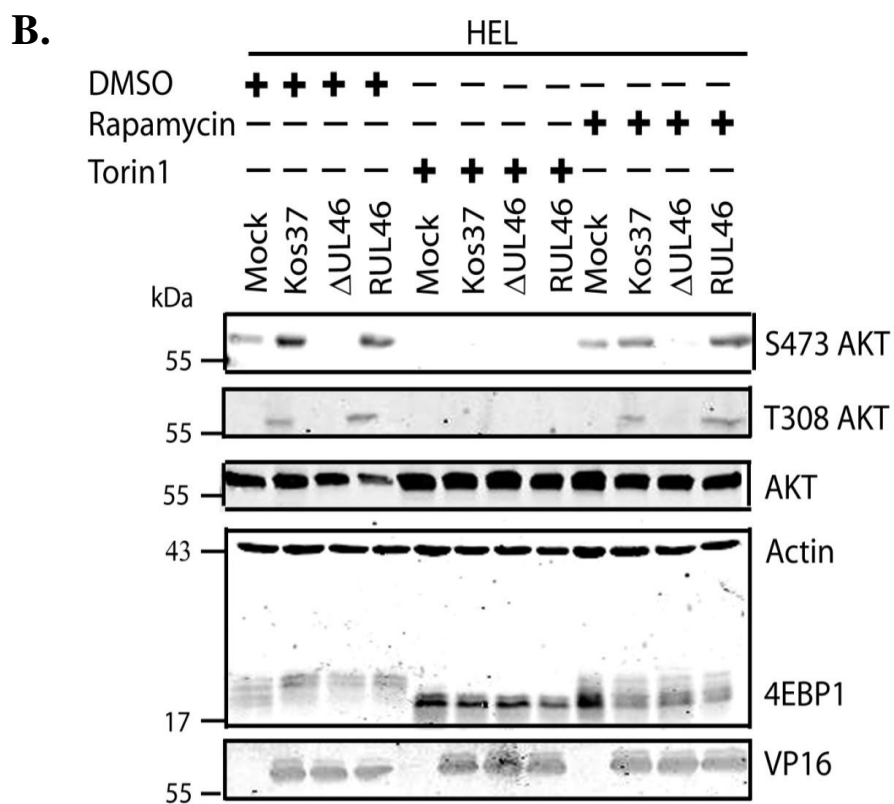
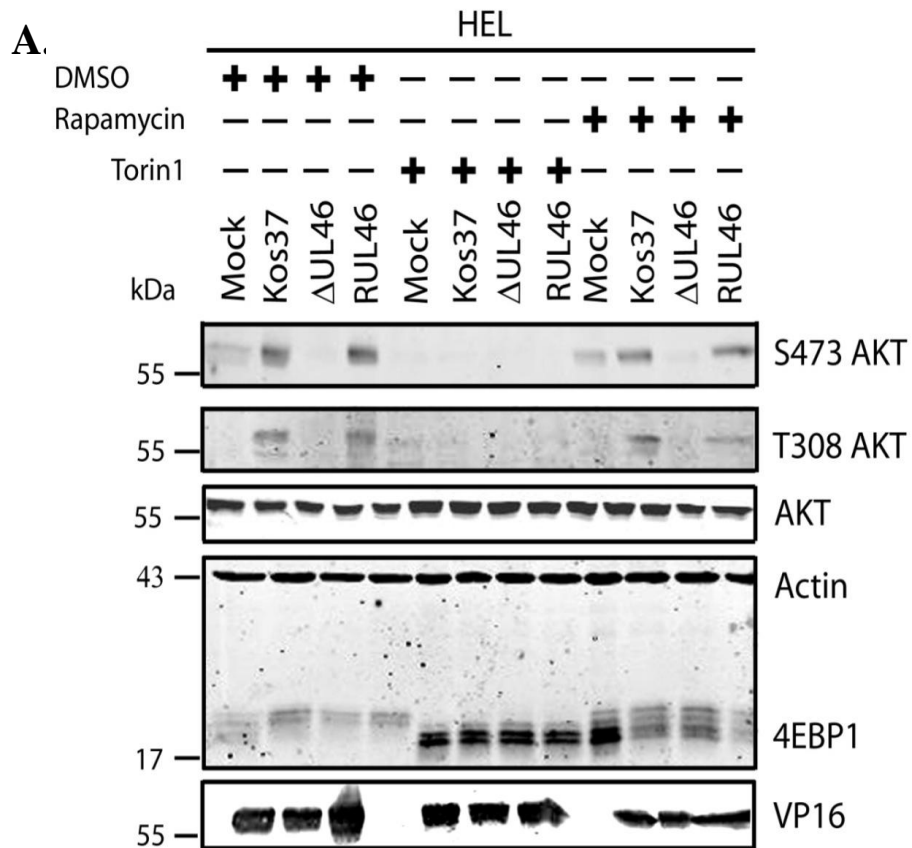
4EBP1 is a direct target of mTORC1 that can exist in several states of phosphorylation, resulting in the appearance of multiple bands on a Western blot. Both rapamycin and torin1 inhibited the phosphorylation of 4EBP1 in mock-infected cells. Reduction in the slowly migrating hyperphosphorylated forms of 4EBP1 is indicative of the effectiveness of these drugs.

AKT phosphorylation remained undetectable in mock-infected cells treated with either drug (Fig. 3.2). As expected, U_L46 was required for HSV-induced AKT phosphorylation, which was restored in RU_L46-infected cells. In the presence of rapamycin, AKT was strongly phosphorylated at residues S473 and T308 during wild type and RU_L46 infection, but not during infection with Δ U_L46 virus. This was expected given that mTORC1 is positioned downstream of AKT and does not positively regulate AKT phosphorylation (79). Regardless of the length of drug treatment, torin1 prevented phosphorylation at both activating residues of AKT during infection with wild type HSV-1 and RU_L46. These data suggest that AKT phosphorylation during HSV-1 infection requires mTORC2. Taken in combination with the results of Wagner and Smiley (247), these results are consistent with the hypothesis that HSV employs VP11/12 to trigger AKT phosphorylation via mechanisms similar to those used by activated growth factor receptors in uninfected cells.

As previously reported (247), in the absence of inhibitors, HSV-1 infection resulted in 4EBP1 hyperphosphorylation. Treatment of infected HEL cells with torin1 or rapamycin caused 4EBP1 to become hypophosphorylated, though this effect was more pronounced in torin1-treated cells. Also as previously reported, 4EBP1 hyperphosphorylation was VP11/12-independent (247). The simplest interpretation of this result is that HSV-1 encodes one or more proteins

other than VP11/12 that promote 4EBP1 hyperphosphorylation in an AKT-independent manner (Fig. 3.2) (247). I next focused my efforts on identifying the additional viral protein(s) involved.

Figure 3.2: mTORC2 is required for AKT activation during HSV-1 infection. Serum-starved HEL cells were either mock-infected or infected with HSV-1 KOS37, the VP11/12-null mutant Δ UL46, and the VP11/12-repaired virus RUL46 for 18 hours. A) During infection, cells were either left in serum-free media, treated with 250 nM torin1 or 100 nM of rapamycin. B) Alternatively, for the last three hours of infection, samples were either left in serum-free media, treated with 250 nM Torin1 or 100 nM of rapamycin. Cell lysates were collected at 18 hpi and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), total AKT, 4EBP1, VP16, and β -actin by Western blotting.



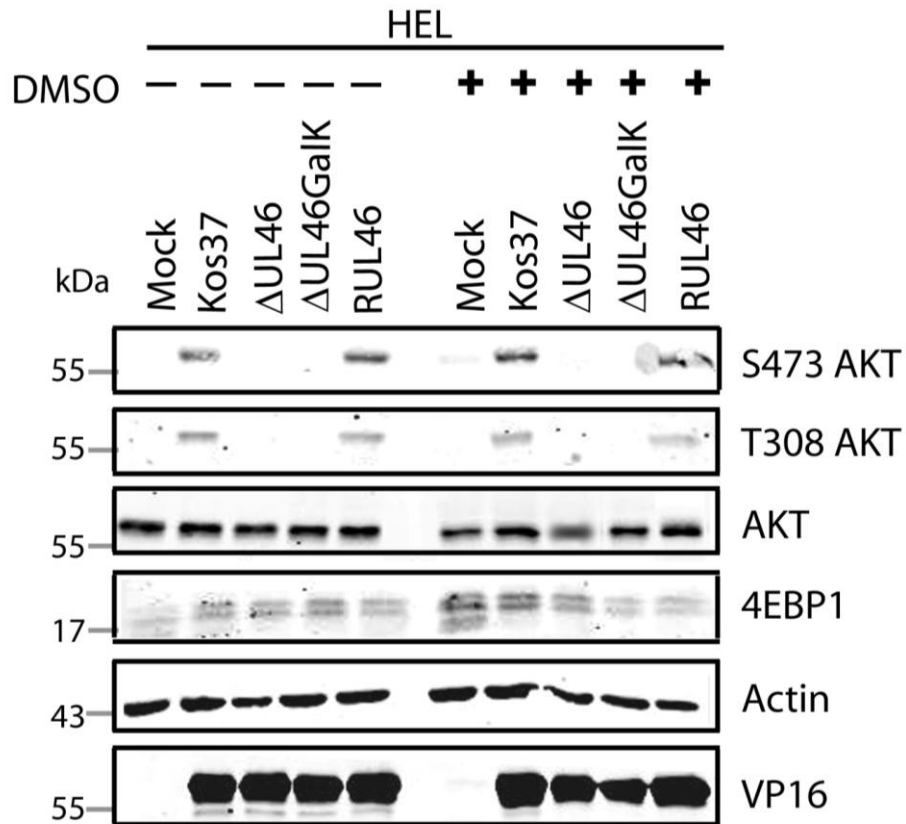


Figure 3.3: DMSO does not dampen AKT activation during HSV-1 infection. Serum-starved HEL cells were mock-infected or infected with wild type HSV-1 KOS37, the VP11/12-null mutants Δ UL46GalK and Δ UL46, or the VP11/12-repaired virus RUL46. During the course of the infection, cells were either left in serum-free media or treated with 1 μ L of DMSO in 1 mL of serum. Cell lysates were collected at 18 hpi and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), total AKT, 4EBP1, VP16, and β -actin by Western blotting.

3.3 *US3 mediates the phosphorylation of AKT targets*

Since U_L46 is required for AKT phosphorylation during HSV-1 infection, the phosphorylation of AKT targets was expected to be U_L46-dependent (247). However, although the AKT targets GSK3 β and 4EBP1 were phosphorylated in HSV-1 infected cells, their phosphorylation did not require U_L46 (247). Therefore, HSV-1 likely encodes one or more proteins other than VP11/12 that promote phosphorylation of AKT targets independently of AKT activation. We considered the possibility that an HSV-1 protein kinase directly phosphorylates AKT targets. HSV-1 encodes two protein kinases, U_S3PK and U_L13PK. Of these, U_S3 has previously been shown to regulate the phosphorylation of BAD on residue S136, the same site that AKT phosphorylates (165). Therefore, I asked whether U_S3 is also required for the phosphorylation of other AKT targets.

Serum-starved HEL cells were mock infected or infected with wild type HSV-1 KOS37 and F strains, the KOS37 strain VP11/12-null mutants Δ U_L46 and Δ U_L46-GalK, the VP11/12-repaired virus RU_L46, the F strain U_S3PK-null mutant R7041, the U_S3PK-repaired virus R7306 or the F strain U_L13-null mutant R7356 virus. Cell lysates were collected at 18 hpi and analyzed for the phosphorylation of GSK3 β and FoxO1 at residues S9 and S256 respectively, as well as for total GSK3 β and FoxO1, VP16, and β -actin by Western blotting (Fig. 3.4).

Compared to mock-infected cells, infection with wild type (KOS37 and F), VP11/12-null mutants (Δ U_L46 and Δ U_L46-GalK) or VP11/12 repaired virus strongly increased GSK3 β and FoxO1 phosphorylation. Phosphorylation of AKT targets during infection was not due to increased protein expression since total GSK3 β was unchanged and total FoxO1 decreased. Phosphorylated FoxO1 was not dramatically increased in Δ U_L46-GalK infected cells, which may be a consequence of gel-loading errors or poor film exposure. In contrast to wild type HSV-1 infection, deletion of U_L13 did not reduce the phosphorylation of AKT targets, whereas deletion of U_S3 abrogated the phosphorylation of FoxO1 and GSK3 β . This result was not due to differences in protein loading as assessed by β -actin levels or a change in the progression of the virus's lytic life cycle assessed by VP16 levels. Phosphorylated GSK3 β and FoxO1 was restored in cells infected

with repaired-U_S3 virus. These data suggest that U_S3 is required for phosphorylation of AKT targets (Fig. 3.4).

During the course of these experiments, the Mohr group published similar results on the phosphorylation of AKT targets by U_S3 (34). These authors showed that U_S3 promoted phosphorylation of AKT targets FoxO1 and GSK3B. Furthermore, like AKT, U_S3 directly phosphorylates TSC2, which led to constitutive activation of mTORC1 and subsequent phosphorylation of S6K and 4EBP1. Phosphorylation of S6K and 4EBP1 resulted in enhanced translation and viral replication. Interestingly, S6K and 4EBP1 phosphorylation was only partially attenuated in Δ U_S3-infected cells, and was further reduced in the presence of an AKT drug inhibitor. These data suggest that HSV-1 activates mTORC1 through both AKT-dependent and AKT-independent mechanisms (34). From Dr. Wagner's findings, we know that AKT is activated by U_L46 (247). Thus, I examined whether U_L46-activated AKT and U_S3 signal in parallel to phosphorylate AKT targets.

HEL cells are a difficult cell line to culture because they had a finite lifespan. Therefore, I developed a new cell system before studying the effects of HSV on AKT signaling.

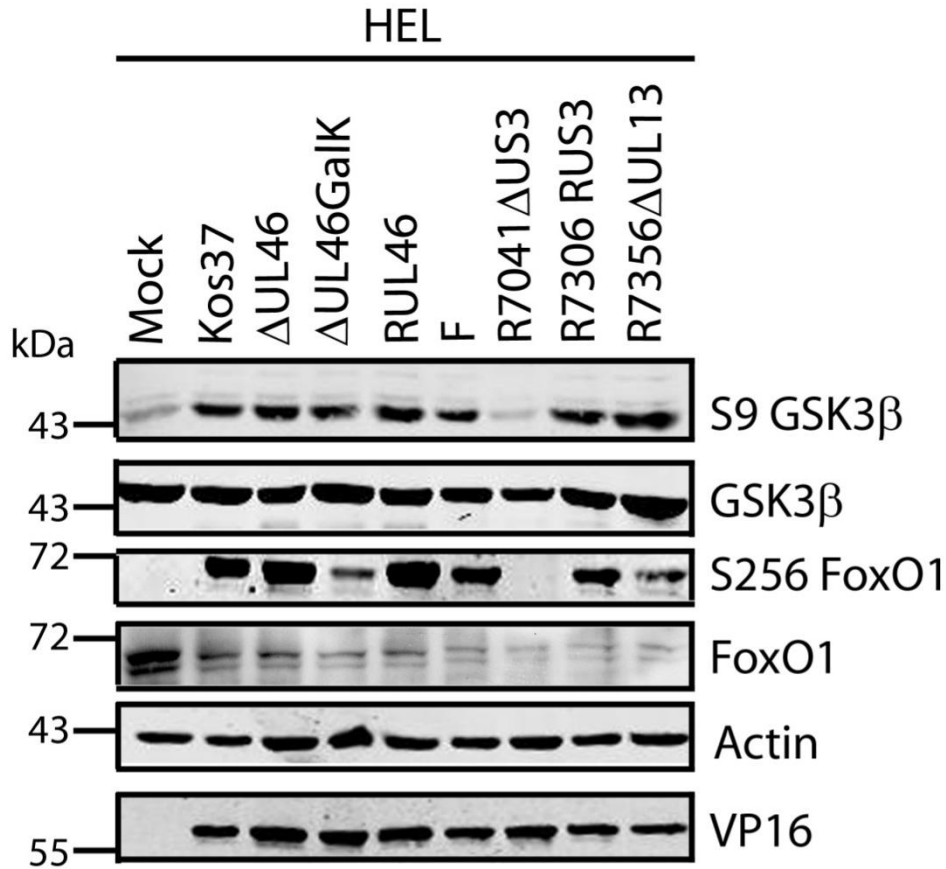


Figure 3.4: The AKT targets, GSK3 β and FoxO1, are phosphorylated in a U_L46-independent fashion, but require U_S3. Serum-starved HELs were mock infected or infected with wild type HSV-1 KOS37 and F, the VP11/12-null mutants Δ UL46GalK and Δ UL46, the VP11/12-repaired virus RUL46, the US3-null mutant R7041 Δ US3, the US3-repaired virus R7306 RUS3, and the UL13-null mutant R7356 Δ UL13. Cell lysates were collected at 18 hpi and analyzed for the inhibiting phosphorylation of GSK3 β and FoxO1 at residues S9 (anti- GSK3 β) and S256 (anti-FoxO1) respectively, total GSK3 β and FoxO1, VP16, and β -actin by Western blotting.

3.4 HSV-1 also activates AKT in Human Foreskin Fibroblasts in a U_L46-dependent fashion

HEL cells have a finite lifespan and therefore cannot be passaged indefinitely in cell culture. In order to provide a more convenient model for AKT activation in non-transformed human fibroblasts, I asked if HSV is also able to promote AKT phosphorylation in a VP11/12-dependent fashion during infection of telomerase-immortalized human foreskin fibroblasts (HFF-tel12) (24), kindly provided Wade Bresnahan). HFF cells were used by the Mohr group to examine the phosphorylation of AKT targets by U_S3 (34). Like HEL cells, HFF-tel12 cells (hereafter referred to as HFF cells) are non-transformed fibroblasts that lack constitutively active AKT. However, unlike HEL cells, they are immortal and therefore able to proliferate indefinitely.

To examine AKT phosphorylation during infection, serum-starved HFFs were mock-infected or infected with wild type HSV-1 KOS37 or the VP11/12-null mutant Δ U_L46. Cell lysates were collected at regular intervals of 3 hours until 15 hpi and analyzed for the activating phosphorylation of AKT at residue S473 (anti-S473), total AKT, VP16, and β -actin by Western blotting. As previously observed for HEL cells, wild type HSV-1 KOS37 enhanced phosphorylation on residue S473 of AKT relative to the levels observed in mock-infected cells and this effect was abrogated by the VP11/12-null mutation. Interestingly, there was a noticeable induction of AKT phosphorylation detected as early as 3 hpi that diminished by 6 hpi and reappeared by 15 hpi (Fig. 3.5A).

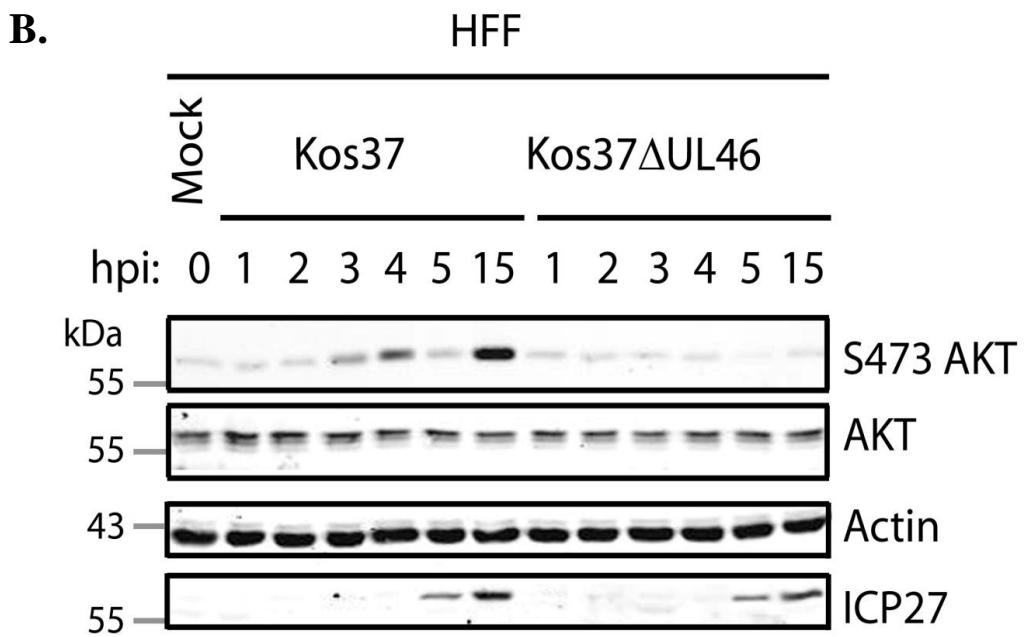
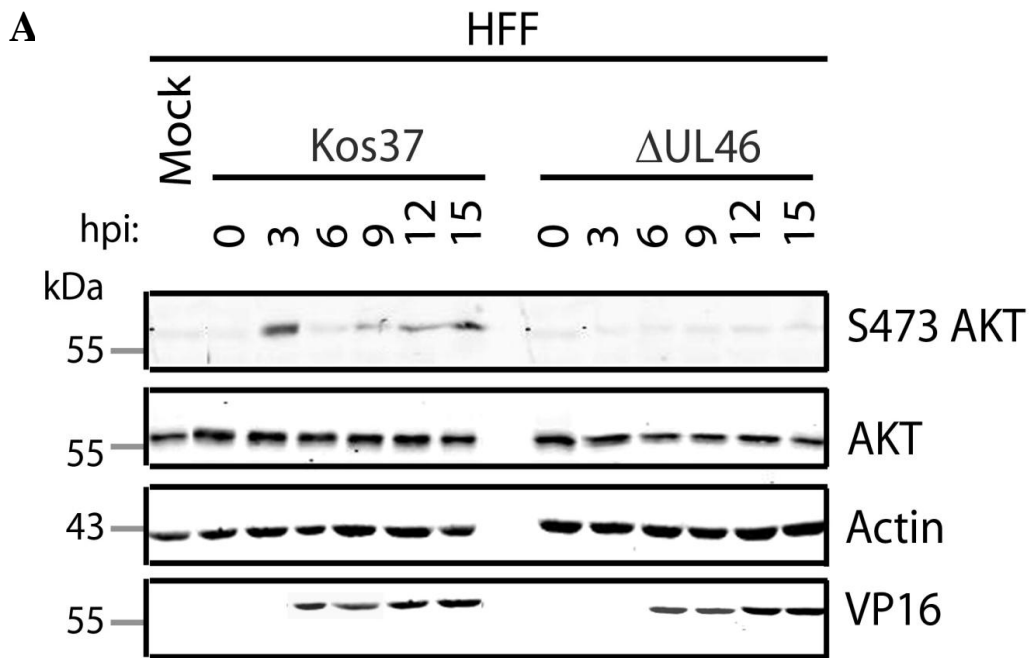
I next examined the kinetics of AKT activation at early times post-infection in greater detail by harvesting cells every hour during the first 5 hpi (Fig. 3.5B). A sample was also harvested at 15 hpi. Samples were analyzed by western blot as before. Actin served as a loading control, and HSV-1 ICP27 was used as a control for early virus infection. In infected HFFs, VP11/12-dependent AKT phosphorylation was not sustained during infection but rather occurred as a biphasic induction. AKT activation was seen at 3 and 4 hpi, diminished at 5 hpi and reappeared at 15 hpi. In this experiment, AKT activation was strongest at late

times post-infection. This was likely due to an accumulation of *de novo* VP11/12 during late infection.

Despite the presence of VP11/12 in the virion tegument, there was no increase in AKT phosphorylation at 1 hpi, suggesting that the phosphorylation of AKT depends on newly synthesized VP11/12 (Fig. 3.5B). However, the experiments performed were not designed to examine whether VP11/12 molecules from incoming virions are sufficient to induce AKT phosphorylation. Overall these data indicate that HSV-1 triggers bi-phasic AKT phosphorylation in a VP11/12-dependent fashion during infection of HFF cells. HFF cells are a suitable cell model to examine AKT signaling during HSV-1 infection. Thus, I was able to examine if U_L46-activated AKT and U_S3 signal in parallel to phosphorylate AKT targets.

AKT activation by HSV-1 was a common phenomenon seen in multiple cell types, including HeLa and Vero cells (Appendix A).

Figure 3.5: HSV-1 activates AKT in a UL46-dependent manner at early and late times post infection. Confluent monolayers of HFF cells were serum-starved and mock-infected or infected with wild type HSV-1 KOS37 or the VP11/12-null mutants Δ UL46. Cell lysates were collected at various times and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), total AKT, VP16 or ICP27, and β -actin by Western blotting.



3.5 U_S3 mediates the phosphorylation of AKT targets despite activation of AKT by U_L46

In HSV-1 infected HEL cells, AKT is phosphorylated at late times in a U_L46-dependent manner (247). In addition to HEL cells, HFFs can be used to study U_L46-dependent AKT activation. Infected HFFs have a biphasic induction (4 and 15 hpi) of AKT phosphorylation that is dependent upon U_L46. Evidence obtained using a combination of HSV-1 strains F and KOS and their mutants, suggested that the phosphorylation of AKT targets requires U_S3 (Fig. 3.4). However, it is unclear if both U_L46-activated AKT and U_S3 signal in parallel to promote the phosphorylation of AKT targets. To compare the roles of U_S3 and U_L46, Δ U_S3- and Δ U_S3 Δ U_L46-mutant viruses were constructed in the same KOS37 background as the U_L46 mutants (as described in the Methods. Wu, Fred, unpublished data). If U_L46-activated AKT signals are transduced to downstream targets, then the phosphorylation of AKT targets should be reduced in cells infected with a Δ U_S3 Δ U_L46-virus compared to cells infected with a Δ U_S3-virus. I examined the relative contributions of U_S3 and U_L46 over the course of infection. Due to the biphasic induction of AKT phosphorylation, I looked at AKT signaling at 4 and 15 hpi.

Serum-starved HFFs were mock-infected or infected with wild type HSV-1 KOS37, the KOS37 strains VP11/12-null mutant Δ U_L46, the U_S3PK-null mutant Δ U_S3, or the VP11/12 and U_S3PK-double null mutant Δ U_L46 Δ U_S3 virus. Cell lysates were collected at 4 hpi and 15 hpi, and analyzed for the phosphorylation of AKT at residues S473 and T308, the phosphorylation of GSK3 β and BAD at residues S9 and S136 respectively, as well as for total AKT, GSK3 β and BAD, and 4EBP1 (Fig. 3.6, 3.7). Lysates harvested at the late times were also analyzed for the phosphorylation of FoxO1 at residue S256 and total FoxO1 (Fig. 3.7). Actin was used as a loading control and HSV-1 ICP27 or VP16 was used as a control for virus infection (Fig. 3.6, 3.7).

For each virus treatment, the level of phosphorylated GSK3 β and 4EBP1 were measured in order to determine whether U_L46-activated AKT signals could reach AKT targets. Phosphorylated GSK3 β levels were then divided by the values

obtained by anti- β -actin to give normalized values; this accommodated for any changes in cellular protein levels. Phosphorylation of 4EBP1 was determined by the ratio of hyperphosphorylated 4EBP1 to hypophosphorylated forms. Experiments pertaining to the first phase of AKT activation were replicated three times, while the experiments pertaining to the second phase of AKT activation were replicated seven times. The quantified values from the infrared signals were statistically analyzed by a two tailed un-paired t-test.

At early (Fig. 3.6A) and late (Fig. 3.7A) times of infection, AKT was strongly phosphorylated in an U_L46 -dependent manner; thus, AKT phosphorylation was attenuated in ΔU_L46 - and $\Delta U_L46\Delta U_S3$ -infections. AKT was phosphorylated in ΔU_S3 -infected cells compared to wild type-infected cells (Fig. 3.6A, 3.7A). In fact, a previous finding states that U_S3 negatively regulates AKT phosphorylation, and the absence of U_S3 enhances and prolongs AKT activation during infection (13). Surprisingly, the phosphorylation of some AKT targets was reduced in ΔU_S3 -infected cells even though AKT was phosphorylated.

At early times of infection, AKT targets (GSK3 β , BAD and 4EBP1) were not overtly phosphorylated in mock-infected cells (Fig. 3.6A). Similar to HSV-1 infection in HEL cells, HFFs infected with wild type-virus exhibited a significant increase in phosphorylated GSK3 β ($p < 0.001$), as well as, an increase in phosphorylated BAD and 4EBP1 and total BAD (Fig 3.6A, B). Although ΔU_L46 -infected cells did not have activated AKT, the AKT targets 4EBP1, BAD and GSK3 β ($p < 0.001$) were strongly phosphorylated. The absence of U_S3 resulted in a reduction in total protein levels of BAD and both phosphorylated BAD and GSK3 β ($p < 0.05$), despite the fact AKT was phosphorylated. These data imply that activated AKT was not required for the phosphorylation of AKT targets. Cells infected with a $\Delta U_L46\Delta U_S3$ -virus displayed a weak induction of phosphorylated GSK3 β and total BAD that was comparable to levels seen in mock-infected cells. The amount of phosphorylated GSK3 β was statistically similar between ΔU_S3 - and $\Delta U_L46\Delta U_S3$ -infections ($p > 0.05$) (Fig. 3.6A, B). This indicates that the phosphorylation of GSK3 β was primarily dependent on U_S3 , with no significant contribution from U_L46 -activated AKT. 4EBP1 was also hyperphosphorylated

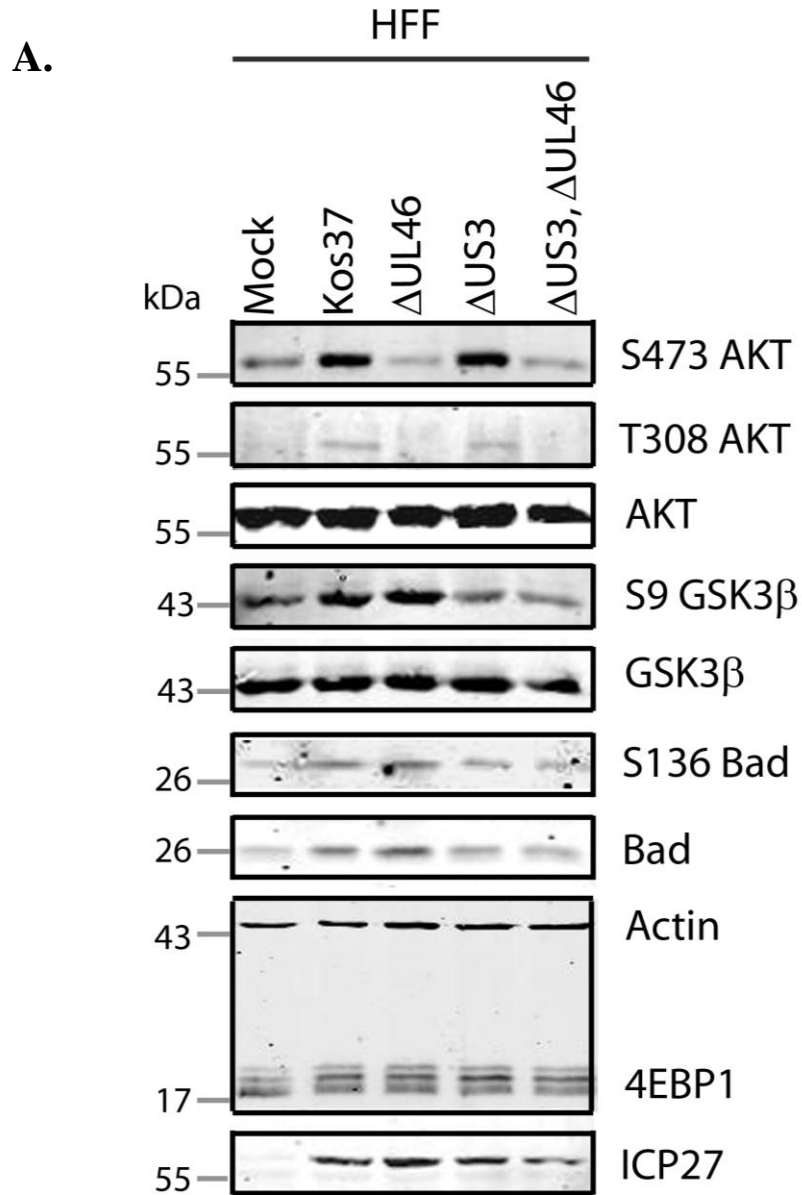
during wild-type virus infection, but it was difficult to discern whether 4EBP1 was phosphorylated in the absence of U_L46 and U_S3. If 4EBP1 was hyperphosphorylated in Δ U_L46 Δ U_S3-infected cells, then the data suggests HSV-1 encodes one or more proteins (other than U_L46 and U_S3) that promote 4EBP1 phosphorylation at early times of infection.

During late infection with wild-type virus, the phosphorylation of AKT targets was enhanced compared to mock-infected cells. The AKT targets GSK3 β ($p < 0.005$) and 4EBP1 ($p < 0.001$) were significantly more phosphorylated in wild-type and Δ U_L46-virus infection (Fig. 3.7A, B, C). In Δ U_S3- or Δ U_L46 Δ U_S3-infection, there was an attenuation of both total BAD and phosphorylation of AKT targets, even though AKT was phosphorylated (Fig. 3.7A). Δ U_S3- and Δ U_L46 Δ U_S3- infected cells had significantly less phosphorylated 4EBP1 and GSK3 β ($p < 0.05$) compared to wild-type infection. Less phosphorylated 4EBP1 could be a result of poor recognition of hyperphosphorylated 4EBP1 species. Cells infected with Δ U_S3-virus showed reduced phosphorylation of GSK3 β and 4EBP1, and these levels were further diminished during Δ U_L46 Δ U_S3-infection. However, this reduction did not correspond to a significant difference between Δ U_S3- and Δ U_L46 Δ U_S3-virus infections ($p > 0.05$, Fig. 3.7B, C). Two conclusions were drawn from these data. First, the phosphorylation of 4EBP1 and GSK3 β are primarily U_S3-dependent with no significant contribution from U_L46-activated AKT. Second, at early times of infection, U_S3 selectively promotes the phosphorylation of some AKT targets and at late times of infection this selection more clearly includes 4EBP1 (Fig. 3.7).

Mohr and colleagues reported that 4EBP1 was degraded during infection in a U_S3 dependent manner (34). My data confirm this finding showing that total 4EBP1 proteins levels were reduced in wild-type and Δ U_L46- infection ($p < 0.05$) and U_S3 was required for this reduction ($p > 0.05$). Cells infected with a Δ U_L46 Δ U_S3- or Δ U_S3-virus had comparable 4EBP1 protein levels ($p > 0.05$) indicating U_L46 does not affect 4EBP1 protein levels (Fig. 3.7D). In summary, these data indicate that U_L46-activated AKT signals do not contribute to the phosphorylation of AKT targets. Furthermore, the

phosphorylation of AKT targets relies upon U_S3, regardless of whether AKT is phosphorylated. Thus, phosphorylated AKT signals are either blocked or redirected during HSV-1 infection.

Figure 3.6: Despite U_L46-activated AKT, GSK3 β is mainly phosphorylated in a U_S3-dependent fashion at early times post infection. Serum-starved HFF cells were mock infected or infected with wild type HSV-1 KOS37, the VP11/12-null mutant Δ UL46, the US3-null mutant Δ US3, and the VP11/12-US3-null mutant Δ UL46 Δ US3. A) Cell lysates were collected at 15 hpi and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), the inhibiting phosphorylation of GSK3 β , FoxO1 and BAD at residues S9 (anti-GSK3 β), S256 (anti-FoxO1), and S136 (anti-BAD) respectively, 4EBP1, total proteins AKT, GSK3 β , FoxO1 and BAD, VP16, and β -actin by Western blotting. B) For each sample, the infrared signal obtained with the anti-GSK3 β phospho-S9 antibody for panel A was divided by the signal obtained with the anti- β -actin antibody, and the ratios were divided to yield a proportional value. Compared to mock infected cells, KOS37- and Δ UL46-infected cells had significantly ($p < 0.001$) more detectable phosphorylated GSK3 β on residue S9. Additionally, Δ US3-infection had significantly ($p < 0.05$) more phosphorylated GSK3 β on residue S9. Relative to Δ US3-infected cells, Δ UL46 Δ US3 infection did not have significantly less phosphorylated GSK3 β on residue S9 ($p > 0.05$).



B.

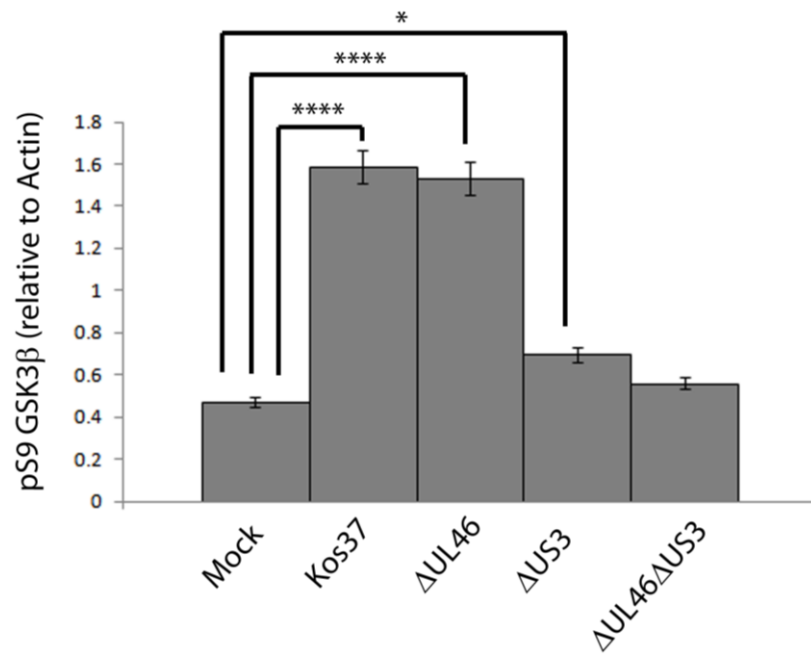
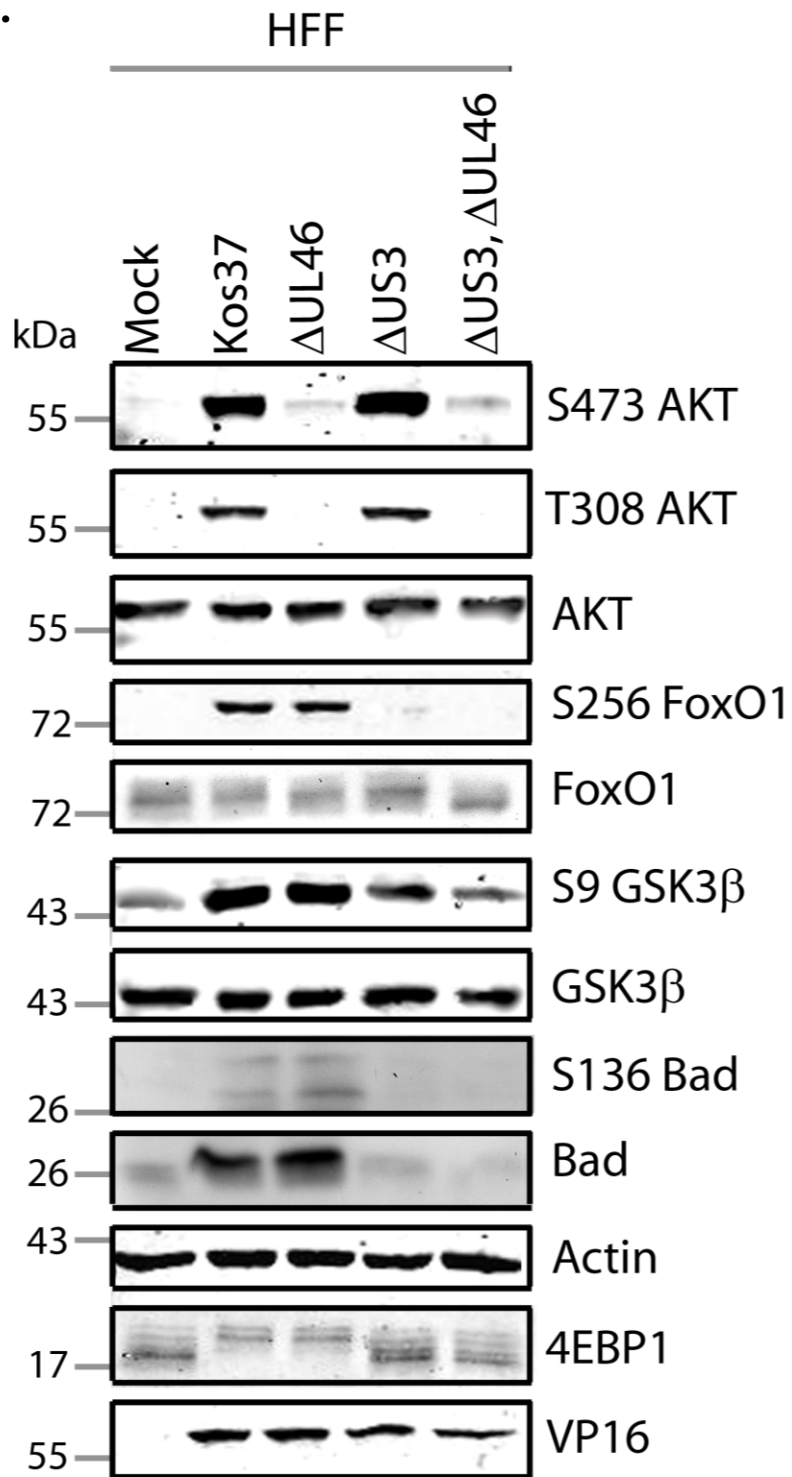
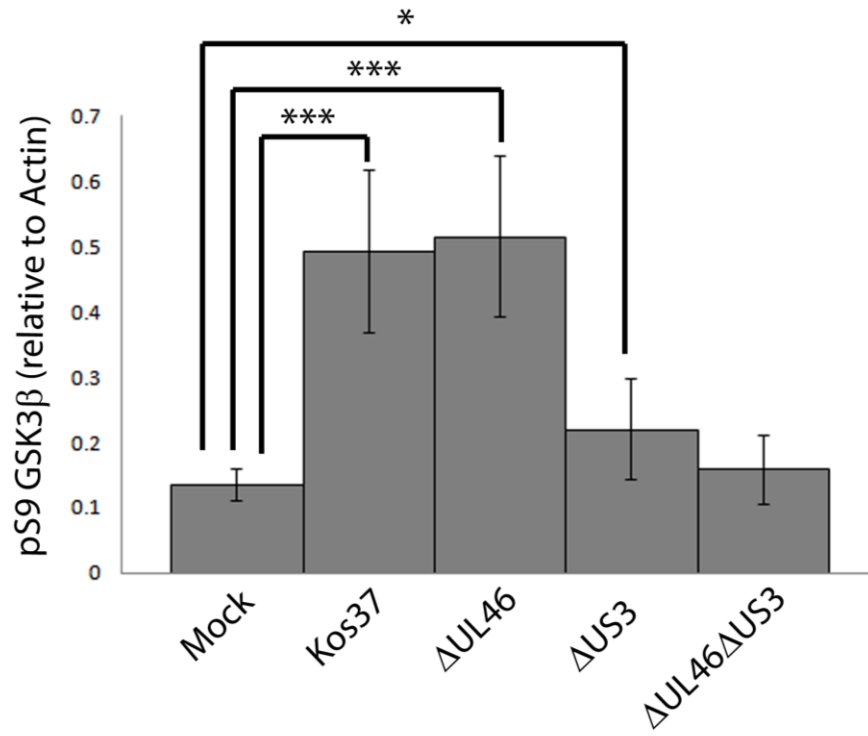


Figure 3.7: Multiple AKT targets are phosphorylated in a Us3-dependent fashion at late times post infection. Serum-starved HFF cells were mock infected or infected with wild type HSV-1 KOS37, the VP11/12-null mutant Δ UL46, the US3-null mutant Δ US3, and the VP11/12-US3-null mutant Δ UL46 Δ US3. Cell lysates were collected at 15 hpi and A) analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), the inhibiting phosphorylation of GSK3 β , FoxO1 and BAD at residues S9 (anti-GSK3 β), S256 (anti-FoxO1), and S136 (anti-BAD) respectively, 4EBP1, total proteins AKT, GSK3 β , FoxO1 and BAD, VP16, and β -actin by Western blotting. B) For each sample, the infrared signal obtained with the anti-GSK3 β phospho-S9 for panel A was divided by the signal obtained with the anti- β -actin antibody, and the ratios were normalized to yield a proportional value. Compared to mock infected cells, Kos37- and Δ UL46-infected cells had significantly ($p < 0.005$) more detectable phosphorylated GSK3 β at residue S9. Additionally, Δ US3-infection had significantly ($p < 0.05$) more phosphorylated GSK3 β at residue S9. Relative to Δ US3-infected cells, Δ UL46 Δ US3 infection did not have significantly less phosphorylated GSK3 β at residue S9 ($p > 0.05$). C) The infrared signal for the two upper and two lower bands of 4EBP1 were divided to yield a proportional value of phosphorylated 4EBP1. Compared to mock infected cells, KOS37- and Δ UL46-infected cells had significantly ($p < 0.001$) more phosphorylated 4EBP1. Relative to Δ US3-infected cells, Δ UL46 Δ US3 infection had similar levels of phosphorylated 4EBP1 ($p > 0.05$). D) The infrared signals obtained with anti-4EBP1 from panel A was divided by the signal obtained with anti- β -actin, and the ratios were normalized to yield a proportional value. Compared to mock infected cells, KOS37- and Δ UL46-infected cells had significantly ($p < 0.05$) more detectable 4EBP1. Compared to wild type infected cells, both Δ US3- and Δ UL46 Δ US3- infection had significantly less 4EBP1 ($p > 0.05$).

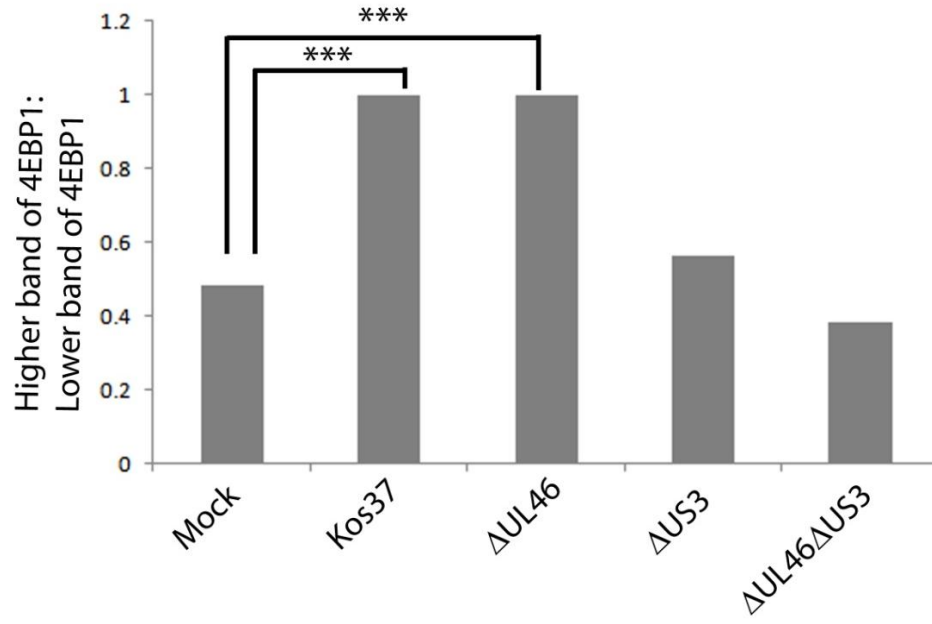
A.



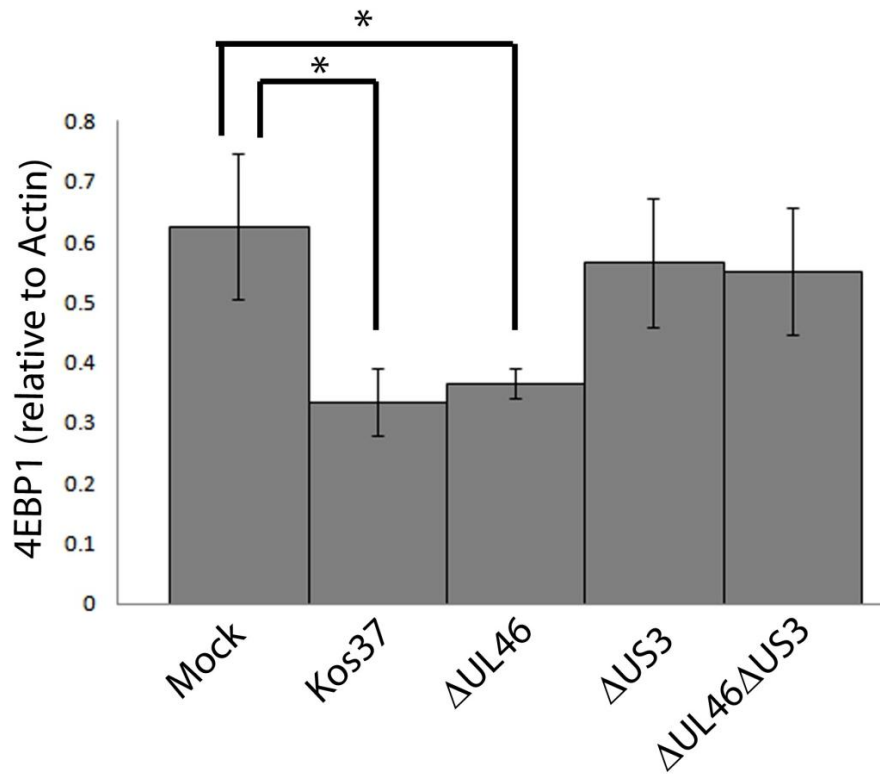
B.



C.



D.



3.6 Serum induces AKT activation, but there is no significant activation signal transduced to AKT targets at late times of infection

HSV-1 infection requires U_L46 for the phosphorylation of AKT, while U_s3 is required for the phosphorylation of AKT targets. Deletion of U_L46, and hence activated AKT, does not affect the phosphorylation of AKT targets. These data indicate that AKT is unable to phosphorylate some of its targets during HSV-1 infection, implying that its activity is either blocked or redirected. If there is a signaling block between AKT and its targets, then stimulating the pathway with external growth factors should not enhance the phosphorylation of AKT targets. The AKT pathway can be stimulated by serum, which contains numerous growth factors that bind and activate cell receptors. In uninfected cells, serum activates some receptors that recruit PI3K to the plasma membrane, which is followed by the phosphorylation of AKT and AKT targets. Since there are two phases of AKT phosphorylation (4 and 15 hpi), I asked whether the signaling dynamics of the AKT pathway changed over the course of infection.

Prior to examining activated AKT signaling during infection, serum was titrated to determine a concentration that would yield maximal AKT phosphorylation. Serum-starved HFFs were exposed to an increasing amount of serum from 0% to 15%. Cell lysates were collected after 20 minutes and analyzed for AKT phosphorylation at residue S473, phosphorylated GSK3 β at residue S9, as well as, total proteins AKT and GSK3 β . β -actin served as a loading control. Serum-starved cells lacked phosphorylated AKT and GSK3 β . As serum concentrations increased, so did the amount of phosphorylated AKT, however, serum concentrations greater than 10% did not induce more phosphorylated AKT (Fig. 3.8B). Phosphorylated GSK3 β was also enhanced with greater serum concentrations (Fig. 3.9A).

Additional control experiments were done to determine the optimal duration of serum exposure that would maximize AKT phosphorylation. Serum-starved HFFs were exposed to 10% serum for increasing lengths of time between 5 minutes and 2 hours. Cell lysates were analyzed for the same proteins noted in Fig. 3.8. AKT was most strongly phosphorylated between 15 and 30 minutes of

serum treatment, and AKT phosphorylation decreased after 30 minutes of exposure (Fig. 3.9). After 5 minutes of serum treatment, GSK3 β was phosphorylated and its phosphorylation was not enhanced with longer serum exposure. Therefore, AKT is phosphorylated most strongly with 10% serum between 15 and 30 minutes.

As mentioned previously, I examined if a signaling block existed between AKT and its targets during infection. If there is a block in signaling between AKT and its targets, then stimulating cells with serum should not result in the phosphorylation of AKT targets. To test this, serum-starved HFFs were infected with the viruses shown in Fig. 3.10 and 3.11. Samples were incubated for either 4 or 15 hpi (Fig. 3.10, 3.11). Prior to harvesting lysates, cells were treated with 10% serum for 20 minutes. The lysates were collected after 20 minutes of serum exposure, and analyzed for the phosphorylation of AKT, GSK3 β and 4EBP1 by western blotting as before (Fig. 3.10, 3.11). β -actin served as a loading control and HSV-1 VP16 or ICP27 were used as controls for virus infection. To discern whether activated growth factor signals could be propagated to AKT targets, the level of phosphorylated GSK3 β was measured. Phospho-GSK3 β levels were then divided by the values obtained by anti- β -actin to give normalized values. The quantified values were statistically analyzed using a two-tailed t-test from three replicates.

During early infection, HSV-1 induced AKT phosphorylation was U_L46-dependent. U_S3 was required for the phosphorylation of AKT targets, excluding 4EBP1, which remained in a hyperphosphorylated form in the absence of U_S3 and U_L46. Therefore, in Δ U_L46 Δ U_S3-infected cells, neither AKT nor the majority of its targets were unphosphorylated in the absence of serum. In the presence of serum, AKT and its targets were phosphorylated in mock, wild-type, Δ U_L46, Δ U_S3 and Δ U_L46 Δ U_S3-infections. Since the addition of serum caused AKT and its targets to become phosphorylated in Δ U_L46 Δ U_S3-infected cells, the data indicates serum-activated AKT signals were propagated to AKT targets. This suggests a signaling block between AKT and its targets is non-existent at early times of infection (Fig. 3.10).

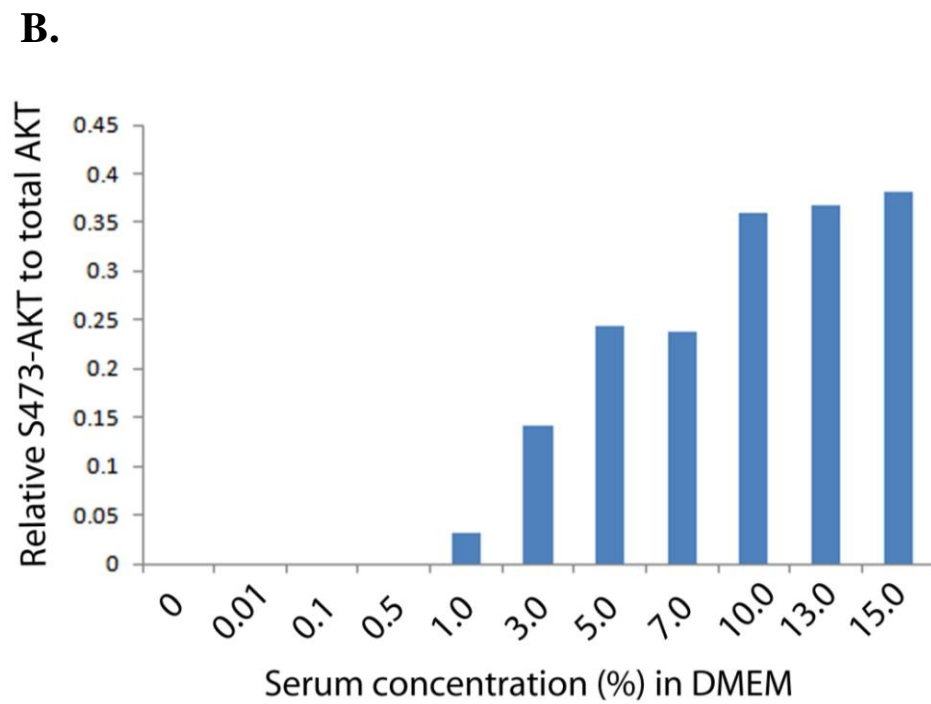
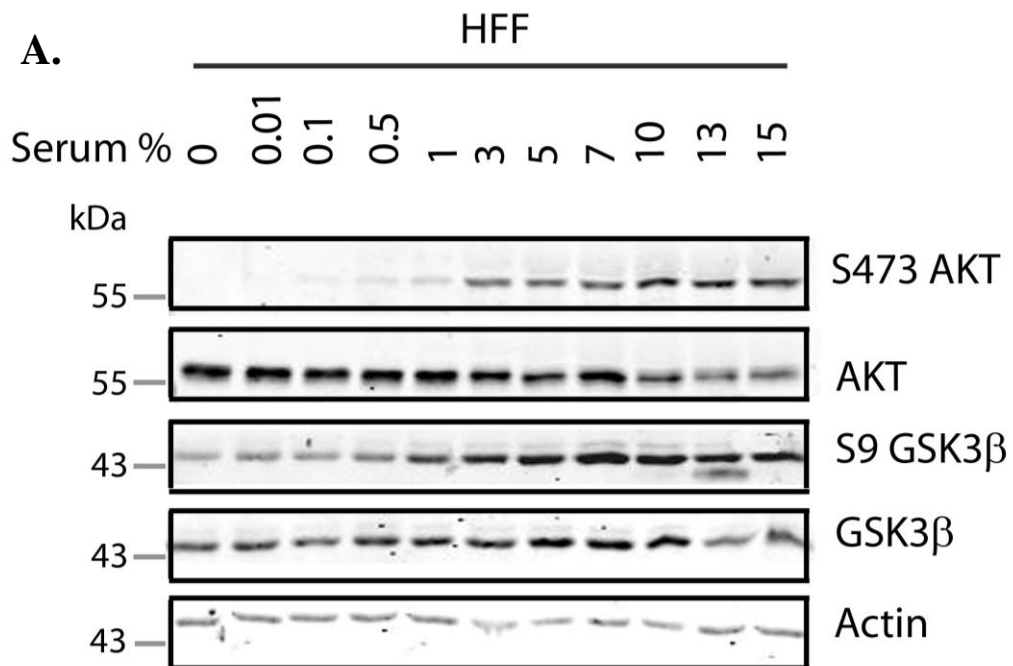
During late infection, AKT was strongly phosphorylated in a U_L46-dependent manner. In the presence of serum, AKT was strongly phosphorylated at the hydrophobic motif in all samples. Contrary to the hydrophobic motif, Δ U_L46-infected cells had less AKT phosphorylation at residue T308 compared to mock-infection. There were similar levels of phosphorylated T308 of AKT between mock- and Δ U_L46 Δ U_S3-infected cells. Thus, only in the absence of U_S3 does serum stimulation result in the phosphorylation of AKT at residue T308. These data suggest that U_S3 is negatively regulating AKT phosphorylation, specifically at the T308 residue, and this effect only exists during late infection (Fig. 3.11). Since serum was able to induce activated AKT in Δ U_L46 Δ U_S3-infected cells, I was able to test whether activated AKT signals could be transduced to downstream targets.

At late times of infection, phosphorylated 4EBP1 and GSK3 β were significantly enhanced in wild-type and Δ U_L46-infected cells ($p < 0.001$), but not in Δ U_S3- or Δ U_L46 Δ U_S3- infections. This supports the theory that U_S3 is required for the phosphorylation of AKT targets during infection. Serum stimulation resulted in comparable levels of phosphorylated 4EBP1 and GSK3 β between mock, wild-type and Δ U_L46-infected cells. Although serum-stimulation enhanced the phosphorylation of 4EBP1 and GSK3 β in Δ U_S3- and Δ U_L46 Δ U_S3-infected cells, these virus infections had significantly less phosphorylated GSK3 β than mock and wild-type infections ($p > 0.05$). Because AKT is phosphorylated in Δ U_L46 Δ U_S3-infected cells, but the signal is not significantly transduced to AKT targets, activated AKT signals are attenuated at late times during infection. Furthermore, this signaling block is independent of U_S3 and U_L46 (Fig. 3.11A, B).

Serum contains numerous growth factors that bind a variety of cell receptors and activate cell signaling pathways. These cell receptors can potentially cross-talk with the AKT pathway. Hence, it is unclear which specific growth factor is contributing to AKT signaling. Thus, an alternative method of testing a signaling block during infection is by using defined growth factors of the PI3K-AKT pathway.

Figure 3.8: 10% serum induces maximal AKT activation after 20 minutes.

Confluent monolayers HFF cells were serum-starved for 24 hours and A) titrated with an increasing concentration of serum in 1 mL DMEM. Cell lysates were collected after 20 minutes of treatment and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473), the inhibiting phosphorylation of GSK3 β at residue S9 (anti- GSK3 β), total proteins AKT and GSK3 β , and β -actin by Western blotting. B) The infrared signal obtained with the anti-S473 AKT for panel A was divided by the signal obtained with the anti-AKT antibody, and the ratios were normalized to yield a proportional value.



C.

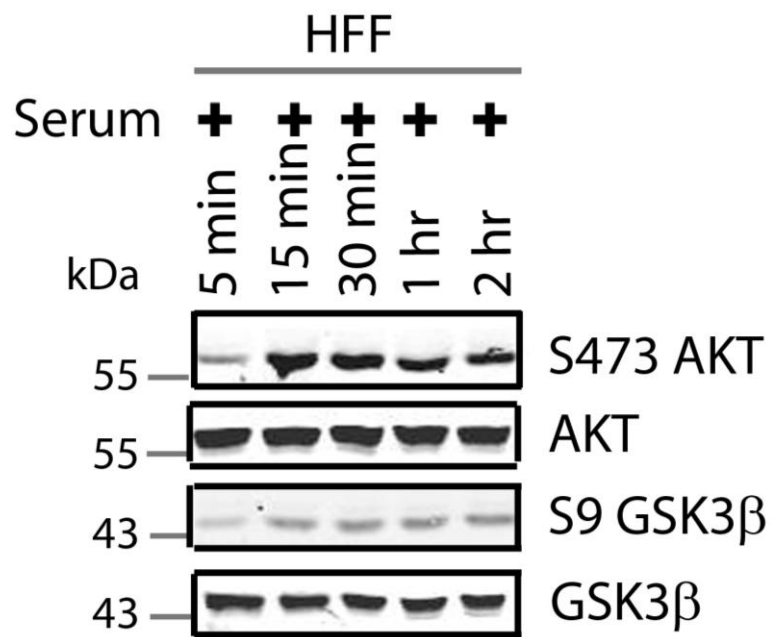


Figure 3.9: Serum induces AKT activation and downstream signaling in infected cells at early infection. Serum-starved HFF cells were mock infected or infected with wild type HSV-1 KOS37, the VP11/12-null mutant Δ UL46, the US3-null mutant Δ US3, and the VP11/12-US3-null mutant Δ UL46 Δ US3. Samples were either left in serum-free media or treated with 10% serum-DMEM for 20 minutes prior to harvesting cells. Cell lysates were collected at 15 hpi and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), the inhibiting phosphorylation of GSK3 β at residue S9 (anti- GSK3 β), 4EBP1, total proteins AKT and GSK3 β , ICP27, and β -actin by Western blotting.

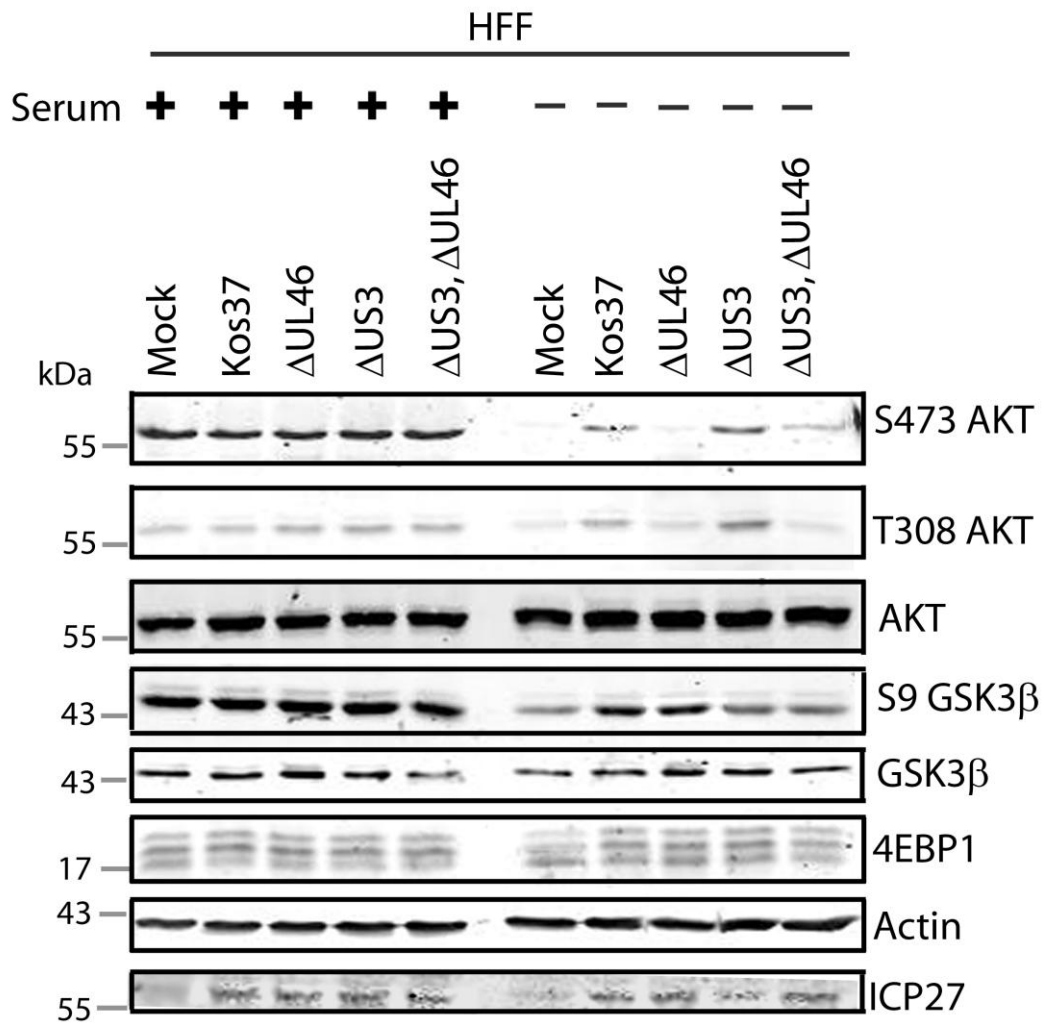
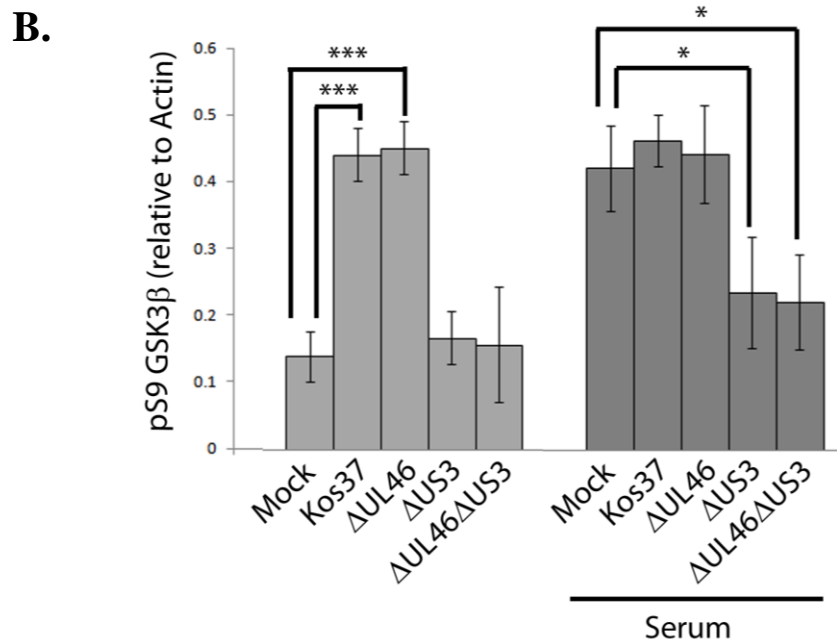
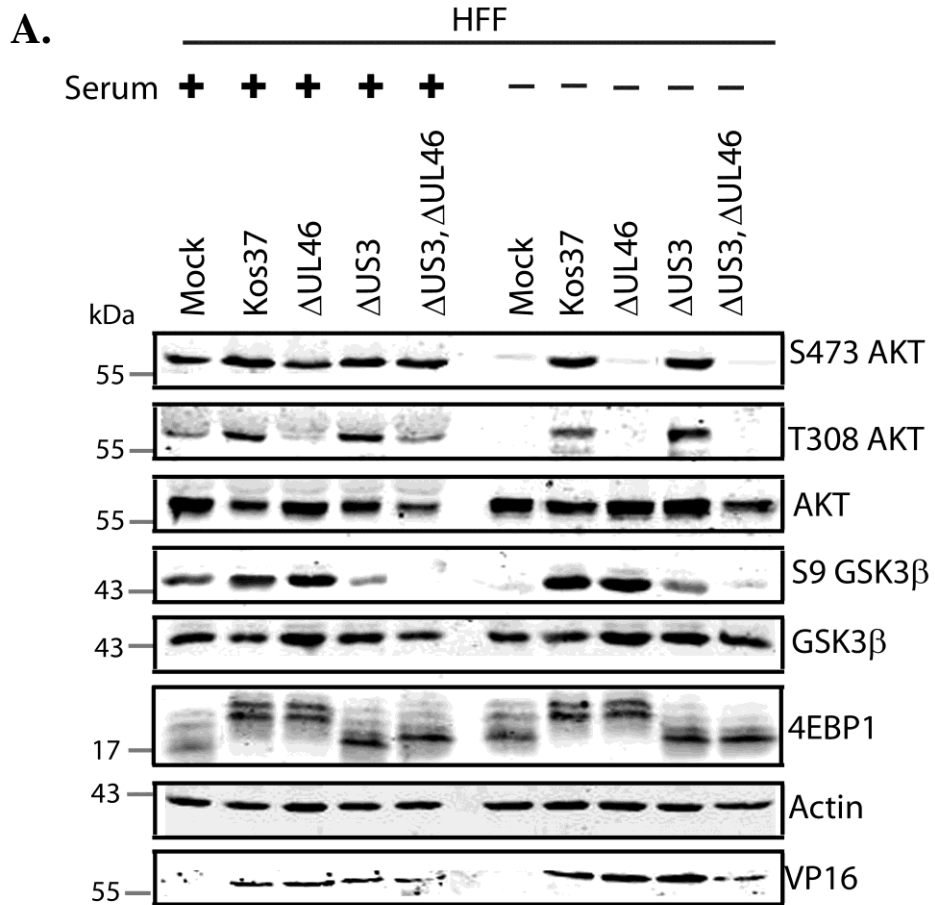


Figure 3.10: Serum induces AKT activation and, but not downstream signaling in infected cells at late times post infection. Serum-starved HFF cells were mock infected or infected with wild type HSV-1 KOS37, the VP11/12-null mutant Δ UL46, the US3-null mutant Δ US3, and the VP11/12-US3-null mutant Δ UL46 Δ US3. Samples were either left in serum-free media or treated with 10% serum-DMEM for 20 minutes prior to harvesting cells. A) Cell lysates were collected at 15 hpi and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), the inhibiting phosphorylation of GSK3 β at residue S9 (anti- GSK3 β), 4EBP1, total proteins AKT and GSK3 β , ICP27, and β -actin by Western blotting. B) The infrared signal obtained with the anti-GSK3 β phospho-S9 antibody for panel A was divided by the signal obtained with the anti- β -actin antibody, and the ratios were normalized to yield a proportional value. Compared to mock infected cells, KOS37- and Δ UL46-infected cells had significantly ($p < 0.001$) more detectable phosphorylated GSK3 β at residue S9. Under serum-stimulation mock infected cells were significantly ($p < 0.05$) more phosphorylated than stimulated Δ US3- and Δ UL46 Δ US3 infected cells.



3.7 PDGF BB induces AKT activation, but the signal is not transduced to AKT targets

Activated PI3K leads to the phosphorylation of AKT and AKT targets. U_L46 is required for AKT activation, but U_L46-activated AKT signals are not transduced to AKT targets during infection; this indicates that AKT signals are either blocked or redirected. As shown in section 3.6, activated AKT signals are not propagated during late times of infection even in the presence of serum, providing evidence for a signaling block. Serum contains a plethora of growth factors and several of these factors could contribute to AKT signaling. Thus, I asked if HSV infection inhibits signaling downstream of AKT when AKT is activated by a defined growth factor, in the same way that HSV blocks signaling downstream of AKT when AKT is activated by serum. PDGF BB ligands are one of many growth factors that activate the PI3K-AKT pathway. When PDGF BB binds to its cognate receptor, it induces dimerization, which is followed by the recruitment and activation of PI3K (5, 119, 259). I examined AKT signaling during late times of infection when cells are stimulated with PDGF BB.

PDGF BB was titrated to give a maximal amount of AKT activation, which happened to be equal to the amount of phosphorylated AKT induced by 10% serum (Fig. 3.8). Serum-starved HFFs were exposed to an increasing concentration of PDGF BB from 1.0 pg/ μ L to 4.0 pg/ μ L, or 10% serum (Fig. 3.12A). Since 20 minutes of serum treatment was sufficient to induce maximal AKT phosphorylation (Fig. 3.8), cell lysates were collected after 20 minutes of PDGF BB treatment. Cell lysates were analyzed for the phosphorylation of AKT and GSK3 β (Fig. 3.12A). In the absence of PDGF BB, AKT and GSK3 β were not phosphorylated. Stimulating cells with 2.5 pg/ μ L of PDGF BB was sufficient to induce phosphorylation of AKT that was equivalent to the levels given by 10% serum (Fig. 3.12B). Phosphorylated GSK3 β was seen in cells treated with a minimum of 1.0 pg/ μ L PDGF BB (Fig. 3.12A). AKT is phosphorylated most strongly with a minimum of 2.5 pg/ μ L PDGF BB for 20 minutes.

As previously stated, if PDGF BB signals are not propagated to AKT targets, then there is evidence for a signaling block. To test this, serum-starved

HFFs were infected with the viruses shown in Fig. 3.13. Cells were incubated for 15 hours and treated with 20.0 pg/ μ L of PDGF BB for 20 minutes; this concentration was more than sufficient to induce phosphorylation of AKT and AKT targets. Cell lysates were collected after exposure to PDGF BB for 20 minutes and analyzed for the phosphorylation of AKT and GSK3 β , and total proteins AKT, GSK3 β and 4EBP1 (Fig. 3.13). β -actin was used as a loading control, and HSV-1 VP16 served as a control for virus infection. For each virus treatment, the level of phosphorylated GSK3 β was measured in order to determine whether PDGF BB activation signals could reach AKT targets. Phospho-GSK3 β levels were then divided by the values obtained by anti- β -actin to give normalized values; this accommodated for any changes in cellular protein levels. The quantified values were statistically analyzed using a two-tailed t-test from three replicates.

As expected, in the absence of PDGF BB, AKT was phosphorylated in a U_L46-dependent fashion. In all samples, PDGF BB treatment greatly enhanced AKT phosphorylation at both activating residues. Treatment with PDGF BB resulted in more phosphorylated AKT in wild-type infected cells compared to unstimulated HSV-1 infected cells. An excessive amount of PDGF BB was added to cells, which could have caused the enhancement of AKT phosphorylation. Interestingly, under the influence of PDGF BB, Δ U_L46-infected cells had equivalent levels of phosphorylated AKT at residue T308 when compared to mock-infected cells (Fig. 3.13A). This result was different from Δ U_L46-infected cells that were treated with serum, which showed less T308 phosphorylation compared to mock-infection (Fig. 3.11A). Δ U_L46-virus reduces T308 phosphorylation in the presence of serum, while PDGF BB-treated cells did not display this inhibition. An excessive amount PDGF BB was added to infected cells, which may compensate for any negative effect on AKT signaling. Since PDGF BB was able to stimulate AKT phosphorylation in Δ U_S3- and Δ U_L46 Δ U_S3-infected cells, I asked if these activation signals could be propagated downstream.

As expected, U_S3 was required for the phosphorylation of AKT targets during HSV-1 infection. AKT targets were phosphorylated in mock, wild-type

and ΔU_L46 -infected cells that were treated with PDGF BB. In the presence of PDGF BB, ΔU_S3 - and $\Delta U_L46\Delta U_S3$ -infected cells displayed an increase in phosphorylated GSK3 β compared to their unstimulated counterparts (Fig. 3.13A). However, this increased GSK3 β phosphorylation was significantly less than levels seen in mock-infected cells treated with PDGF BB ($p < 0.005$) (Fig. 3.13B). In ΔU_S3 - and $\Delta U_L46\Delta U_S3$ -infected cells, 4EBP1 remained in a hypophosphorylated form in the presence of PDGF BB (Fig. 3.13A). Because PDGF BB-activated AKT signals were not significantly propagated downstream during infection, there is a block in signaling between AKT and some of its targets. Furthermore, PDGF signals were attenuated in virus infections lacking U_S3 and U_L46 , which infers that these two viral proteins are not responsible for the signaling block.

It would be interesting to examine whether or not HSV-1 blocks activation signals from other defined growth factors. PDGF BB is one member from a family of PDGF ligands that are specific for activating the PI3K-AKT pathway. Thus, I examined AKT signaling in the presence of other PDGF ligands during HSV-1 infection.

Figure 3.11: PDGF BB activates AKT. Confluent monolayers of HFF cells were serum-starved for 24 hours and titrated with an increasing concentration of PDGF BB in 1 mL DMEM. A) Cell lysates were collected after 20 minutes of treatment and analyzed for the activating phosphorylation of AKT at residue S473 (anti-S473), the inhibiting phosphorylation of GSK3 β at residue S9 (anti-GSK3 β), total protein AKT by Western blotting. B) The infrared signal obtained with the anti-S473 AKT for panel A was divided by the signal obtained with the anti- β -actin antibody, and the ratios were normalized to yield a proportional value.

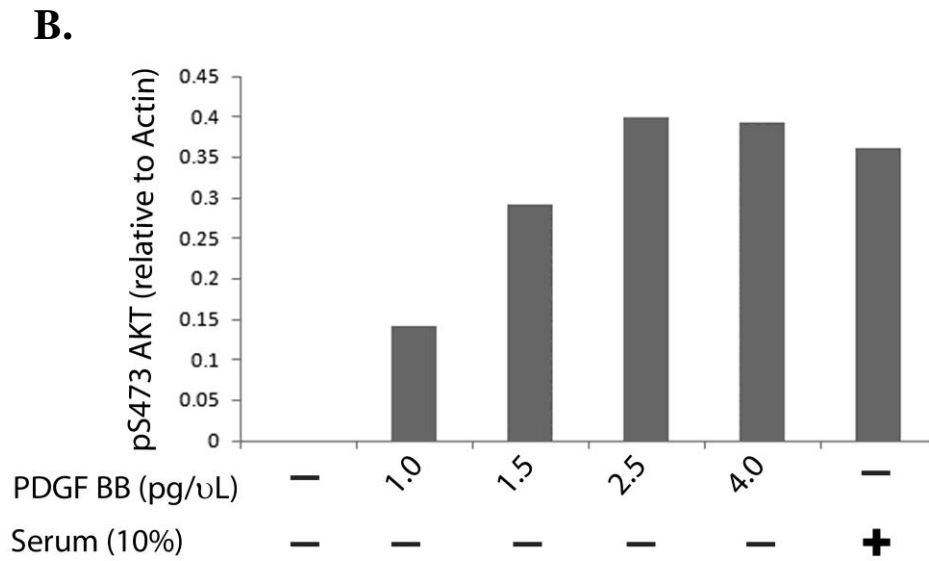
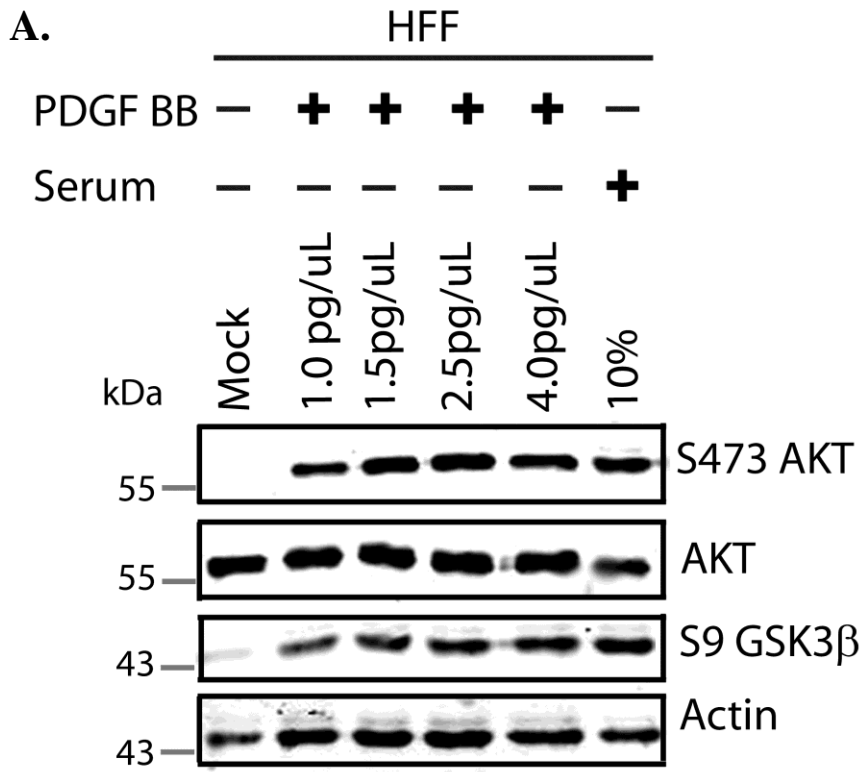
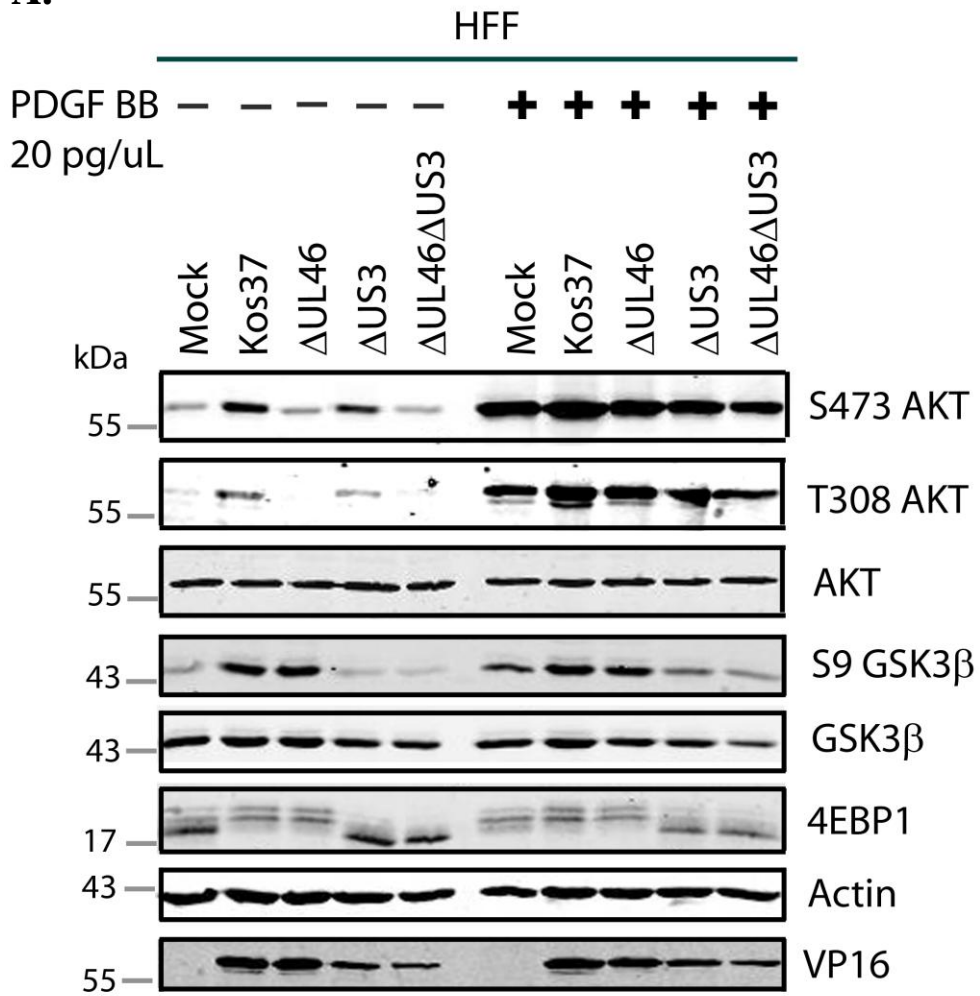
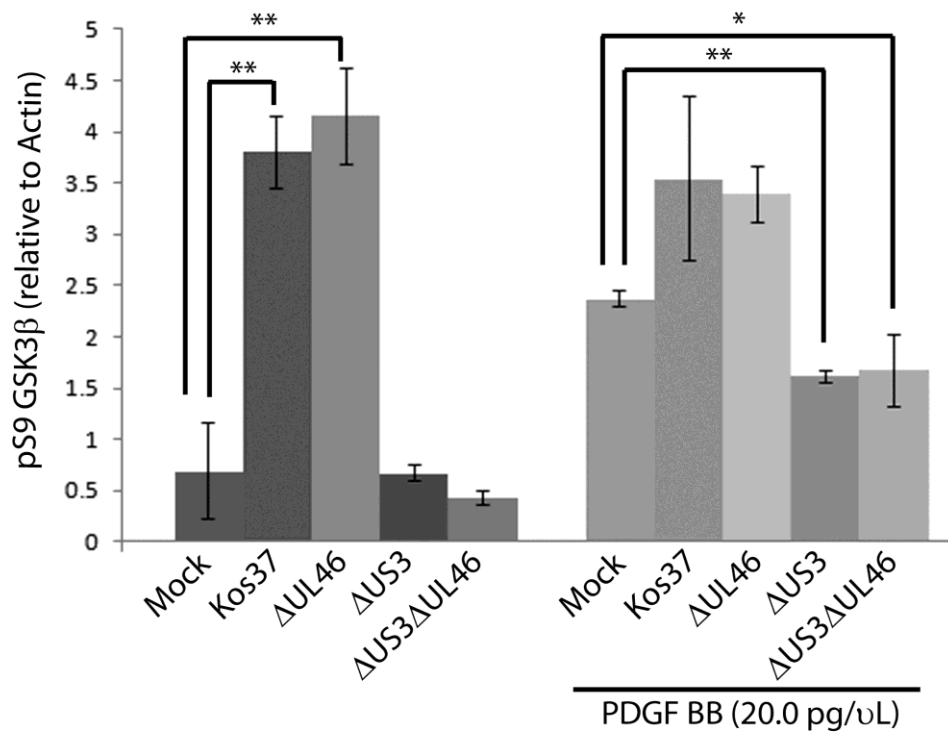


Figure 3.12: PDGF BB induces AKT activation, but the signal is not propagated to AKT targets during late times post infection. Serum-starved HFF cells were mock infected or infected with wild type HSV-1 KOS37, the VP11/12-null mutant Δ UL46, the US3-null mutant Δ US3, and the VP11/12-US3-null mutant Δ UL46 Δ US3. Samples were either left in serum-free media or treated with 20.0 pg/ μ L PDGF BB for 20 minutes prior to harvesting cells. A) Cell lysates were collected at 15 hpi and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), the inhibiting phosphorylation of GSK3 β at residue S9 (anti- GSK3 β), 4EBP1, total proteins AKT and GSK3 β , VP16, and β -actin by Western blotting. B) The infrared signal obtained with the anti-S9 GSK3 β for panel A was divided by the signal obtained with the anti- β -actin antibody, and the ratios were normalized to yield a proportional value. Kos37- and Δ UL46-infected cells had significantly ($p < 0.005$) more detectable phosphorylated GSK3 β at residue S9 than mock-infection. Upon PDGF BB stimulation mock- infected cells were significantly more phosphorylated than stimulated Δ US3- ($p < 0.005$) and Δ UL46 Δ US3 ($p < 0.05$) infected cells.

A.



B.



3.8 Titration of PDGF AA, AB and BB

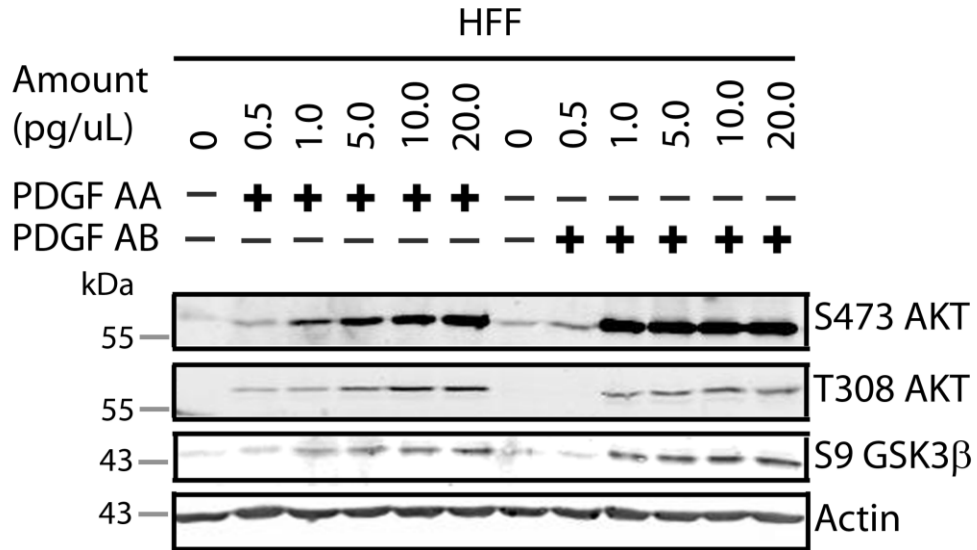
PDGF ligands exist in three isoforms, (AA, AB and BB) and each of them differs in their receptor-binding specificity (85, 226). AA has the ability to bind the $\alpha\alpha$ - receptor; AB the $\alpha\beta$ - and $\alpha\alpha$ - receptor; BB the $\beta\beta$ -, $\alpha\beta$ -, and $\alpha\alpha$ -receptor (62, 69, 90, 91, 218). Once the dimer-ligands are bound to their receptors, PDGF-receptors undergo homo- or heterodimerization and auto-tyrosine phosphorylation, which triggers the recruitment and activation of PI3K (5, 119, 259).

The PDGF ligands were titrated for an equal level of AKT phosphorylation at both activating residues. To accomplish this, serum-starved HFFs were treated with increasing amounts of PDGFs between 0.5 pg/ μ L and 50.0 pg/ μ L for 20 minutes (Fig. 3.14A, B, C). Cell lysates were collected after 20 minutes of PDGF treatment and analyzed for the phosphorylation of AKT and GSK3 β (Fig. 3.14). β -actin was used as a loading control. These experiments were replicated three times for both phosphorylated residues of AKT, S473 and T308.

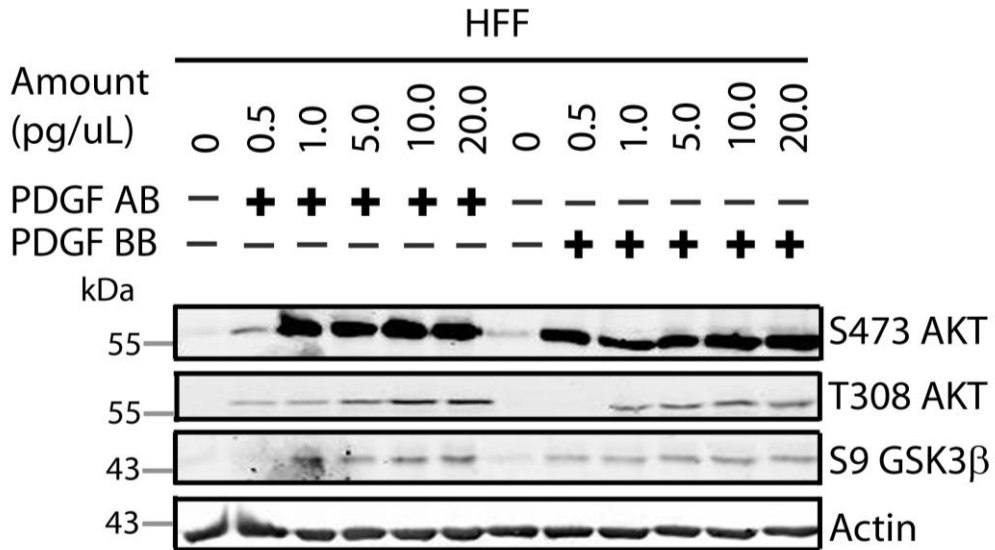
When cells were treated with equal concentrations of PDGFs, PDGF AA was unable to induce S473- and T308- phosphorylation as strongly as, PDGF AB and BB (Fig. 3.14D, E). Stimulating cells with PDGF AB produced the most phosphorylated AKT compared to PDGF AA and BB (Fig. 3.14 A, B, C). It is interesting that PDGF AB triggers stronger AKT phosphorylation than PDGF BB at the same concentration, even though PDGF BB binds all three PDGF receptors and PDGF AB only binds two (62, 69, 90, 91, 218). Equivalent levels of phosphorylated AKT at residue S473 were achieved at 30.0 pg/ μ L PDGF AA, 10.0 pg/ μ L PDGF AB, and 20.0 pg/ μ L PDGF BB (Fig. 3.14D). Equivalent levels of phosphorylated AKT at residue T308 were achieved at 20.0 pg/ μ L PDGF AA, 5.0 pg/ μ L PDGF AB, and 10.0 pg/ μ L PDGF BB (Fig. 3.14E). Regardless of the type of PDGF, the phosphorylation of GSK3 β was enhanced with greater PDGF concentrations (Fig. 3.14 A, B, C). Using PDGF concentrations that gave equal levels of phosphorylated AKT, I asked whether PDGF AA, AB and BB signals could be propagated to AKT and AKT targets during infection.

Figure 3.13: Differing concentrations of PDGF AA, AB and BB induce comparable levels of AKT activation. Confluent monolayers of HFF cells were serum-starved for 24 hours and titrated with an increasing concentration of PDGF in 1 mL DMEM. A), B), C) Cell lysates were collected after 20 minutes of treatment and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), the inhibiting phosphorylation of GSK3 β at residue S9 (anti- GSK3 β), total proteins AKT and GSK3 β , and β -actin by Western blotting. The infrared signal obtained with the D) anti-S473 and E) anti-T308 AKT for panel D was divided by the signal obtained with the anti- β -actin antibody, and the ratios were normalized to yield a proportional value.

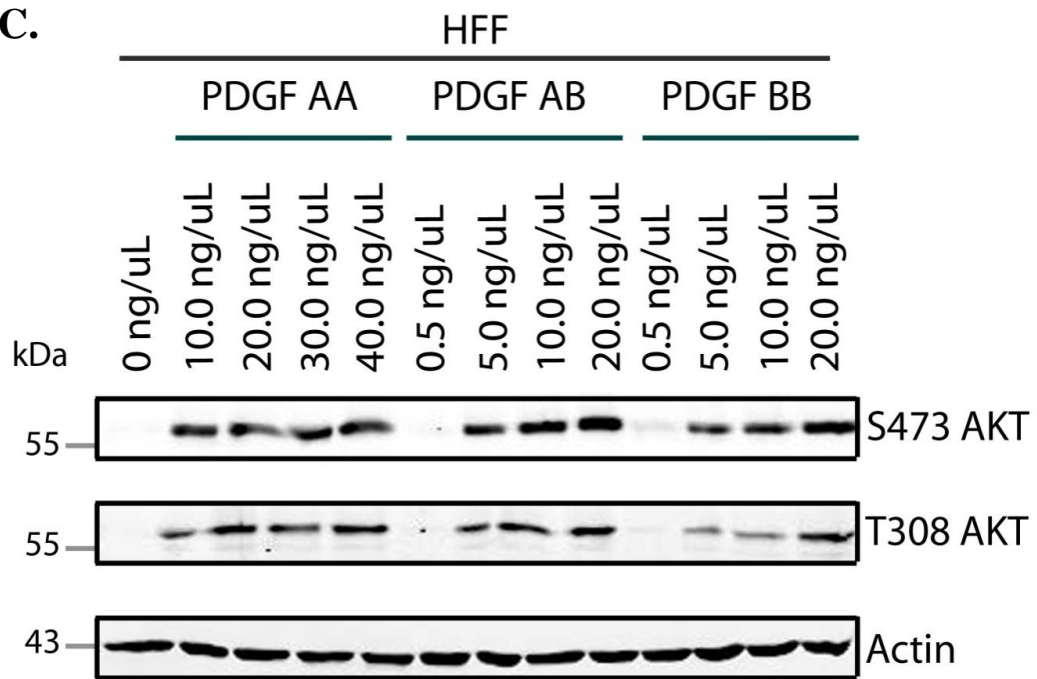
A.

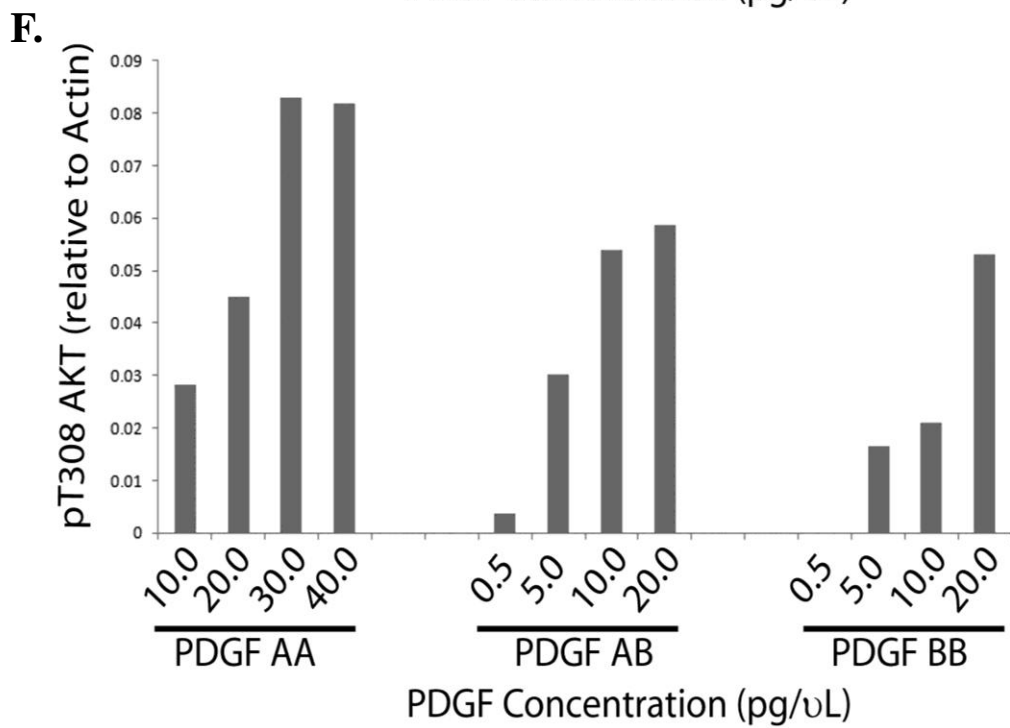
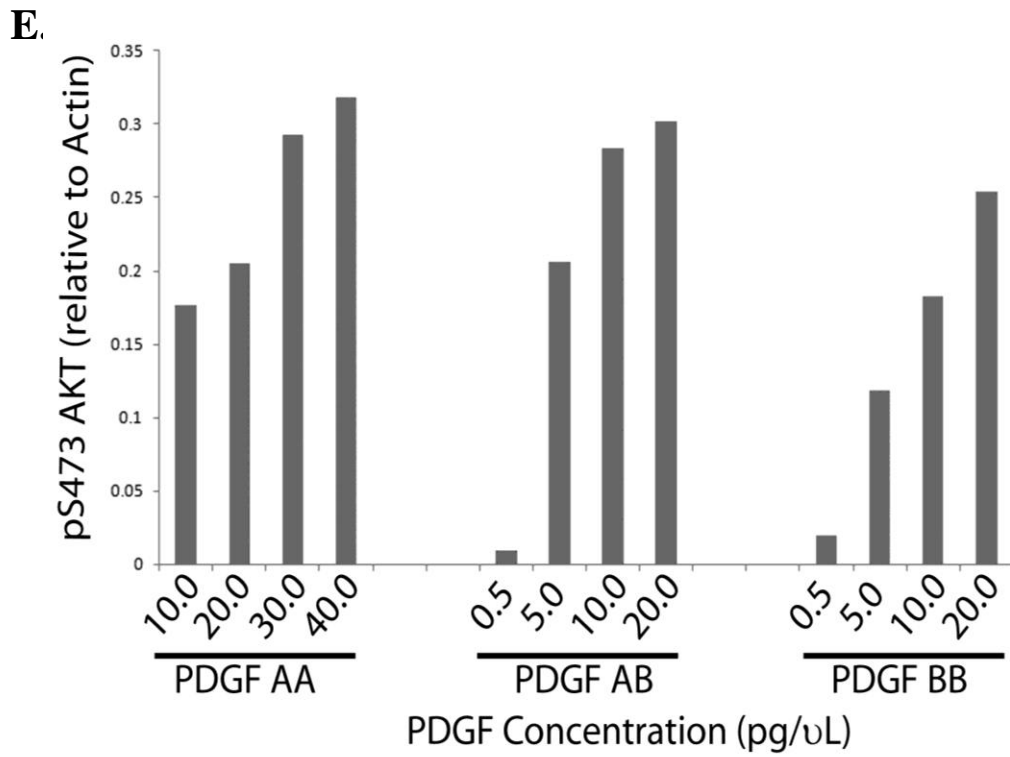


B.



C.





3.9 PDGF ligands do not perform equally at activating AKT during infection

In uninfected cells, the presence of PDGF BB results in AKT activation and the successive phosphorylation of AKT targets. In HSV-1 infected cells, PDGF BB signals enhanced AKT phosphorylation, but those phosphorylated signals were not propagated downstream (Fig. 3.13). Thus, there is a block in signaling between AKT and its targets, which is observed when infected cells are stimulated with PDGF BB. I examined whether HSV-1 blocks signaling between AKT and AKT targets when cells are stimulated with other types of PDGF ligands (AA, AB and BB).

Serum-starved cells were infected with the viruses indicated in Fig. 3.15 and 3.16. Cells were incubated for 15 hours and then treated with PDGF AA, AB or BB. Because the PDGF ligands do not perform equally at stimulating AKT phosphorylation on either residue, cells were treated with PDGFs that gave comparable levels of AKT phosphorylation either at S473 or T308 (as noted in Section 3.9, Fig. 3.14). Cell lysates were collected after 20 minutes of PDGF treatment and analyzed for the phosphorylation of AKT and AKT targets (Fig. 3.15, 3.16). HSV-1 VP16 was used as a control for viral infection, and β -actin was used as a loading control. These experiments were replicated three times using PDGF concentrations that gave equal levels of AKT phosphorylation either at residue S473 or T308.

In mock-infected cells, all three PDGFs gave equal levels of phosphorylated AKT at residues S473 and T308. Since the PDGFs gave similar levels of phosphorylated AKT, any change in AKT signaling would likely be a result of virus infection. As expected, phosphorylated AKT was increased during HSV-1 infection in a U_L46 -dependent fashion. In wild-type and ΔU_S3 -infected cells, all three PDGF ligands were able to further enhance the phosphorylation of AKT in contrast to their unstimulated counterparts. Interestingly, during HSV-1 infection, PDGF AA produced less phosphorylated AKT compared to PDGF AB or BB stimulation, while PDGF BB gave the greatest amount of phosphorylated AKT. This observation was most apparent in cells infected with a virus lacking U_L46 . Cells infected with ΔU_L46 - or $\Delta U_L46\Delta U_S3$ -virus displayed a lack of AKT

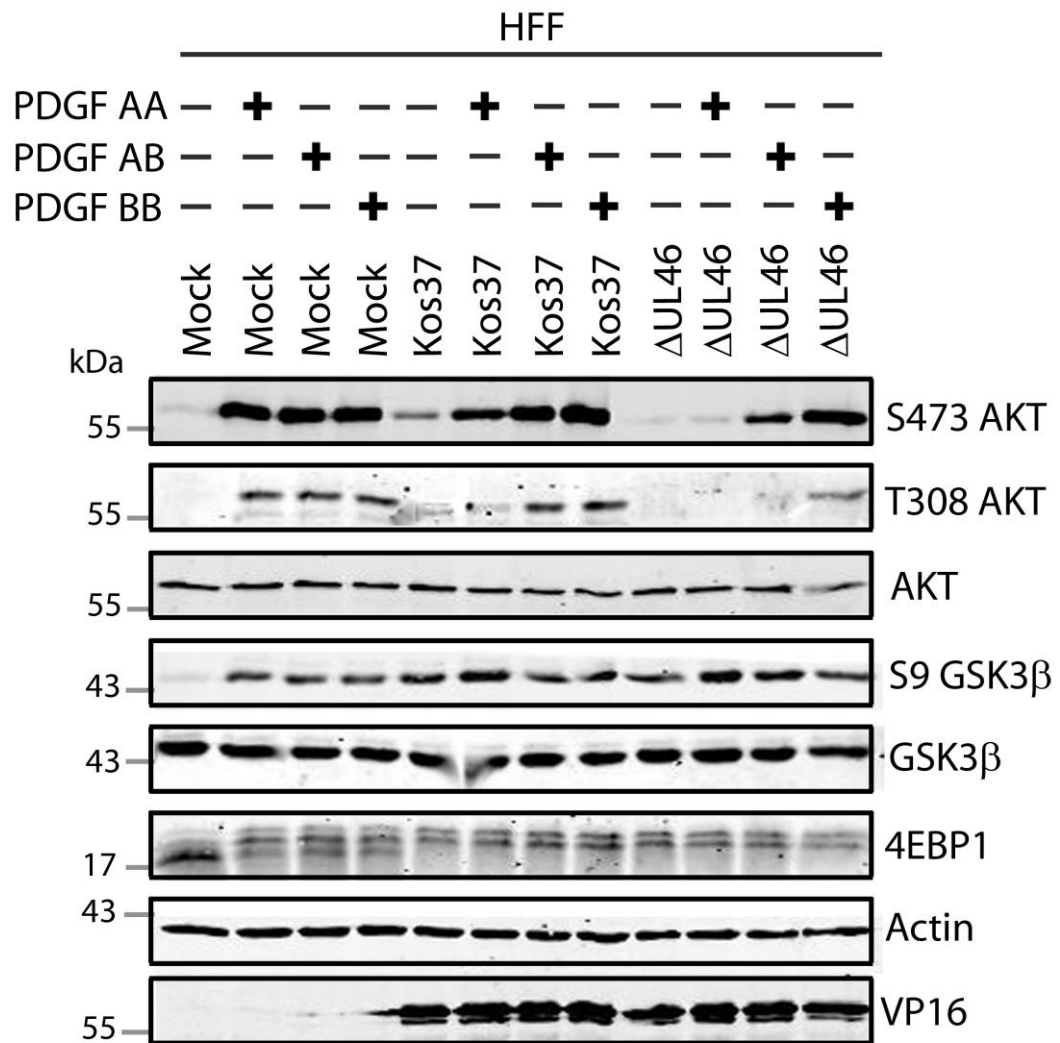
phosphorylation, and stimulation with PDGF AA or AB had either little (Fig. 3.15) or no effect (Fig. 3.16) at inducing AKT phosphorylation. In the event that AKT phosphorylation was enhanced by PDGFs during infection, PDGF AB had a stronger effect at inducing AKT phosphorylation compared to PDGF AA (Fig. 3.15). On the other hand, phosphorylated AKT was strongest in cells stimulated with PDGF BB (Fig. 3.15, 3.16). Therefore, these data suggest that HSV-1 infection dampens signaling from certain PDGF receptors to AKT. It is unclear whether the block is at the receptor, or between the receptor and AKT. Interestingly, treatment with PDGF AB induced less AKT phosphorylation in ΔU_L46 -infected cells compared to $\Delta U_L46\Delta U_S3$ -infected cells. Thus, U_S3 may have a marginal role in modulating growth factor signaling at the receptor. However, U_L46 and U_S3 were obviously not the main factors for this signaling block, which indicates other viral protein(s) are involved. As a result of this receptor-block, PDGF AA and AB were not suitable candidates to assess signaling between AKT and AKT targets.

In the presence of each PDGF, AKT targets were phosphorylated in mock-infected cells. In wild type and ΔU_L46 -infected cells, AKT targets were noticeably phosphorylated and this phosphorylation was not enhanced in the presence of an external ligand. As expected, the phosphorylation of AKT targets were dependent on U_S3 , thus phosphorylation was attenuated in the ΔU_S3 - and $\Delta U_L46\Delta U_S3$ -infected cells. In ΔU_S3 - and $\Delta U_L46\Delta U_S3$ -infected cells, PDGF AA and PDGF AB had no apparent effect on the phosphorylated levels of 4EBP1 and GSK3 β . Stimulating cells with PDGF BB increased phosphorylated GSK3 β in ΔU_S3 - and $\Delta U_L46\Delta U_S3$ -infected cells, but as witnessed in Section 3.8, this phosphorylation was less than those levels seen in mock-infected cells that were stimulated with PDGF BB. As stated previously in Section 3.8, activated AKT signals are attenuated during HSV-1 infection and this is independent of U_L46 and U_S3 (Fig. 3.15, 3.16).

The initial aim of this experiment was to examine if PDGF AA, AB and BB signals could be propagated to AKT targets during infection. Rather, I witnessed that HSV-1 inhibits signaling through some receptors for unknown

reasons (Fig. 3.15, 3.16). Regardless of this inhibition, HSV-1 encodes U_L46 to activate AKT, yet these activated signals are not propagated to the examined downstream targets. Rather, HSV-1 encodes U_S3 to cherry-pick and phosphorylate AKT targets. These data indicate that activated AKT signals are either attenuated or redirected during infection.

Figure 3.14: PDGF ligands do not perform equally at phosphorylating S473 AKT during infection. Serum-starved HFF cells were mock infected or infected with wild type HSV-1 KOS37, the VP11/12-null mutant Δ UL46, the US3-null mutant Δ US3, and the VP11/12-US3-null mutant Δ UL46 Δ US3. Samples were either left in serum-free media or treated with 30.0 pg/ μ L PDGF AA, 1.0 pg/ μ L PDGF AB, and 20.0 pg/ μ L PDGF BB for 20 minutes prior to harvesting. Cell lysates were collected at 15 hpi and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), the inhibiting phosphorylation of GSK3 β at residue S9 (anti-GSK3 β), 4EBP1, total proteins AKT and GSK3 β , VP16, and β -actin by Western blotting.



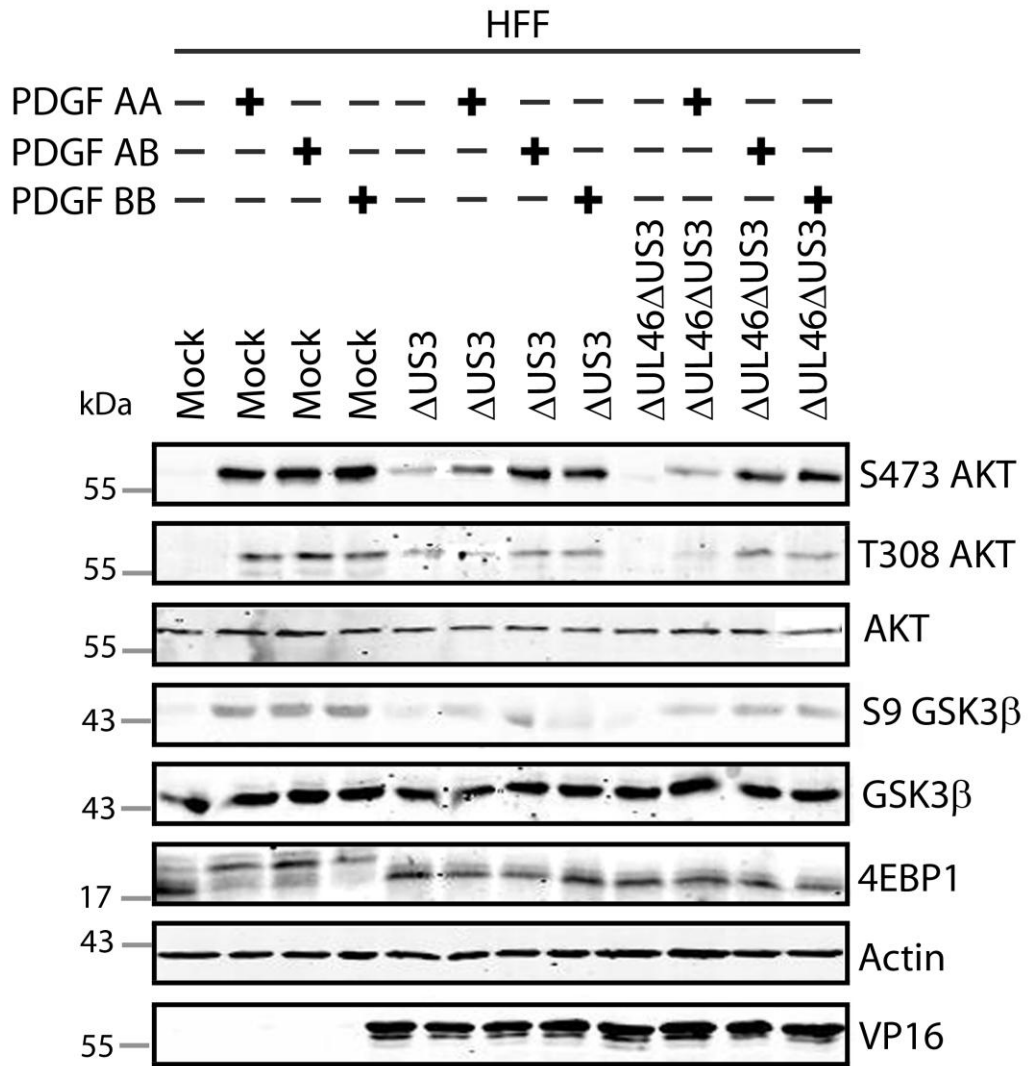
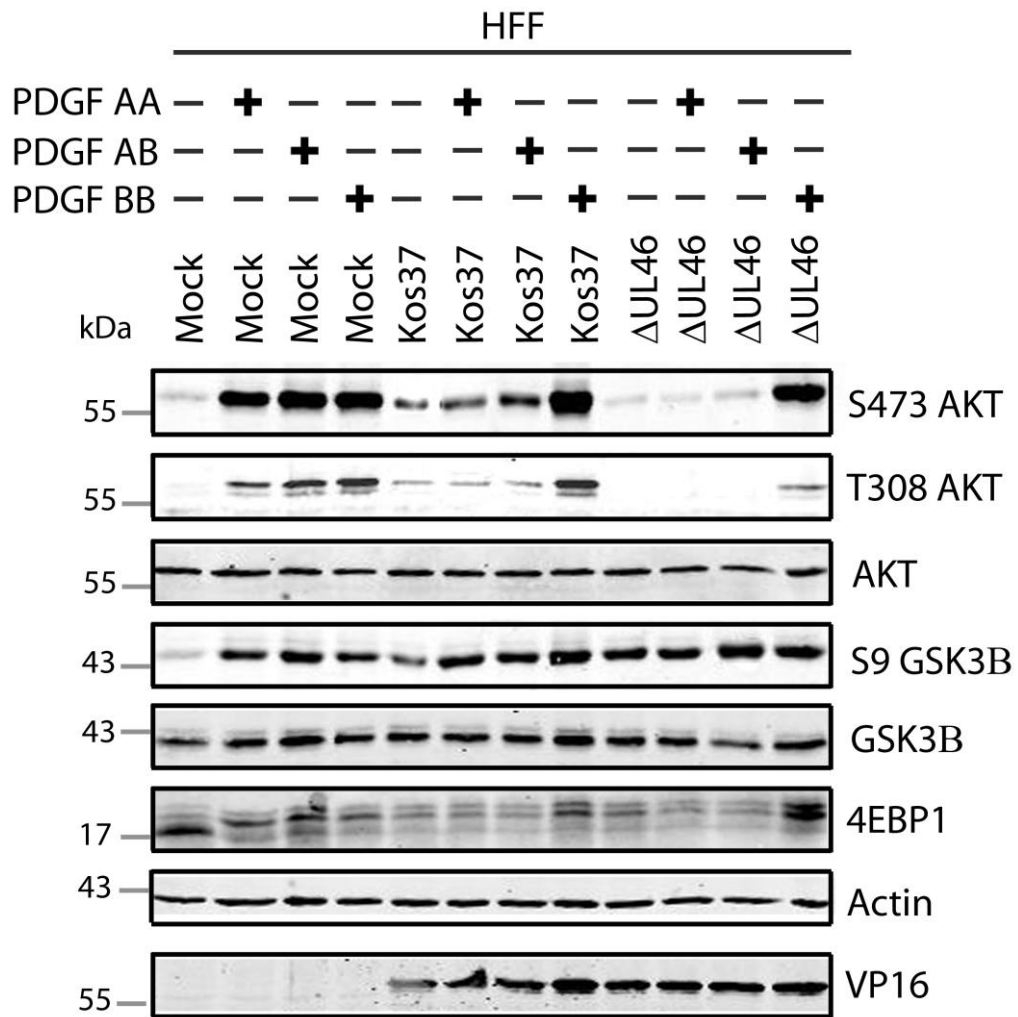
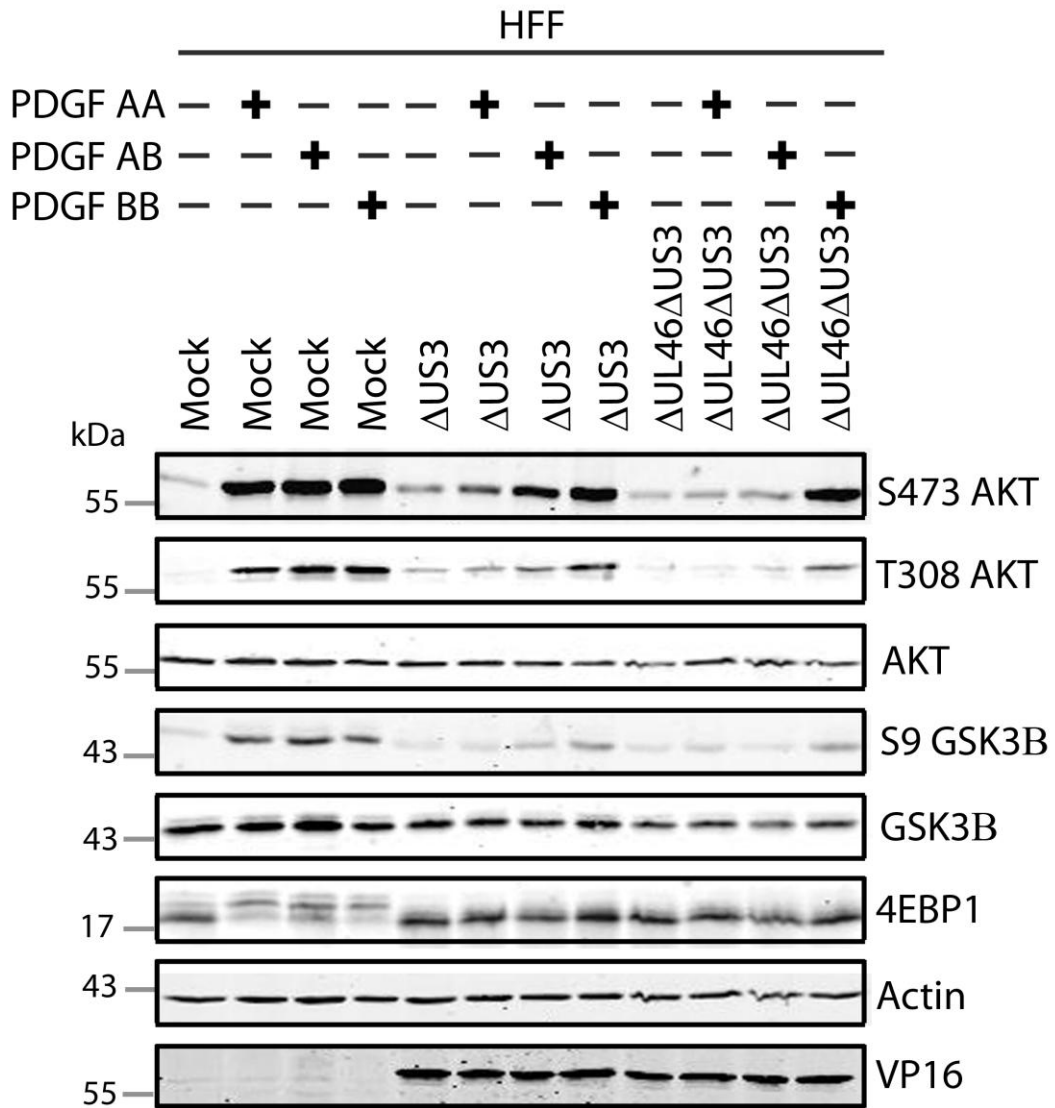


Figure 3.15: PDGF ligands do not perform equally at phosphorylating T308 AKT during infection. Serum-starved HFF cells were mock infected or infected with wild type HSV-1 KOS37, the VP11/12-null mutant Δ UL46, the US3-null mutant Δ US3, and the VP11/12-US3-null mutant Δ UL46 Δ US3. Samples were either left in serum-free media or treated with 20.0 pg/ μ L PDGF AA, 5.0 pg/ μ L PDGF AB, and 10.0 pg/ μ L PDGF BB for 20 minutes prior to harvesting. Cell lysates were collected at 15 hpi and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), the inhibiting phosphorylation of GSK3 β at residue S9 (anti- GSK3 β), 4EBP1, total proteins AKT and GSK3 β , VP16, and β -actin by Western blotting.





CHAPTER 4: DISCUSSION

4.1 Thesis Summary

The results in this communication have highlighted the complexity in AKT signaling during HSV-1 infection. The data support a model where the HSV-1 tegument protein VP11/12 is required for the phosphorylation of AKT (247). Although a viral protein is devoted to activating AKT, signaling from activated AKT is severely attenuated. Rather, the phosphorylation of AKT targets in HSV-infected cells depends predominantly on the viral AKT mimic, U_S3 (Fig. 4.1).

In addition to attenuating activated-AKT signals, HSV-1 prevents signaling from select PDGF receptors that normally initiate AKT signaling (Fig. 4.2). Although HSV-1 infection induces two blocks in AKT signaling, AKT is still activated for unknown reasons. Interestingly, neither U_S3 nor U_L46 are required for either of these signaling blocks indicating that other viral proteins may be involved in AKT signaling. My work in this thesis has shed light on many aspects of AKT regulation.

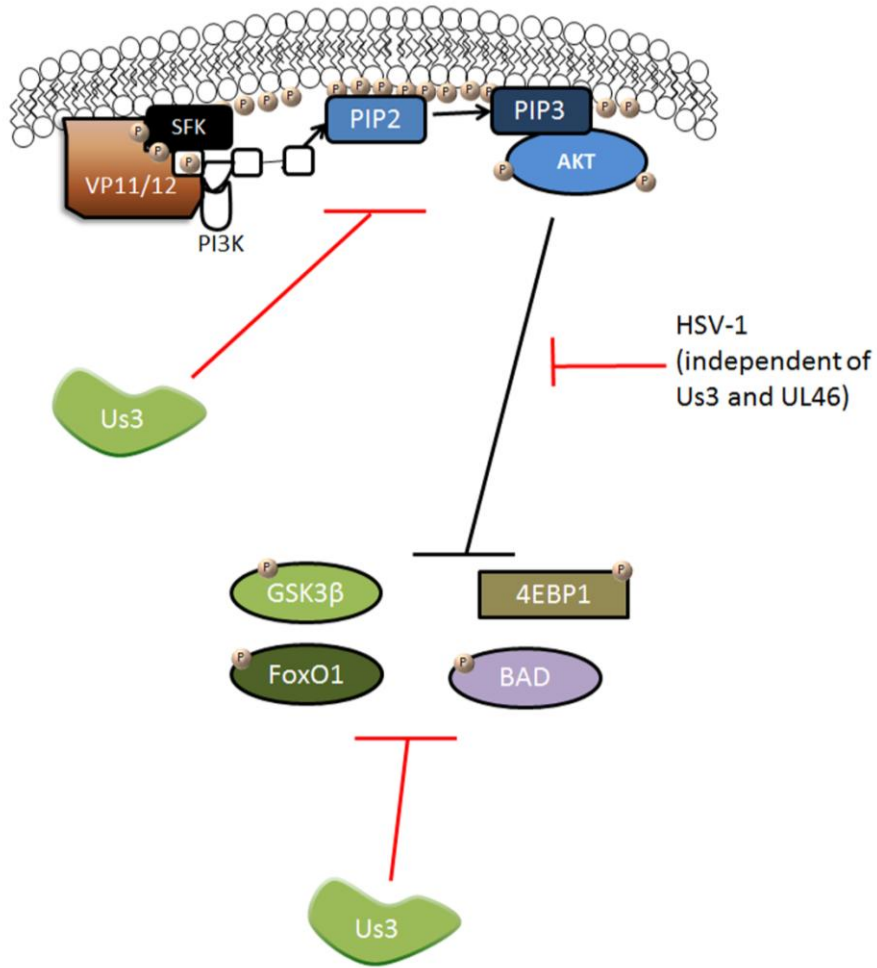


Figure 4.1: U_L46 promotes AKT activation, while U_S3 negatively regulates AKT signaling. AKT is activated in a U_L46-dependent manner during HSV-1 infection, while the phosphorylation of AKT targets is primarily U_S3-dependent. During infection, the phosphorylation of AKT targets does not require U_L46, but rather depends on U_S3. Since AKT is activated, but the signal is not propagated, HSV-1 appears to attenuate signaling between AKT and its downstream targets. Interestingly, neither U_L46 nor U_S3 are required for attenuation of an activated AKT signal. Although U_S3 does not block the signaling between AKT and its targets, U_S3 appears to inhibit the phosphorylation of residue T308 on AKT.

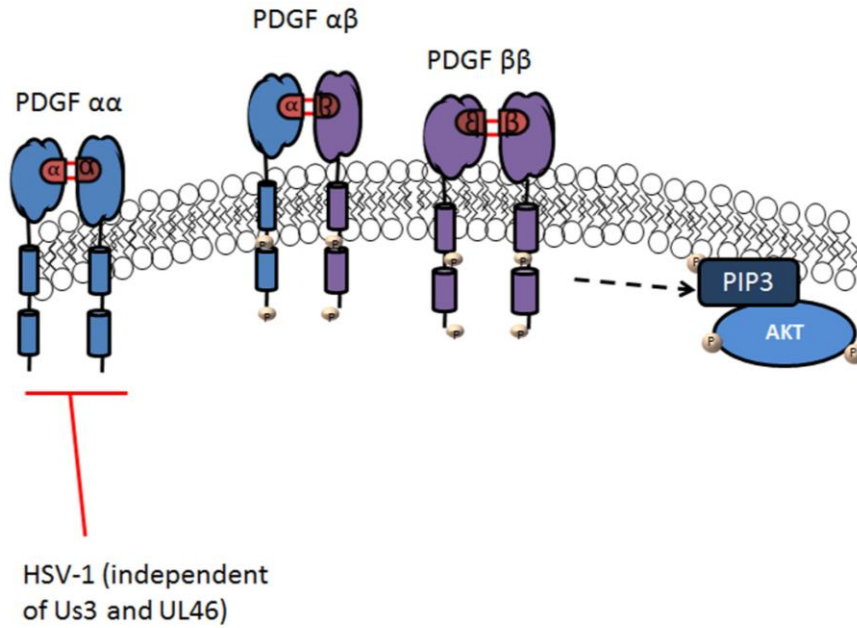


Figure 4.2: HSV-1 blocks AKT activation downstream of specific receptors. AKT is phosphorylated during HSV-1 infection. During infection, the phosphorylation of AKT can be further enhanced when cells are stimulated with PDGF $\beta\beta$, and to a lesser extent PDGF $\alpha\beta$. However, stimulating infected cells with PDGF $\alpha\alpha$ does not enhance the phosphorylation of AKT. Thus, HSV-1 selectively blocks signaling to AKT from select receptors.

4.2 The HSV-1 kinase U_S3 masquerades as an AKT mimic to regulate cellular processes

While U_S3 negatively regulates AKT activation, I, as well as the Mohr group (34), found that U_S3 is required for the phosphorylation of several AKT targets (Fig. 3.6, 3.7). Though U_S3 has no sequence homology to AKT, Mohr and colleagues (34) found that it behaves analogously to AKT by directly phosphorylating the targets TSC2, FoxO1, and GSK3 β on the same residues as AKT. A similar phenomenon is found in another Herpesvirus. The VZV U_S3 homolog ORF 66 phosphorylates the same residues and targets as PKA (61). These data suggest that it is advantageous for a virus to bypass cellular signaling to directly regulate selective target proteins. For example, the activation of AKT normally requires external growth factors, while U_S3 is able to phosphorylate AKT targets in serum-starved conditions (Fig. 3.6, 3.7). Furthermore, the activation of AKT is biphasic and transient during infection (Fig. 3.5), whereas U_S3 promotes the phosphorylation of AKT targets as early as 3 hours after infection and continues until as late as 21 hpi (Appendix C).

Furthermore, it is possible that U_S3 does not undergo the same negative regulation as AKT. Firstly, inhibition from PLC and the phosphatase PTEN must be overcome in order for AKT to be phosphorylated (124, 141). Secondly, activated AKT is dephosphorylated, and thus inactivated, by active PP2A (13). Thirdly, AKT exists in a negative feedback loop with downstream proteins. For example, it was suggested that U_S3 is unaffected by the AKT-S6K-IRS1 negative feedback loop (34, 188). Mohr suggested (34) that since U_S3 is unrelated to AKT at the sequence level, it is unresponsive to cellular pathways that limit AKT activity.

Another possible advantage of encoding a viral AKT mimic is that U_S3 can selectively phosphorylate specific AKT targets (34). By modulating AKT targets, U_S3 hijacks cellular regulation of processes such as apoptosis and protein translation.

4.3 HSV-1 promotes cap-dependent translation

HSV-1 protein synthesis relies on host translational machinery. We and the Mohr group (34) have independently found that U_{S3} promotes the phosphorylation and degradation of the translational repressor 4EBP1 (Fig. 3.7) by phosphorylating an upstream regulator, TSC2 (34). Phosphorylated TSC2 leads to the activation of mTORC1, which is required for the hyperphosphorylation of 4EBP1 (29).

Mohr and colleagues (249) previously found a complete reduction in the hyperphosphorylation of 4EBP1 when HSV-1-infected cells were treated with rapamycin, which is consistent with viral manipulation of TSC2 upstream of mTORC1. On the other hand, HCMV continually induces hyperphosphorylation of 4EBP1 without being impaired by rapamycin, indicating that other Herpesviruses can affect translation initiation downstream of mTORC1 (160). We found that treating cells with rapamycin partially reduced 4EBP1 phosphorylation (Appendix B), suggesting that HSV-1 manipulates both upstream regulators of 4EBP1 and 4EBP1 directly, reconciling these reports.

Interestingly, although 4EBP1 was hyperphosphorylated at both early (Fig. 3.6) and late (Fig. 3.7) time points during wildtype infection, U_{S3} was required for 4EBP1 hyperphosphorylation only at late times. It was unclear whether U_{S3} was required for 4EBP1 hyperphosphorylation at early times. Thus, it is possible that another viral protein maintains 4EBP1 hyperphosphorylation at early times of infection. ICP0 is a likely candidate that has been shown to enhance the assembly of translational machinery (249). ICP0 not only stimulates 4EBP1 phosphorylation, but also targets 4EBP1 for proteasomal degradation (21, 249). Although the phosphorylation of 4EBP1 is ICP0-dependent at 8 hpi (249), it would be interesting to determine if ICP0 is required for the phosphorylation of 4EBP1 that we observed at 4 hpi.

4.4 A potential role for U_{L46} in promoting cell survival

In addition to regulating protein translation, it has long been known that U_{S3} promotes cell survival in response to variety of apoptotic stimuli, including the stress of virus infection (4, 134). Accordingly, we found that infection with

ΔU_{S3} -virus resulted in cells bearing an apoptotic morphology (Appendix D, Kevin Quach and Heather Eaton, unpublished data).

Though U_{L46} is required for AKT activation, U_{L46} -activated AKT signals are not transduced to GSK3 β , FoxO1, and 4EBP1 during HSV-1 infection (Fig. 3.11). This is a stark contrast to other Herpesviruses. For instance, VZV encodes ORF 12 to activate AKT, resulting in phosphorylation of FoxO1 (XueQiao, et al., unpublished data). Given this information, the purpose of U_{L46} -activated AKT is a conundrum that has yet to be solved.

Given that U_{S3} and U_{L46} both appear to promote AKT signaling, we examined whether they collaborate to suppress apoptosis. Using light-microscope analysis to examine cell morphology, we found that, in contrast to ΔU_{S3} -infection, ΔU_{L46} -infected cells were not phenotypically apoptotic. However, deleting both ΔU_{L46} and ΔU_{S3} increased the frequency of apoptotic cells compared to ΔU_{S3} -infection alone. These data indicate that U_{L46} may also contribute to cell survival.

If U_{L46} promotes cell survival, then cells infected with ΔU_{L46} -virus should be more sensitive to pro-apoptotic stimuli (such as UV exposure or treatment with drugs that induce apoptosis) compared to wild type-infected cells. For example, we would expect to find increased cleavage of the cellular proteins PARP and caspase-3 by Western blot analysis, DNA fragmentation by TUNEL staining, and the appearance of apoptotic cell bodies using immunofluorescence imaging. In order to address whether U_{L46} inhibits apoptosis through an AKT-dependent mechanism, we could test whether the presence of an AKT inhibitor, AKT VIII, impairs cell survival in a U_{L46} -competent virus infection. If the putative pro-survival function of U_{L46} requires AKT, then this may be directed to selective AKT targets such as p53 and MDM2 (reviewed in (144, 221)). Alternatively, if inhibiting AKT does not impair cell survival, then U_{L46} may promote cell survival through another signaling pathway. For example, the VZV U_{L46} homolog ORF12 activates ERK signaling to prevent apoptosis (137).

4.5 HSV-1 encodes proteins other than U_{L46} and U_{S3} to modulate AKT signaling

External growth stimuli resulted in activation of AKT, but these signals were not transduced to the examined AKT targets in ΔU_{S3} - or $\Delta U_{L46}\Delta U_{S3}$ -

infected cells (Fig. 3.11, 3.13). Therefore, both U_S3 and U_L46 are not required for HSV-1 to attenuate AKT signaling, suggesting that another viral protein(s) prevents activated AKT signals from being transduced downstream. One potential candidate is the HSV-1 protein kinase U_L13, as the VZV orthologs of U_L13 and U_S3, ORF 47 and ORF 66 respectively, have been shown to collaborate to modulate AKT signaling (189). Interestingly, the U_L13 gene product is conserved between HSV-1 and VZV with a 79% sequence similarity. In contrast to VZV, we found that U_L13 deficiency did not affect the phosphorylation of AKT targets (Fig. 3.4). However, the results do not exclude the possibility that U_L13 is involved in AKT signaling. It would be interesting to examine if serum-induced activated-AKT signals result in the phosphorylation of AKT targets during infection with viral mutants lacking U_L13. These viral mutants must also be deficient in U_S3 in order to observe the downstream effects of activated AKT.

4.6 HSV-1 permits signaling to AKT from specific receptors

HSV-1 has previously been shown to downregulate expression of EGFR (135). In addition to EGFR, our studies show that HSV-1 regulates signaling from other growth factor receptors. Specifically, we found that HSV-1 infection selectively impairs signaling from PDGF receptors that include the α -subunit (Fig. 3.15, 3.16, 4.2). Neither U_L46 nor U_S3 are the main contributors to this signaling impairment through the α -chain, suggesting that another viral protein is involved. ICP0 is a potential candidate given the precedence for ICP0-mediated inhibition of EGFR signaling. In addition to transcriptional regulation of the EGFR gene, ICP0 was found to form a complex with the cellular proteins Cin85 and Cbl to mediate a reduction in the levels of cell surface and total EGFR through internalization and degradation (135). Similar to EGFR, PDGFRs are receptor tyrosine kinases that can form a complex with Cin85 and Cbl (227). Consequently, PDGFRs undergo negative regulation by endocytosis and degradation or recycling (53). Thus, it would be interesting to determine whether ICP0 mediates downregulation of the α -PDGFR chain during HSV-1 infection. Though it remains unknown what the biological outcomes of EGFR or PDGFR downregulation are during HSV-1 infection, it is interesting to speculate that

excessive signaling or selective signaling pathways are not beneficial for viral infection. Our findings that HSV-1 utilizes U_{S3} to bypass AKT signaling and directly regulate AKT targets lend further support to this idea.

4.7 Other aspects of AKT activation that are modulated by HSV-1

My work has also shed light on the molecular mechanisms by which U_{L46} and U_{S3} modulate the PI3K-AKT pathway.

4.7.1 A mechanism for the phosphorylation of AKT by HSV-1

We have previously shown that the phosphorylation of AKT is U_{L46}-dependent. However, the role of mTORC2 in phosphorylating the hydrophobic motif of AKT during HSV-1 infection was unclear. We found that HSV-1 induced AKT phosphorylation was prevented in the presence of torin1. Since use of drugs could have non-specific effects on cell signaling, RNA silencing of mTORC2 subunits can be used to verify that mTORC2 is required for AKT phosphorylation during infection. Knockdown of the mTORC2 subunits mTOR and rictor were previously used by Sabatini and colleagues to prevent AKT phosphorylation in uninfected cells (204). Though mTORC2 is required for the phosphorylation of AKT, these results do not tell us whether U_{L46} activates AKT by promoting mTORC2 activity. To get an indication of this, we can examine whether infection with ΔU_{L46}-virus reduces rictor expression levels, impairs the association between rictor and mTOR, or impairs the phosphorylation of rictor.

4.7.2 U_{S3} negatively regulates the phosphorylation of the T308 residue of AKT

Though we and others have shown that U_{S3} antagonizes AKT phosphorylation at both the S473 and T308 residues (13), the mechanism by which this occurs was unclear. By using serum to stimulate AKT phosphorylation, we have shown that U_{S3} specifically antagonizes phosphorylation of T308. This implies that U_{S3} interferes with a step upstream of PDK1 phosphorylation of AKT (Fig. 3.11, 4.2). For instance, U_{S3} may be interfering with the generation of PIP3 species by activating PLC, inhibiting PI3K, or activating phosphatases such as PTEN, and inositol polyphosphate 5'-phosphatases (INPP5) (including SHIP-2 and type II inositol polyphosphate 5-phosphatase) (7, 35, 193). Potential activation of INPP5 species by U_{S3} would increase cleavage of PIP3 (109, 148,

170), preventing binding of AKT and PDK1 at the plasma membrane, and consequently phosphorylation of T308 on AKT. Alternatively, U_{S3} may disrupt interactions of the PH domain of PDK1 and AKT with PIP3, or impair PDK1 activity.

4.8 Future Directions

Previous work done by Dr. Melany Wagner examined the interactions between U_{L46} and SFK, and their effect on AKT signaling (247). Her data has clearly shown that U_{L46}-dependent activated AKT signals are not required for the phosphorylation of downstream targets. In fact, we found that U_{L46}-activated AKT signals are attenuated during infection. Future work should aim to identify cellular or viral factors that prevent activated AKT signals from being transduced. Although U_{L46} is required for AKT activation, we have yet to determine the biological function(s) of U_{L46} during infection. Although we suspect that U_{L46} promotes cell survival, future studies should verify this possibility and determine whether U_{L46} promotes cell survival through an AKT-dependent mechanism.

HSV-1 not only attenuates signaling between AKT and its targets, but also inhibits signaling from select growth factor receptors. The viral proteins involved in attenuating PDGFR α -receptor signaling have not been explored. Future experiments should examine gene and cell surface expression of PDGFRs during infection.

The investigations from this thesis add to our knowledge of how HSV infection induces changes to cell signaling. Work in our lab presently examines other signaling pathways targeted by U_{L46}, the anti-apoptotic roles played by U_{L46} and U_{S3}, the molecular mechanisms governing U_{L46}-mediated activation of SFKs, and inhibition of AKT phosphorylation by U_{S3}. Although these future directions should add to our understanding of HSV-modulated AKT signaling, many questions remained unanswered, such as reasons for encoding an AKT mimic, resolving how AKT signals are attenuated, the purpose of attenuating AKT signaling, and other viral proteins involved in AKT signaling.

BIBLIOGRAPHY

1. **Ahmed, N. N., H. L. Grimes, A. Bellacosa, T. O. Chan, and P. N. Tsichlis.** 1997. Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. *Proceedings of the National Academy of Sciences of the United States of America* **94**:3627-3632.
2. **Alessi, D. R., F. B. Caudwell, M. Andjelkovic, B. A. Hemmings, and P. Cohen.** 1996. Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett* **399**:333-338.
3. **Alessi, D. R., M. Deak, A. Casamayor, F. B. Caudwell, N. Morrice, D. G. Norman, P. Gaffney, C. B. Reese, C. N. MacDougall, D. Harbison, A. Ashworth, and M. Bownes.** 1997. 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the *Drosophila* DSTPK61 kinase. *Current biology* : CB **7**:776-789.
4. **Asano, S., T. Honda, F. Goshima, D. Watanabe, Y. Miyake, Y. Sugiura, and Y. Nishiyama.** 1999. US3 protein kinase of herpes simplex virus type 2 plays a role in protecting corneal epithelial cells from apoptosis in infected mice. *The Journal of general virology* **80** (Pt 1):51-56.
5. **Auger, K. R., L. A. Serunian, S. P. Soltoff, P. Libby, and L. C. Cantley.** 1989. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell* **57**:167-175.
6. **Avruch, J., C. Belham, Q. Weng, K. Hara, and K. Yonezawa.** 2001. The p70 S6 kinase integrates nutrient and growth signals to control translational capacity. *Prog Mol Subcell Biol* **26**:115-154.
7. **Backers, K., D. Blero, N. Paternotte, J. Zhang, and C. Erneux.** 2003. The termination of PI3K signalling by SHIP1 and SHIP2 inositol 5-phosphatases. *Advances in enzyme regulation* **43**:15-28.
8. **Balendran, N., R. L. Clough, J. R. Arguello, R. Barber, C. Veal, A. B. Jones, J. L. Rosbotham, A. M. Little, A. Madrigal, J. N. Barker, S. H. Powis, and R. C. Trembath.** 1999. Characterization of the major susceptibility region for psoriasis at chromosome 6p21.3. *J Invest Dermatol* **113**:322-328.
9. **Batterson, W., and B. Roizman.** 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of alpha genes. *Journal of virology* **46**:371-377.
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. *The Journal of biological chemistry* **273**:17050-17055.
11. **Benetti, L., J. Munger, and B. Roizman.** 2003. The herpes simplex virus 1 US3 protein kinase blocks caspase-dependent double cleavage and activation of the proapoptotic protein BAD. *Journal of virology* **77**:6567-6573.

12. **Benetti, L., and B. Roizman.** 2004. Herpes simplex virus protein kinase US3 activates and functionally overlaps protein kinase A to block apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **101**:9411-9416.
13. **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. *Journal of virology* **80**:3341-3348.
14. **Berridge, M. J., and R. F. Irvine.** 1989. Inositol phosphates and cell signalling. *Nature* **341**:197-205.
15. **Binder, P. S.** 1977. Herpes simplex keratitis. *Surv Ophthalmol* **21**:313-331.
16. **Biondi, R. M.** 2004. Phosphoinositide-dependent protein kinase 1, a sensor of protein conformation. *Trends in biochemical sciences* **29**:136-142.
17. **Biondi, R. M., P. C. Cheung, A. Casamayor, M. Deak, R. A. Currie, and D. R. Alessi.** 2000. Identification of a pocket in the PDK1 kinase domain that interacts with PIF and the C-terminal residues of PKA. *The EMBO journal* **19**:979-988.
18. **Bloom, D. C., N. V. Giordani, and D. L. Kwiatkowski.** Epigenetic regulation of latent HSV-1 gene expression. *Biochim Biophys Acta* **1799**:246-256.
19. **Boehmer, P. E., and I. R. Lehman.** 1993. Physical interaction between the herpes simplex virus 1 origin-binding protein and single-stranded DNA-binding protein ICP8. *Proceedings of the National Academy of Sciences of the United States of America* **90**:8444-8448.
20. **Bottomley, M. J., K. Salim, and G. Panayotou.** 1998. Phospholipid-binding protein domains. *Biochim Biophys Acta* **1436**:165-183.
21. **Boutell, C., S. Sadis, and R. D. Everett.** 2002. Herpes simplex virus type 1 immediate-early protein ICP0 and its isolated RING finger domain act as ubiquitin E3 ligases in vitro. *Journal of virology* **76**:841-850.
22. **Brazil, D. P., and B. A. Hemmings.** 2001. Ten years of protein kinase B signalling: a hard Akt to follow. *Trends in biochemical sciences* **26**:657-664.
23. **Brazil, D. P., Z. Z. Yang, and B. A. Hemmings.** 2004. Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends in biochemical sciences* **29**:233-242.
24. **Bresnahan, W. A., G. E. Hultman, and T. Shenk.** 2000. Replication of wild-type and mutant human cytomegalovirus in life-extended human diploid fibroblasts. *J Virol* **74**:10816-10818.

25. **Bronstein, J. C., S. K. Weller, and P. C. Weber.** 1997. The product of the UL12.5 gene of herpes simplex virus type 1 is a capsid-associated nuclease. *Journal of virology* **71**:3039-3047.
26. **Burgering, B. M., and P. J. Coffe.** 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**:599-602.
27. **Burgering, B. M., and R. H. Medema.** 2003. Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. *J Leukoc Biol* **73**:689-701.
28. **Burke, J. E., O. Vadas, A. Berndt, T. Finegan, O. Perisic, and R. L. Williams.** Dynamics of the phosphoinositide 3-kinase p110delta interaction with p85alpha and membranes reveals aspects of regulation distinct from p110alpha. *Structure* **19**:1127-1137.
29. **Burnett, P. E., R. K. Barrow, N. A. Cohen, S. H. Snyder, and D. M. Sabatini.** 1998. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proceedings of the National Academy of Sciences of the United States of America* **95**:1432-1437.
30. **Cai, W., and P. A. Schaffer.** 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. *Journal of virology* **66**:2904-2915.
31. **Calleja, V., D. Alcor, M. Laguerre, J. Park, B. Vojnovic, B. A. Hemmings, J. Downward, P. J. Parker, and B. Larijani.** 2007. Intramolecular and intermolecular interactions of protein kinase B define its activation in vivo. *PLoS Biol* **5**:e95.
32. **Carpenter, C. L., B. C. Duckworth, K. R. Auger, B. Cohen, B. S. Schaffhausen, and L. C. Cantley.** 1990. Purification and characterization of phosphoinositide 3-kinase from rat liver. *The Journal of biological chemistry* **265**:19704-19711.
33. **Chuluunbaatar, U., and I. Mohr.** 2011. A herpesvirus kinase that masquerades as Akt: you don't have to look like Akt, to act like it. *Cell Cycle* **10**:2064-2068.
34. **Chuluunbaatar, U., R. Roller, M. E. Feldman, S. Brown, K. M. Shokat, and I. Mohr.** Constitutive mTORC1 activation by a herpesvirus Akt surrogate stimulates mRNA translation and viral replication. *Genes & development* **24**:2627-2639.
35. **Cockcroft, S., and G. M. Thomas.** 1992. Inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. *The Biochemical journal* **288** (Pt 1):1-14.
36. **Conley, A. J., D. M. Knipe, P. C. Jones, and B. Roizman.** 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive

- mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides. *Journal of virology* **37**:191-206.
37. **Costanzo, F., G. Campadelli-Fiume, L. Foa-Tomasi, and E. Cassai.** 1977. Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. *Journal of virology* **21**:996-1001.
 38. **Coughlin, S. R., J. A. Escobedo, and L. T. Williams.** 1989. Role of phosphatidylinositol kinase in PDGF receptor signal transduction. *Science* **243**:1191-1194.
 39. **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**:785-789.
 40. **Crute, J. J., E. S. Mocarski, and I. R. Lehman.** 1988. A DNA helicase induced by herpes simplex virus type 1. *Nucleic acids research* **16**:6585-6596.
 41. **Daikoku, T., R. Kurachi, T. Tsurumi, and Y. Nishiyama.** 1994. Identification of a target protein of US3 protein kinase of herpes simplex virus type 2. *The Journal of general virology* **75 (Pt 8)**:2065-2068.
 42. **Daikoku, T., Y. Yamashita, T. Tsurumi, and Y. Nishiyama.** 1995. The US3 protein kinase of herpes simplex virus type 2 is associated with phosphorylation of the UL12 alkaline nuclease in vitro. *Arch Virol* **140**:1637-1644.
 43. **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. *Journal of virology* **75**:6135-6142.
 44. **Datta, S. R., A. Brunet, and M. E. Greenberg.** 1999. Cellular survival: a play in three Akts. *Genes & development* **13**:2905-2927.
 45. **Datta, S. R., H. Dudek, X. Tao, S. Masters, H. A. Fu, Y. Gotoh, and M. E. Greenberg.** 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**:231-241.
 46. **Datta, S. R., A. Katsov, L. Hu, A. Petros, S. W. Fesik, M. B. Yaffe, and M. E. Greenberg.** 2000. 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. *Mol Cell* **6**:41-51.
 47. **Dawson, C. W., G. Tramontanis, 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. *The Journal of biological chemistry* **278**:3694-3704.
 48. **de Bruyn Kops, A., S. L. Uprichard, M. Chen, and D. M. Knipe.** 1998. Comparison of the intranuclear distributions of herpes simplex virus proteins involved in various viral functions. *Virology* **252**:162-178.

49. **DeLuca, N., D. J. Bzik, V. C. Bond, S. Person, and W. Snipes.** 1982. Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion, and production of glycoprotein gb (VP7). *Virology* **122**:411-423.
50. **Desbois-Mouthon, C., A. Cadoret, M. J. Blivet-Van Eggelpeol, F. Bertrand, G. Cherqui, C. Perret, and J. Capeau.** 2001. Insulin and IGF-1 stimulate the beta-catenin pathway through two signalling cascades involving GSK-3beta inhibition and Ras activation. *Oncogene* **20**:252-259.
51. **Deshmane, S. L., and N. W. Fraser.** 1989. During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. *Journal of virology* **63**:943-947.
52. **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.
53. **Dikic, I., and S. Giordano.** 2003. Negative receptor signalling. *Curr Opin Cell Biol* **15**:128-135.
54. **Divecha, N., and R. F. Irvine.** 1995. Phospholipid signaling. *Cell* **80**:269-278.
55. **Dobson, A. T., F. Sederati, G. Devi-Rao, W. M. Flanagan, M. J. Farrell, J. G. Stevens, E. K. Wagner, and L. T. Feldman.** 1989. Identification of the latency-associated transcript promoter by expression of rabbit beta-globin mRNA in mouse sensory nerve ganglia latently infected with a recombinant herpes simplex virus. *Journal of virology* **63**:3844-3851.
56. **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**:2795-2809.
57. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effects on social behaviour of infected cells. *The Journal of general virology* **2**:357-364.
58. **Elias, P., M. E. O'Donnell, E. S. Mocarski, and I. R. Lehman.** 1986. A DNA binding protein specific for an origin of replication of herpes simplex virus type 1. *Proceedings of the National Academy of Sciences of the United States of America* **83**:6322-6326.
59. **Elliott, G., and P. O'Hare.** 1998. Herpes simplex virus type 1 tegument protein VP22 induces the stabilization and hyperacetylation of microtubules. *Journal of virology* **72**:6448-6455.
60. **Elliott, G., and P. O'Hare.** 1997. Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* **88**:223-233.

61. **Erazo, A., and P. R. Kinchington.** Varicella-zoster virus open reading frame 66 protein kinase and its relationship to alphaherpesvirus US3 kinases. *Current topics in microbiology and immunology* **342**:79-98.
62. **Eriksson, A., A. Siegbahn, B. Westermarck, C. H. Heldin, and L. Claesson-Welsh.** 1992. PDGF alpha- and beta-receptors activate unique and common signal transduction pathways. *The EMBO journal* **11**:543-550.
63. **Everett, R. D., P. Freemont, H. Saitoh, M. Dasso, A. Orr, M. Kathoria, and J. Parkinson.** 1998. The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. *Journal of virology* **72**:6581-6591.
64. **Fang, Y., M. Vilella-Bach, R. Bachmann, A. Flanigan, and J. Chen.** 2001. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science* **294**:1942-1945.
65. **Feng, J., J. Park, P. Cron, D. Hess, and B. A. Hemmings.** 2004. Identification of a PKB/Akt hydrophobic motif Ser-473 kinase as DNA-dependent protein kinase. *The Journal of biological chemistry* **279**:41189-41196.
66. **Ferguson, K. M., M. A. Lemmon, P. B. Sigler, and J. Schlessinger.** 1995. Scratching the surface with the PH domain. *Nat Struct Biol* **2**:715-718.
67. **Forrester, A., H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and T. Minson.** 1992. Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *Journal of virology* **66**:341-348.
68. **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. *The Journal of general virology* **68 (Pt 10)**:2699-2704.
69. **Fretto, L. J., A. J. Snape, J. E. Tomlinson, J. J. Seroogy, D. L. Wolf, W. J. LaRochelle, and N. A. Giese.** 1993. Mechanism of platelet-derived growth factor (PDGF) AA, AB, and BB binding to alpha and beta PDGF receptor. *The Journal of biological chemistry* **268**:3625-3631.
70. **Frias, M. A., C. C. Thoreen, J. D. Jaffe, W. Schroder, T. Sculley, S. A. Carr, and D. M. Sabatini.** 2006. mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Current biology : CB* **16**:1865-1870.
71. **Friedman, E., A. H. Katcher, and V. J. Brightman.** 1977. Incidence of recurrent herpes labialis and upper respiratory infection: a prospective study of the influence of biologic, social and psychologic predictors. *Oral Surg Oral Med Oral Pathol* **43**:873-878.

72. **Gao, M., and D. M. Knipe.** 1991. Potential role for herpes simplex virus ICP8 DNA replication protein in stimulation of late gene expression. *Journal of virology* **65**:2666-2675.
73. **Gao, X., and D. Pan.** 2001. TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. *Genes & development* **15**:1383-1392.
74. **Geraghty, R. J., C. Krummenacher, G. H. Cohen, R. J. Eisenberg, and P. G. Spear.** 1998. Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* **280**:1618-1620.
75. **Gibson, W.** 1996. Structure and assembly of the virion. *Intervirolgy* **39**:389-400.
76. **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.
77. **Gingras, A. C., S. P. Gygi, B. Raught, R. D. Polakiewicz, R. T. Abraham, M. F. Hoekstra, R. Aebersold, and N. Sonenberg.** 1999. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes & development* **13**:1422-1437.
78. **Gingras, A. C., B. Raught, and N. Sonenberg.** 1999. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem* **68**:913-963.
79. **Gingras, A. C., B. Raught, and N. Sonenberg.** 2001. Regulation of translation initiation by FRAP/mTOR. *Genes & development* **15**:807-826.
80. **Glezen, W. P., G. W. Fernald, and J. A. Lohr.** 1975. Acute respiratory disease of university students with special reference to the etiologic role of Herpesvirus hominis. *Am J Epidemiol* **101**:111-121.
81. **Greenberg, M. E., S. R. Datta, and A. Brunet.** 1999. Cellular survival: a play in three Akts. *Genes & development* **13**:2905-2927.
82. **Gross, A., J. M. McDonnell, and S. J. Korsmeyer.** 1999. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* **13**:1899-1911.
83. **Guertin, D. A., and D. M. Sabatini.** 2007. Defining the role of mTOR in cancer. *Cancer Cell* **12**:9-22.
84. **Guertin, D. A., and D. M. Sabatini.** 2005. An expanding role for mTOR in cancer. *Trends Mol Med* **11**:353-361.
85. **Hammacher, A., U. Hellman, A. Johnsson, A. Ostman, K. Gunnarsson, B. Westermark, A. Wasteson, and C. H. Heldin.** 1988. A major part of platelet-

derived growth factor purified from human platelets is a heterodimer of one A and one B chain. *The Journal of biological chemistry* **263**:16493-16498.

86. **Han, J. Y., D. D. Sloan, M. Aubert, S. A. Miller, C. H. Dang, and K. R. Jerome.** 2007. Apoptosis and antigen receptor function in T and B cells following exposure to herpes simplex virus. *Virology* **359**:253-263.
87. **Hara, K., Y. Maruki, X. Long, K. Yoshino, N. Oshiro, S. Hidayat, C. Tokunaga, J. Avruch, and K. Yonezawa.** 2002. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* **110**:177-189.
88. **Hardwicke, M. A., and R. M. Sandri-Goldin.** 1994. The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection. *Journal of virology* **68**:4797-4810.
89. **Hata, S., A. H. Koyama, H. Shiota, A. Adachi, F. Goshima, and Y. Nishiyama.** 1999. Antiapoptotic activity of herpes simplex virus type 2: the role of US3 protein kinase gene. *Microbes Infect* **1**:601-607.
90. **Heidaran, M. A., J. H. Pierce, R. A. Jensen, T. Matsui, and S. A. Aaronson.** 1990. Chimeric alpha- and beta-platelet-derived growth factor (PDGF) receptors define three immunoglobulin-like domains of the alpha-PDGF receptor that determine PDGF-AA binding specificity. *The Journal of biological chemistry* **265**:18741-18744.
91. **Heidaran, M. A., J. C. Yu, R. A. Jensen, J. H. Pierce, and S. A. Aaronson.** 1992. A deletion in the extracellular domain of the alpha platelet-derived growth factor (PDGF) receptor differentially impairs PDGF-AA and PDGF-BB binding affinities. *The Journal of biological chemistry* **267**:2884-2887.
92. **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. *The Journal of general virology* **75 (Pt 6)**:1211-1222.
93. **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. *Journal of virology* **65**:1090-1098.
94. **Hers, I., E. E. Vincent, and J. M. Tavaré.** Akt signalling in health and disease. *Cell Signal* **23**:1515-1527.
95. **Hinchliffe, K., and R. Irvine.** 1997. Inositol lipid pathways turn turtle. *Nature* **390**:123-124.
96. **Holt, K. H., L. Olson, W. S. Moye-Rowley, and J. E. Pessin.** 1994. Phosphatidylinositol 3-kinase activation is mediated by high-affinity interactions between distinct domains within the p110 and p85 subunits. *Molecular and cellular biology* **14**:42-49.

97. **Homa, F. L., and J. C. Brown.** 1997. Capsid assembly and DNA packaging in herpes simplex virus. *Rev Med Virol* **7**:107-122.
98. **Honess, R. W., and B. Roizman.** 1975. Proteins specified by herpes simplex virus. XIII. Glycosylation of viral polypeptides. *Journal of virology* **16**:1308-1326.
99. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *Journal of virology* **14**:8-19.
100. **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**:1276-1280.
101. **Huang, E. a. K., T. (ed.).** 1993. *Molecular Aspects of Human Cytomegalovirus Diseases.* Springer, Berlin.
102. **Ichaso, N., and S. M. Dilworth.** 2001. Cell transformation by the middle T-antigen of polyoma virus. *Oncogene* **20**:7908-7916.
103. **Ingham, P. W.** 2001. Hedgehog signaling: a tale of two lipids. *Science* **294**:1879-1881.
104. **Ingham, P. W., and A. P. McMahon.** 2001. Hedgehog signaling in animal development: paradigms and principles. *Genes & development* **15**:3059-3087.
105. **Inoki, K., Y. Li, T. Zhu, J. Wu, and K. L. Guan.** 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nature cell biology* **4**:648-657.
106. **Isakoff, S. J., T. Cardozo, J. Andreev, Z. Li, K. M. Ferguson, R. Abagyan, M. A. Lemmon, A. Aronheim, and E. Y. Skolnik.** 1998. Identification and analysis of PH domain-containing targets of phosphatidylinositol 3-kinase using a novel in vivo assay in yeast. *The EMBO journal* **17**:5374-5387.
107. **Jackson, S. A., and N. A. DeLuca.** 2003. Relationship of herpes simplex virus genome configuration to productive and persistent infections. *Proceedings of the National Academy of Sciences of the United States of America* **100**:7871-7876.
108. **Jefferies, H. B., S. Fumagalli, P. B. Dennis, C. Reinhard, R. B. Pearson, and G. Thomas.** 1997. Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. *The EMBO journal* **16**:3693-3704.
109. **Jefferson, A. B., and P. W. Majerus.** 1995. Properties of type II inositol polyphosphate 5-phosphatase. *The Journal of biological chemistry* **270**:9370-9377.
110. **Johnson, D. C., and M. T. Huber.** 2002. Directed egress of animal viruses promotes cell-to-cell spread. *Journal of virology* **76**:1-8.

111. **Jovasevic, V., L. Liang, and B. Roizman.** 2008. Proteolytic cleavage of VP1-2 is required for release of herpes simplex virus 1 DNA into the nucleus. *Journal of virology* **82**:3311-3319.
112. **Kandel, E. S., and N. Hay.** 1999. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Experimental cell research* **253**:210-229.
113. **Kapeller, R., and L. C. Cantley.** 1994. Phosphatidylinositol 3-kinase. *BioEssays : news and reviews in molecular, cellular and developmental biology* **16**:565-576.
114. **Kapeller, R., K. V. Prasad, O. Janssen, W. Hou, B. S. Schaffhausen, C. E. Rudd, and L. C. Cantley.** 1994. Identification of two SH3-binding motifs in the regulatory subunit of phosphatidylinositol 3-kinase. *The Journal of biological chemistry* **269**:1927-1933.
115. **Kaplan, D. R., M. Whitman, B. Schaffhausen, D. C. Pallas, M. White, L. Cantley, and T. M. Roberts.** 1987. Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. *Cell* **50**:1021-1029.
116. **Katan, M., A. Haigh, C. P. Verrijzer, P. C. van der Vliet, and P. O'Hare.** 1990. Characterization of a cellular factor which interacts functionally with Oct-1 in the assembly of a multicomponent transcription complex. *Nucleic acids research* **18**:6871-6880.
117. **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**:2149-2162.
118. **Kazlauskas, A., and J. A. Cooper.** 1989. Autophosphorylation of the PDGF receptor in the kinase insert region regulates interactions with cell proteins. *Cell* **58**:1121-1133.
119. **Kazlauskas, A., and J. A. Cooper.** 1990. Phosphorylation of the PDGF receptor beta subunit creates a tight binding site for phosphatidylinositol 3 kinase. *The EMBO journal* **9**:3279-3286.
120. **Kelly, J. D., B. A. Haldeman, F. J. Grant, M. J. Murray, R. A. Seifert, D. F. Bowen-Pope, J. A. Cooper, and A. Kazlauskas.** 1991. Platelet-derived growth factor (PDGF) stimulates PDGF receptor subunit dimerization and intersubunit trans-phosphorylation. *The Journal of biological chemistry* **266**:8987-8992.
121. **Kennedy, S. G., A. J. Wagner, S. D. Conzen, J. Jordan, A. Bellacosa, P. N. Tsichlis, and N. Hay.** 1997. The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes & development* **11**:701-713.

122. **Kim, D. H., D. D. Sarbassov, S. M. Ali, J. E. King, R. R. Latek, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini.** 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110**:163-175.
123. **Kim, D. H., D. D. Sarbassov, S. M. Ali, R. R. Latek, K. V. Guntur, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini.** 2003. GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Mol Cell* **11**:895-904.
124. **Kim, M. J., E. Kim, S. H. Ryu, and P. G. Suh.** 2000. The mechanism of phospholipase C-gamma1 regulation. *Exp Mol Med* **32**:101-109.
125. **Klippel, A., J. A. Escobedo, W. J. Fantl, and L. T. Williams.** 1992. The C-terminal SH2 domain of p85 accounts for the high affinity and specificity of the binding of phosphatidylinositol 3-kinase to phosphorylated platelet-derived growth factor beta receptor. *Molecular and cellular biology* **12**:1451-1459.
126. **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-hydroxyonyl)adenine]. *The Journal of general virology* **67 (Pt 9)**:2023-2028.
127. **Kristie, T. M., J. H. LeBowitz, and P. A. Sharp.** 1989. The octamer-binding proteins form multi-protein--DNA complexes with the HSV alpha TIF regulatory protein. *The EMBO journal* **8**:4229-4238.
128. **Kristie, T. M., and B. Roizman.** 1987. Host cell proteins bind to the cis-acting site required for virion-mediated induction of herpes simplex virus 1 alpha genes. *Proceedings of the National Academy of Sciences of the United States of America* **84**:71-75.
129. **Kristie, T. M., and B. Roizman.** 1984. Separation of sequences defining basal expression from those conferring alpha gene recognition within the regulatory domains of herpes simplex virus 1 alpha genes. *Proceedings of the National Academy of Sciences of the United States of America* **81**:4065-4069.
130. **Kristie, T. M., and P. A. Sharp.** 1990. Interactions of the Oct-1 POU subdomains with specific DNA sequences and with the HSV alpha-trans-activator protein. *Genes & development* **4**:2383-2396.
131. **Lang, F., and P. Cohen.** 2001. Regulation and physiological roles of serum- and glucocorticoid-induced protein kinase isoforms. *Sci STKE* **2001**:re17.
132. **Leevers, S. J., B. Vanhaesebroeck, and M. D. Waterfield.** 1999. Signalling through phosphoinositide 3-kinases: the lipids take centre stage. *Current Opinion in Cell Biology* **11**:219-225.

133. **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. *Journal of virology* **35**:798-811.
134. **Leopardi, R., C. Van Sant, and B. Roizman.** 1997. The herpes simplex virus 1 protein kinase US3 is required for protection from apoptosis induced by the virus. *Proceedings of the National Academy of Sciences of the United States of America* **94**:7891-7896.
135. **Liang, Y., A. Kurakin, and B. Roizman.** 2005. Herpes simplex virus 1 infected cell protein 0 forms a complex with CIN85 and Cbl and mediates the degradation of EGF receptor from cell surfaces. *Proceedings of the National Academy of Sciences of the United States of America* **102**:5838-5843.
136. **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. *Journal of virology* **62**:1486-1494.
137. **Liu, X., Q. Li, K. Dowdell, E. R. Fischer, and J. I. Cohen.** 2012. Varicella-Zoster virus ORF12 protein triggers phosphorylation of ERK1/2 and inhibits apoptosis. *Journal of virology* **86**:3143-3151.
138. **Loewith, R., E. Jacinto, S. Wullschleger, A. Lorberg, J. L. Crespo, D. Bonenfant, W. Oppliger, P. Jenoe, and M. N. Hall.** 2002. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell* **10**:457-468.
139. **Mackem, S., and B. Roizman.** 1982. Differentiation between alpha promoter and regulator regions of herpes simplex virus 1: the functional domains and sequence of a movable alpha regulator. *Proceedings of the National Academy of Sciences of the United States of America* **79**:4917-4921.
140. **Mackem, S., and B. Roizman.** 1980. Regulation of herpesvirus macromolecular synthesis: transcription-initiation sites and domains of alpha genes. *Proceedings of the National Academy of Sciences of the United States of America* **77**:7122-7126.
141. **Maehama, T., and J. E. Dixon.** 1999. PTEN: a tumour suppressor that functions as a phospholipid phosphatase. *Trends Cell Biol* **9**:125-128.
142. **Manning, B. D., and L. C. Cantley.** 2007. AKT/PKB signaling: navigating downstream. *Cell* **129**:1261-1274.
143. **Manning, B. D., and L. C. Cantley.** 2003. Rheb fills a GAP between TSC and TOR. *Trends in biochemical sciences* **28**:573-576.
144. **Marine, J. C., S. Francoz, M. Maetens, G. Wahl, F. Toledo, and G. Lozano.** 2006. Keeping p53 in check: essential and synergistic functions of Mdm2 and Mdm4. *Cell death and differentiation* **13**:927-934.

145. **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.
146. **Matis, J., and M. Kudelova.** 2001. Early shutoff of host protein synthesis in cells infected with herpes simplex viruses. *Acta Virol* **45**:269-277.
147. **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. *The Journal of general virology* **86**:1979-1985.
148. **Matzaris, M., S. P. Jackson, K. M. Laxminarayan, C. J. Speed, and C. A. Mitchell.** 1994. Identification and characterization of the phosphatidylinositol-(4, 5)-bisphosphate 5-phosphatase in human platelets. *The Journal of biological chemistry* **269**:3397-3402.
149. **Maul, G. G.** 1998. Nuclear domain 10, the site of DNA virus transcription and replication. *BioEssays : news and reviews in molecular, cellular and developmental biology* **20**:660-667.
150. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *The Journal of general virology* **69 (Pt 7)**:1531-1574.
151. **McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon.** 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. *Journal of molecular biology* **181**:1-13.
152. **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. *Journal of virology* **61**:992-1001.
153. **McLean, C., A. Buckmaster, D. Hancock, A. Buchan, A. Fuller, and A. Minson.** 1982. Monoclonal antibodies to three non-glycosylated antigens of herpes simplex virus type 2. *The Journal of general virology* **63**:297-305.
154. **McMillan, J. A., L. B. Weiner, A. M. Higgins, and V. J. Lamparella.** 1993. Pharyngitis associated with herpes simplex virus in college students. *Pediatr Infect Dis J* **12**:280-284.
155. **Mellerick, D. M., and N. W. Fraser.** 1987. Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. *Virology* **158**:265-275.
156. **Mettenleiter, T. C.** 2002. Herpesvirus assembly and egress. *Journal of virology* **76**:1537-1547.

157. **Milburn, C. C., M. Deak, S. M. Kelly, N. C. Price, D. R. Alessi, and D. M. Van Aalten.** 2003. Binding of phosphatidylinositol 3,4,5-trisphosphate to the pleckstrin homology domain of protein kinase B induces a conformational change. *The Biochemical journal* **375**:531-538.
158. **Miller, J. R.** 2002. The Wnts. *Genome Biol* **3**:REVIEWS3001.
159. **Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear.** 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87**:427-436.
160. **Moorman, N. J., and T. Shenk.** Rapamycin-resistant mTORC1 kinase activity is required for herpesvirus replication. *Journal of virology* **84**:5260-5269.
161. **Mora, A., D. Komander, D. M. van Aalten, and D. R. Alessi.** 2004. PDK1, the master regulator of AGC kinase signal transduction. *Semin Cell Dev Biol* **15**:161-170.
162. **Morgan, C., H. M. Rose, M. Holden, and E. P. Jones.** 1959. Electron microscopic observations on the development of herpes simplex virus. *J Exp Med* **110**:643-656.
163. **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. *The EMBO journal* **12**:2257-2264.
164. **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. *Journal of virology* **83**:5181-5191.
165. **Munger, J., A. V. Chee, and B. Roizman.** 2001. The U(S)3 protein kinase blocks apoptosis induced by the d120 mutant of herpes simplex virus 1 at a premitochondrial stage. *Journal of virology* **75**:5491-5497.
166. **Munger, J., and B. Roizman.** 2001. The US3 protein kinase of herpes simplex virus 1 mediates the posttranslational modification of BAD and prevents BAD-induced programmed cell death in the absence of other viral proteins. *Proceedings of the National Academy of Sciences of the United States of America* **98**:10410-10415.
167. **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**:279-289.
168. **Newcomb, W. W., B. L. Trus, F. P. Booy, A. C. Steven, J. S. Wall, and J. C. Brown.** 1993. Structure of the herpes simplex virus capsid. *Molecular*

- composition of the pentons and the triplexes. *Journal of molecular biology* **232**:499-511.
169. **Newton, A. C.** 2003. Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. *The Biochemical journal* **370**:361-371.
170. **Nishizuka, Y.** 1995. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* **9**:484-496.
171. **O'Hare, P., C. R. Goding, and A. Haigh.** 1988. Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. *The EMBO journal* **7**:4231-4238.
172. **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 research* **31**:3635-3641.
173. **Ogg, P. D., P. J. McDonell, B. J. Ryckman, C. M. Knudson, and R. J. Roller.** 2004. The HSV-1 Us3 protein kinase is sufficient to block apoptosis induced by overexpression of a variety of Bcl-2 family members. *Virology* **319**:212-224.
174. **Ojeda, F., H. Folch, M. I. Guarda, B. Jastorff, and H. A. Diehl.** 1995. Induction of apoptosis in thymocytes: new evidence for an interaction of PKC and PKA pathways. *Biol Chem Hoppe Seyler* **376**:389-393.
175. **Patel, A. H., F. J. Rixon, C. Cunningham, and A. J. Davison.** 1996. Isolation and characterization of herpes simplex virus type 1 mutants defective in the UL6 gene. *Virology* **217**:111-123.
176. **Persad, S., S. Attwell, V. Gray, N. Mawji, J. T. Deng, D. Leung, J. Yan, J. Sanghera, M. P. Walsh, and S. Dedhar.** 2001. Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino acids arginine 211 and serine 343. *The Journal of biological chemistry* **276**:27462-27469.
177. **Peterson, R. T., and S. L. Schreiber.** 1999. Kinase phosphorylation: Keeping it all in the family. *Current biology* : CB **9**:R521-524.
178. **Phelan, A., J. Dunlop, A. H. Patel, N. D. Stow, and J. B. Clements.** 1997. Nuclear sites of herpes simplex virus type 1 DNA replication and transcription colocalize at early times postinfection and are largely distinct from RNA processing factors. *Journal of virology* **71**:1124-1132.
179. **Poon, A. P., H. Gu, and B. Roizman.** 2006. ICP0 and the US3 protein kinase of herpes simplex virus 1 independently block histone deacetylation to enable gene expression. *Proceedings of the National Academy of Sciences of the United States of America* **103**:9993-9998.

180. **Poon, A. P., Y. Liang, and B. Roizman.** 2003. Herpes simplex virus 1 gene expression is accelerated by inhibitors of histone deacetylases in rabbit skin cells infected with a mutant carrying a cDNA copy of the infected-cell protein no. 0. *Journal of virology* **77**:12671-12678.
181. **Potter, C. J., L. G. Pedraza, and T. Xu.** 2002. Akt regulates growth by directly phosphorylating Tsc2. *Nature cell biology* **4**:658-665.
182. **Preston, C. M., M. C. Frame, and M. E. Campbell.** 1988. A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence. *Cell* **52**:425-434.
183. **Purifoy, D. J., R. B. Lewis, and K. L. Powell.** 1977. Identification of the herpes simplex virus DNA polymerase gene. *Nature* **269**:621-623.
184. **Purves, F. C., A. D. Deana, F. Marchiori, D. P. Leader, and L. A. Pinna.** 1986. The substrate specificity of the protein kinase induced in cells infected with herpesviruses: studies with synthetic substrates [corrected] indicate structural requirements distinct from other protein kinases. *Biochim Biophys Acta* **889**:208-215.
185. **Purves, F. C., R. M. Longnecker, D. P. Leader, and B. Roizman.** 1987. Herpes simplex virus 1 protein kinase is encoded by open reading frame US3 which is not essential for virus growth in cell culture. *Journal of virology* **61**:2896-2901.
186. **Purves, F. C., and B. Roizman.** 1992. The UL13 gene of herpes simplex virus 1 encodes the functions for posttranslational processing associated with phosphorylation of the regulatory protein alpha 22. *Proceedings of the National Academy of Sciences of the United States of America* **89**:7310-7314.
187. **Purves, F. C., D. Spector, and B. Roizman.** 1991. The herpes simplex virus 1 protein kinase encoded by the US3 gene mediates posttranslational modification of the phosphoprotein encoded by the UL34 gene. *Journal of virology* **65**:5757-5764.
188. **Radimerski, T., J. Montagne, M. Hemmings-Mieszczak, and G. Thomas.** 2002. Lethality of *Drosophila* lacking TSC tumor suppressor function rescued by reducing dS6K signaling. *Genes Dev* **16**:2627-2632.
189. **Rahaus, M., N. Desloges, and M. H. Wolff.** 2007. Varicella-zoster virus requires a functional PI3K/Akt/GSK-3alpha/beta signaling cascade for efficient replication. *Cell Signal* **19**:312-320.
190. **Raptis, L., A. L. Boynton, and J. F. Whitfield.** 1986. Protein kinase C promotes the phosphorylation of immunoprecipitated middle T antigen from polyoma-transformed cells. *Biochemical and biophysical research communications* **136**:995-1000.

191. **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. *Journal of virology* **76**:8939-8952.
192. **Rhee, S. G., and Y. S. Bae.** 1997. Regulation of phosphoinositide-specific phospholipase C isozymes. *The Journal of biological chemistry* **272**:15045-15048.
193. **Rhee, S. G., and K. D. Choi.** 1992. Regulation of inositol phospholipid-specific phospholipase C isozymes. *The Journal of biological chemistry* **267**:12393-12396.
194. **Rice, S. A., and D. M. Knipe.** 1990. Genetic evidence for two distinct transactivation functions of the herpes simplex virus alpha protein ICP27. *Journal of virology* **64**:1704-1715.
195. **Rock, D. L., and N. W. Fraser.** 1983. Detection of HSV-1 genome in central nervous system of latently infected mice. *Nature* **302**:523-525.
196. **Rock, D. L., and N. W. Fraser.** 1985. Latent herpes simplex virus type 1 DNA contains two copies of the virion DNA joint region. *Journal of virology* **55**:849-852.
197. **Roizman, B. (ed.).** 1996. In *Fields Virology*, 3 ed. Lippincott-Raven Publishers, Philadelphia.
198. **Roizman, B., and D. Knipe (ed.).** 2001. In *Field's virology* (eds. D.M. Knipe and P.M. Howley), 4th eds. ed, vol. 2. Lippincott, Williams and Wilkins, Philadelphia.
199. **Ryan K.J., R. C. G. (ed.).** 2004. *Sherris medical microbiology an introduction to infectious diseases*, 4th e.d. ed. McGraw Hill, New York.
200. **Ryckman, B. J., and R. J. Roller.** 2004. Herpes simplex virus type 1 primary envelopment: UL34 protein modification and the US3-UL34 catalytic relationship. *Journal of virology* **78**:399-412.
201. **Samaniego, L. A., A. L. Webb, and N. A. DeLuca.** 1995. Functional interactions between herpes simplex virus immediate-early proteins during infection: gene expression as a consequence of ICP27 and different domains of ICP4. *Journal of virology* **69**:5705-5715.
202. **Sano, K., Y. Takai, J. Yamanishi, and Y. Nishizuka.** 1983. A role of calcium-activated phospholipid-dependent protein kinase in human platelet activation. Comparison of thrombin and collagen actions. *The Journal of biological chemistry* **258**:2010-2013.
203. **Sarbassov, D. D., S. M. Ali, D. H. Kim, D. A. Guertin, R. R. Latek, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini.** 2004. Rictor, a novel

- binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Current biology : CB* **14**:1296-1302.
204. **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**:1098-1101.
 205. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B2) in virion infectivity. *Journal of virology* **29**:1149-1158.
 206. **Sawtell, N. M.** 1997. Comprehensive quantification of herpes simplex virus latency at the single-cell level. *Journal of virology* **71**:5423-5431.
 207. **Scheid, M. P., P. A. Marignani, and J. R. Woodgett.** 2002. Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. *Molecular and cellular biology* **22**:6247-6260.
 208. **Sheppard, K., K. M. Kinross, B. Solomon, R. B. Pearson, and W. A. Phillips.** Targeting PI3 kinase/AKT/mTOR signaling in cancer. *Crit Rev Oncog* **17**:69-95.
 209. **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. *The Journal of cell biology* **116**:1273-1281.
 210. **Ship, II, V. J. Brightman, and L. L. Laster.** 1967. The patient with recurrent aphthous ulcers and the patient with recurrent herpes labialis: a study of two population samples. *J Am Dent Assoc* **75**:645-654.
 211. **Ship, II, M. F. Miller, and C. Ram.** 1977. A retrospective study of recurrent herpes labialis (RHL) in a professional population, 1958-1971. *Oral Surg Oral Med Oral Pathol* **44**:723-730.
 212. **Shukla, D., and P. G. Spear.** 2001. Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. *J Clin Invest* **108**:503-510.
 213. **Simmons, A.** 2002. Clinical manifestations and treatment considerations of herpes simplex virus infection. *J Infect Dis* **186 Suppl 1**:S71-77.
 214. **Smalley, M. J., and T. C. Dale.** 1999. Wnt signalling in mammalian development and cancer. *Cancer Metastasis Rev* **18**:215-230.
 215. **Sodeik, B., M. W. Ebersold, and A. Helenius.** 1997. Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *The Journal of cell biology* **136**:1007-1021.
 216. **Soliman, T. M., R. M. Sandri-Goldin, and S. J. Silverstein.** 1997. Shuttling of the herpes simplex virus type 1 regulatory protein ICP27 between the nucleus and

- cytoplasm mediates the expression of late proteins. *Journal of virology* **71**:9188-9197.
217. **Song, G., G. Ouyang, and S. Bao.** 2005. The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med* **9**:59-71.
218. **Soriano, P.** 1997. The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. *Development* **124**:2691-2700.
219. **Spear, P. G., and R. Longnecker.** 2003. Herpesvirus entry: an update. *Journal of virology* **77**:10179-10185.
220. **Spear, P. G., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. *Journal of virology* **9**:143-159.
221. **Speidel, D.** Transcription-independent p53 apoptosis: an alternative route to death. *Trends Cell Biol* **20**:14-24.
222. **Spencer, C. A., M. E. Dahmus, and S. A. Rice.** 1997. Repression of host RNA polymerase II transcription by herpes simplex virus type 1. *Journal of virology* **71**:2031-2040.
223. **Stagno, S., R. F. Pass, G. Cloud, W. J. Britt, R. E. Henderson, P. D. Walton, D. A. Veren, F. Page, and C. A. Alford.** 1986. Primary cytomegalovirus infection in pregnancy. Incidence, transmission to fetus, and clinical outcome. *JAMA* **256**:1904-1908.
224. **Stevens, J. G., and M. L. Cook.** 1971. Latent herpes simplex virus in spinal ganglia of mice. *Science* **173**:843-845.
225. **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**:567-570.
226. **Stroobant, P., and M. D. Waterfield.** 1984. Purification and properties of porcine platelet-derived growth factor. *The EMBO journal* **3**:2963-2967.
227. **Szymkiewicz, I., K. Kowanetz, P. Soubeyran, A. Dinarina, S. Lipkowitz, and I. Dikic.** 2002. CIN85 participates in Cbl-b-mediated down-regulation of receptor tyrosine kinases. *The Journal of biological chemistry* **277**:39666-39672.
228. **Tal-Singer, R., C. Peng, M. Ponce De Leon, W. R. Abrams, B. W. Banfield, F. Tufaro, G. H. Cohen, and R. J. Eisenberg.** 1995. Interaction of herpes simplex virus glycoprotein gC with mammalian cell surface molecules. *Journal of virology* **69**:4471-4483.

229. **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.
230. **Tapon, N., N. Ito, B. J. Dickson, J. E. Treisman, and I. K. Hariharan.** 2001. The *Drosophila* tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell* **105**:345-355.
231. **Tatman, J. D., V. G. Preston, P. Nicholson, R. M. Elliott, and F. J. Rixon.** 1994. Assembly of herpes simplex virus type 1 capsids using a panel of recombinant baculoviruses. *The Journal of general virology* **75 (Pt 5)**:1101-1113.
232. **Taylor, T. J., M. A. Brockman, E. E. McNamee, and D. M. Knipe.** 2002. Herpes simplex virus. *Front Biosci* **7**:d752-764.
233. **Tengelsen, L. A., N. E. Pederson, P. R. Shaver, M. W. Wathen, and F. L. Homa.** 1993. Herpes simplex virus type 1 DNA cleavage and encapsidation require the product of the UL28 gene: isolation and characterization of two UL28 deletion mutants. *Journal of virology* **67**:3470-3480.
234. **Thomas, G., and M. N. Hall.** 1997. TOR signalling and control of cell growth. *Curr Opin Cell Biol* **9**:782-787.
235. **Thoreen, C. C., S. A. Kang, J. W. Chang, Q. Liu, J. Zhang, Y. Gao, L. J. Reichling, T. Sim, D. M. Sabatini, and N. S. Gray.** 2009. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *The Journal of biological chemistry* **284**:8023-8032.
236. **Toker, A., and A. C. Newton.** 2000. Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *The Journal of biological chemistry* **275**:8271-8274.
237. **Triezenberg, S. J., K. L. LaMarco, and S. L. McKnight.** 1988. Evidence of DNA: protein interactions that mediate HSV-1 immediate early gene activation by VP16. *Genes & development* **2**:730-742.
238. **Uprichard, S. L., and D. M. Knipe.** 1996. Herpes simplex ICP27 mutant viruses exhibit reduced expression of specific DNA replication genes. *Journal of virology* **70**:1969-1980.
239. **Vanhaesebroeck, B., and D. R. Alessi.** 2000. The PI3K-PDK1 connection: more than just a road to PKB. *Biochemical Journal* **346**:561-576.
240. **Vanhaesebroeck, B., S. J. Leever, K. Ahmadi, J. Timms, R. Katso, P. C. Driscoll, R. Woscholski, P. J. Parker, and M. D. Waterfield.** 2001. Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem* **70**:535-602.

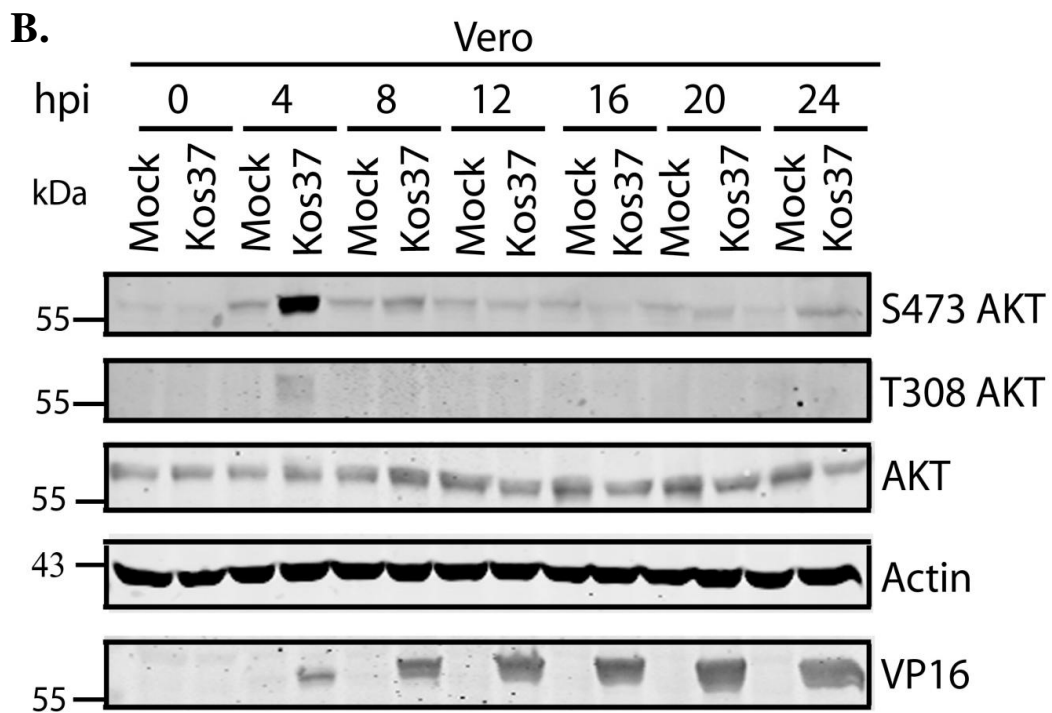
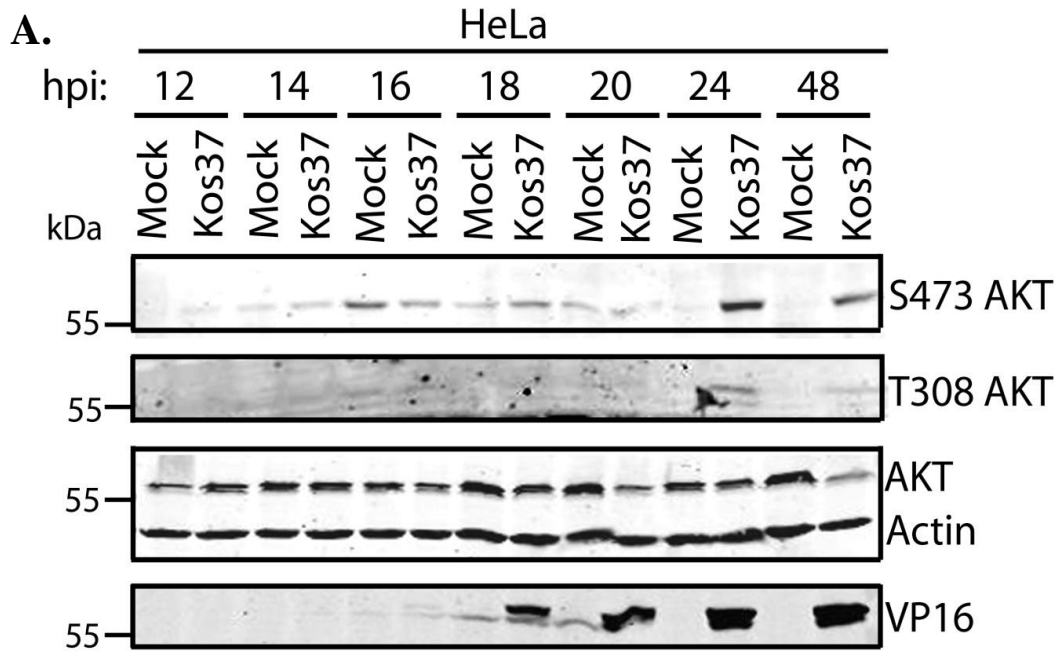
241. **Vanhaesebroeck, B., S. J. Leever, G. Panayotou, and M. D. Waterfield.** 1997. Phosphoinositide 3-kinases: A conserved family of signal transducers. *Trends in biochemical sciences* **22**:267-272.
242. **Vanhaesebroeck, B., S. J. Leever, G. Panayotou, and M. D. Waterfield.** 1997. Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends in biochemical sciences* **22**:267-272.
243. **Vanhaesebroeck, B., and M. D. Waterfield.** 1999. Signaling by distinct classes of phosphoinositide 3-kinases. *Experimental cell research* **253**:239-254.
244. **Virdee, K., P. A. Parone, and A. M. Tolkovsky.** 2000. Phosphorylation of the pro-apoptotic protein BAD on serine 155, a novel site, contributes to cell survival. *Curr Biol* **10**:1151-1154.
245. **Volsky, D. J., and A. Loyter.** 1978. Role of Ca⁺⁺ in virus-induced membrane fusion. Ca⁺⁺ accumulation and ultrastructural changes induced by Sendai virus in chicken erythrocytes. *The Journal of cell biology* **78**:465-479.
246. **von Gise, A., P. Lorenz, C. Wellbrock, B. Hemmings, F. Berberich-Siebelt, U. R. Rapp, and J. Troppmair.** 2001. Apoptosis suppression by Raf-1 and MEK1 requires MEK- and phosphatidylinositol 3-kinase-dependent signals. *Mol Cell Biol* **21**:2324-2336.
247. **Wagner, M. J., and J. R. Smiley.** Herpes simplex virus requires VP11/12 to activate Src family kinase-phosphoinositide 3-kinase-Akt signaling. *Journal of virology* **85**:2803-2812.
248. **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. *Journal of virology* **83**:12452-12461.
249. **Walsh, D., and I. Mohr.** 2004. Phosphorylation of eIF4E by Mnk-1 enhances HSV-1 translation and replication in quiescent cells. *Genes & development* **18**:660-672.
250. **Warner, M. S., R. J. Geraghty, W. M. Martinez, R. I. Montgomery, J. C. Whitbeck, R. Xu, R. J. Eisenberg, G. H. Cohen, and P. G. Spear.** 1998. A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus. *Virology* **246**:179-189.
251. **Watson, R. J., and J. B. Clements.** 1980. A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. *Nature* **285**:329-330.
252. **Watson, R. J., C. M. Preston, and J. B. Clements.** 1979. Separation and characterization of herpes simplex virus type 1 immediate-early mRNA's. *Journal of virology* **31**:42-52.

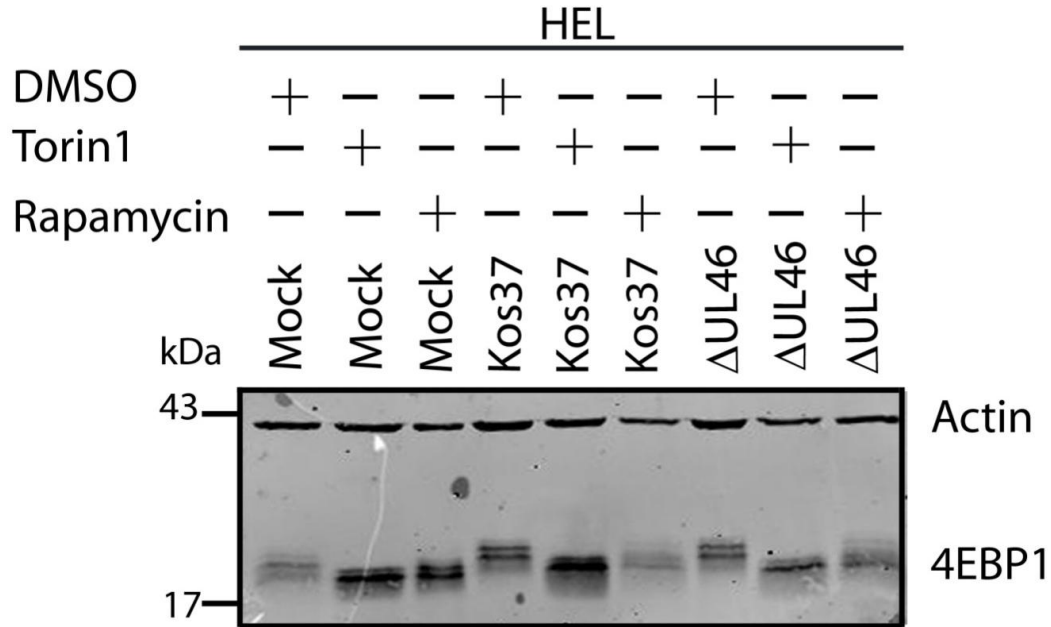
253. **Whitley, R. J. (ed.)**. 1996. Herpesviruses. in: Baron's Medical Microbiology, 4th ed. ed. Univ of Texas Medical Branch, Galveston, Texas.
254. **Whitley, R. J., D. W. Kimberlin, and B. Roizman**. 1998. Herpes simplex viruses. *Clin Infect Dis* **26**:541-553; quiz 554-545.
255. **Whitley, R. J. a. G., J.W. (ed.)**. 1993. The epidemiology and clinical manifestations of herpes simplex virus infections. Raven Press, New York.
256. **Wildy, P., W. C. Russell, and R. W. Horne**. 1960. The morphology of herpes virus. *Virology* **12**:204-222.
257. **Wilkie, N. M., R. Cortini, and J. B. Clements**. 1977. Structural studies and physical maps for the herpes simplex virus genome. *The Journal of antimicrobial chemotherapy* **3 Suppl A**:47-62.
258. **Willard, M**. 2002. Rapid directional translocations in virus replication. *Journal of virology* **76**:5220-5232.
259. **Williams, L. T**. 1989. Signal transduction by the platelet-derived growth factor receptor. *Science* **243**:1564-1570.
260. **Woscholski, R., and P. J. Parker**. 1997. Inositol lipid 5-phosphatases--traffic signals and signal traffic. *Trends in biochemical sciences* **22**:427-431.
261. **WuDunn, D., and P. G. Spear**. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *Journal of virology* **63**:52-58.
262. **Wymann, M. P., and L. Pirola**. 1998. Structure and function of phosphoinositide 3-kinases. *Biochim Biophys Acta* **1436**:127-150.
263. **Xu, Y., K. W. Harder, N. D. Huntington, M. L. Hibbs, and D. M. Tarlinton**. 2005. Lyn tyrosine kinase: accentuating the positive and the negative. *Immunity* **22**:9-18.
264. **Yang, J., P. Cron, V. Thompson, V. M. Good, D. Hess, B. A. Hemmings, and D. Barford**. 2002. Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation. *Mol Cell* **9**:1227-1240.
265. **Yarden, Y., and A. Ullrich**. 1988. Growth factor receptor tyrosine kinases. *Annu Rev Biochem* **57**:443-478.
266. **Yu, D., A. K. Sheaffer, D. J. Tenney, and S. K. Weller**. 1997. Characterization of ICP6::lacZ insertion mutants of the UL15 gene of herpes simplex virus type 1 reveals the translation of two proteins. *Journal of virology* **71**:2656-2665.
267. **Yu, J., C. Wjasow, and J. M. Backer**. 1998. Regulation of the p85/p110alpha phosphatidylinositol 3'-kinase. Distinct roles for the n-terminal and c-terminal SH2 domains. *The Journal of biological chemistry* **273**:30199-30203.

268. **Yu, J., Y. Zhang, J. McIlroy, T. Rordorf-Nikolic, G. A. Orr, and J. M. Backer.** 1998. Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. *Molecular and cellular biology* **18**:1379-1387.
269. **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. *Journal of virology* **82**:6098-6108.
270. **Zhang, X., O. Vadas, O. Perisic, K. E. Anderson, J. Clark, P. T. Hawkins, L. R. Stephens, and R. L. Williams.** Structure of lipid kinase p110beta/p85beta elucidates an unusual SH2-domain-mediated inhibitory mechanism. *Mol Cell* **41**:567-578.
271. **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. *Journal of virology* **67**:1482-1492.
272. **Zhou, Z. H., D. H. Chen, J. Jakana, F. J. Rixon, and W. Chiu.** 1999. Visualization of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions. *Journal of virology* **73**:3210-3218.

APPENDICES

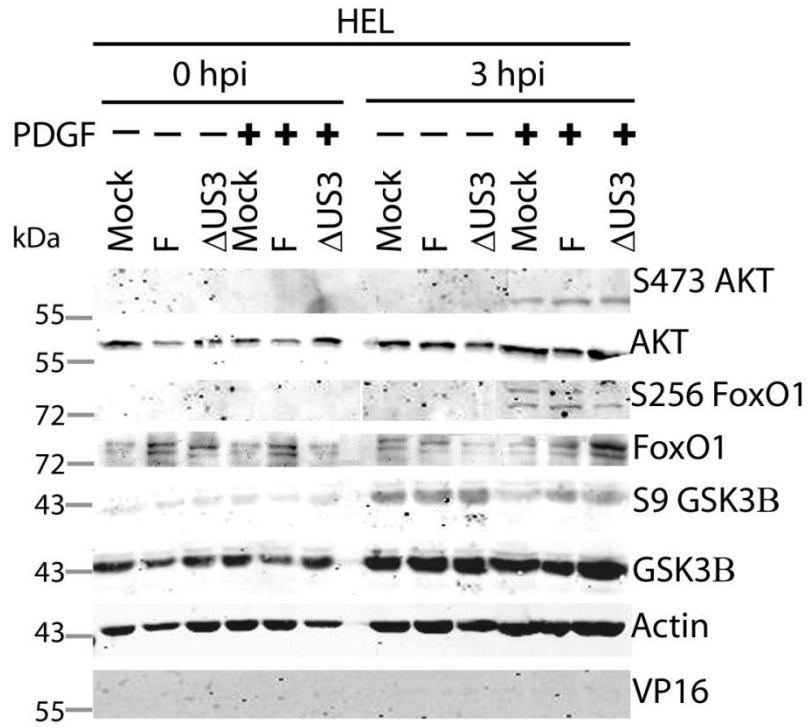
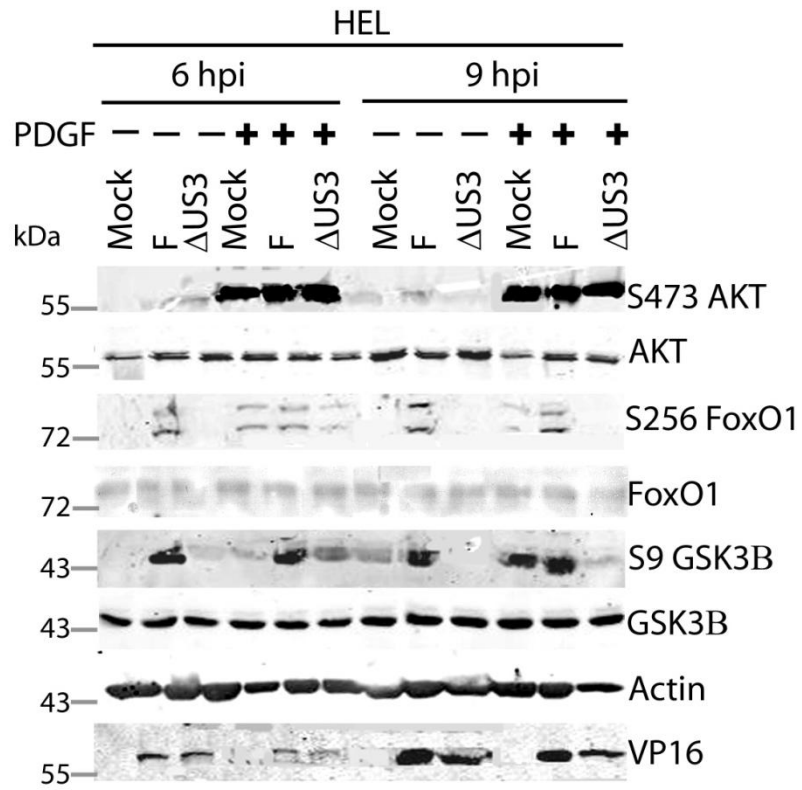
Appendix A: AKT is activated in HSV-1 infected HeLa and Vero cells.
Confluent monolayers of a) HeLa and b) Vero cells were serum-starved and mock-infected or infected with wild type HSV-1 KOS37. Cell lysates were collected at various times and analyzed for the activating phosphorylation of AKT at residues S473 (anti-S473) and T308 (anti-T308), total AKT, VP16, and β -actin by Western blotting.

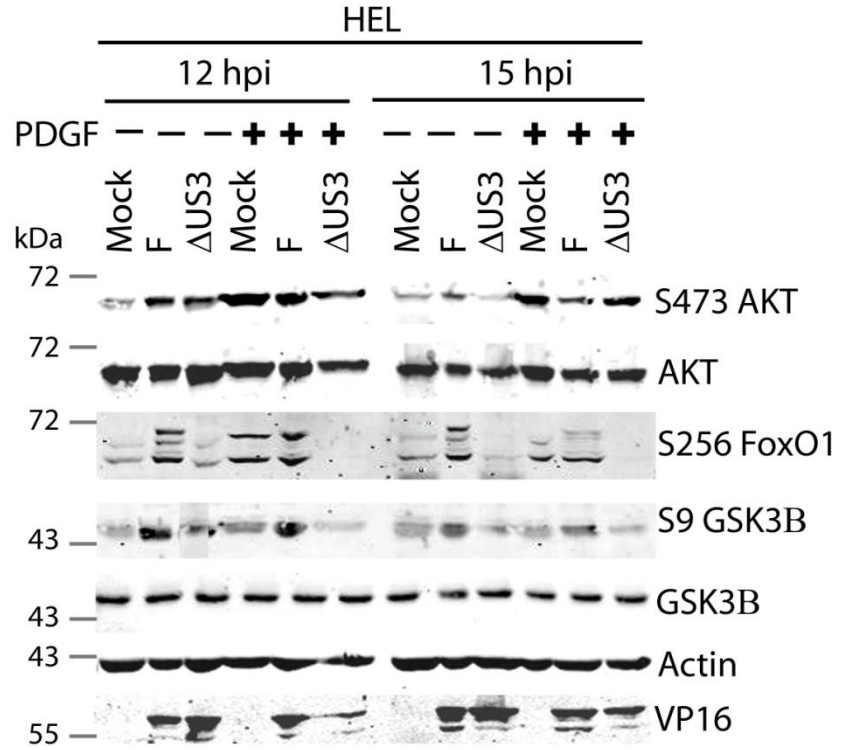
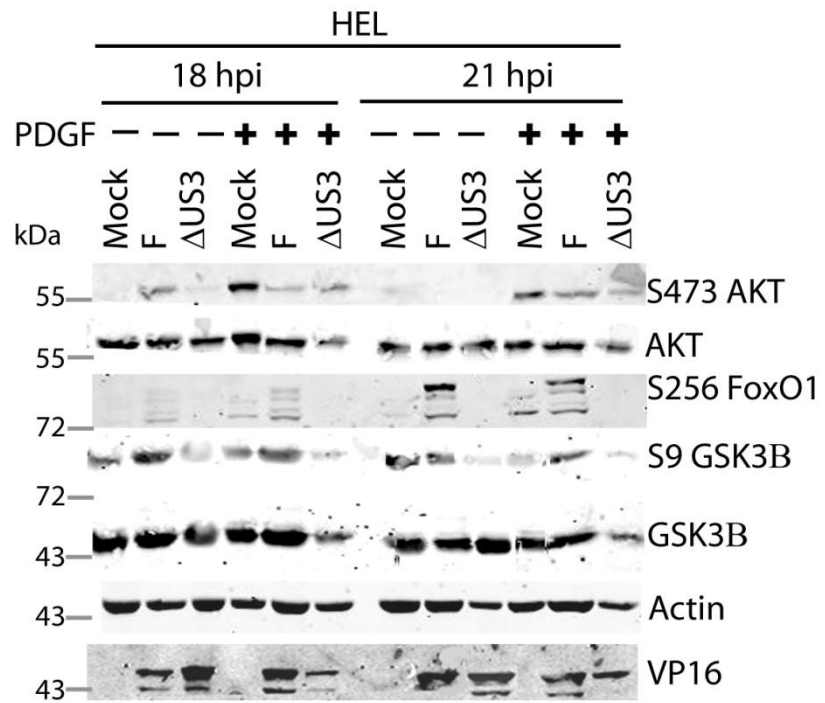




Appendix B: Torin1 and rapamycin reduce 4EBP1 hyperphosphorylated patterns regardless of infection. Confluent monolayers of HEL cells were serum-starved and infected with mock, wild type HSV-1 KOS37, or the VP11/12-null mutant Δ UL46. For the last three hours of infection, cells were either left in serum-free media or treated with 250nM of torin1 or 100nM of rapamycin diluted in 1 mL of serum free DMEM. Cell lysates were collected at 18 hpi and analyzed for 4EBP1 and β -actin by Western blotting.

Appendix C: U_S3 promotes the phosphorylation of AKT targets as early as 6 hpi. Confluent monolayers of HEL cells were serum-starved and mock-infected or infected with wild type HSV-1 F or the U_S3-null mutant. Cell lysates were collected at various times and analyzed for the activating phosphorylation of AKT at residue S473 (anti-S473), phosphorylated FoxO1 and GSK3 β at serine 256 and serine 9 respectively, total AKT, FoxO1 and GSK3 β , VP16, and β -actin by Western blotting.

A.**B.**

C.**D.**

Appendix D: Cells infected with ΔU_S3 - and $\Delta U_L46\Delta U_S3$ - virus are more apoptotic than wild type infection. Using light-microscope analysis to examine cell morphology, cells infected with ΔU_S3 -virus were more apoptotic compared to wild type-infected cells. Cells that were infected with $\Delta U_L46\Delta U_S3$ -virus appeared even more apoptotic than ΔU_S3 -infected cells. These data indicated that U_L46 and U_S3 are potentially involved in preventing apoptosis

