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Søren Ottosen

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PHOSPHORYLATION OF THE TRANSCRIPTIONAL ACTIVATOR VP16 DURING LYTIC INFECTION BY HERPES SIMPLEX TYPE 1

By

Søren Ottosen

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ABSTRACT

PHOSPHORYLATION OF THE TRANSCRIPTIONAL ACTIVATOR VP16 DURING LYTIC INFECTION BY HERPES SIMPLEX TYPE 1

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Phosphorylation of a protein frequently serves to regulate the activity of the protein. Because the understanding of its regulation can be the key to understanding the function of the protein, efforts are often made to map the patterns of phosphorylation. VP16, the activator of the immediate early genes of herpes simplex, type 1, is phosphorylated during infection. This activator is viewed as a key factor not only in activation of the immediate early genes, but also in the control of the virion host shut-off function and in the assembly of virion. Because these functions occur at different stages in infection, it raises the question of how the virus program manages them during infection. Phosphorylation is a likely possibility and the identification of the phosphorylation sites of VP16 during infection could improve our understanding of how herpes simplex bypasses the host cells defense mechanisms and how it recruits the cellular systems to ensure its own survival.

The main question addressed in this thesis was whether specific phosphorylation events were involved in regulation of the function of VP16 during infection. In order to answer that question, it was first necessary to map sites of phosphorylation during infection. The mapping positively identified three sites of phosphorylation (Ser18, Ser353, Ser452) and indicated a fourth (Ser411) as a possible site. A site that had been

reported to be phosphorylated outside of the context of infection (Ser375) was found not to be phosphorylated under the conditions tested.

The functions of the Ser375 and Ser411 in infection were tested. A virus strain with the Ser375Ala mutation displayed a significant reduction of immediate early activation when protein synthesis was inhibited. In contrast, when protein synthesis was allowed, the immediate early gene expression was only marginally affected, as compared to the wild type. The strain did not exhibit discernible deficiencies in virus proliferation, nor did the mutation of the site affect the phosphorylation of VP16 derived from infection by this virus. It was concluded that while the mutation affects one mode of activation by VP16, the IE genes are redundantly activated. The results favors a model in which Ser375 plays a functional role but does not serve as a phosphorylation site, and is therefore probably not as a site of regulation.

Virus strains carrying alanine, glutamate and threonine substitutions at Ser411 were found to be unaffected in proliferation. The strains were also found to support wild type levels of immediate early gene activation, regardless of whether protein expression was allowed in infection or not. The over-all phosphorylation of VP16 from these strains was comparable to wild type. It was concluded that Ser411 was not an essential site for VP16 function in infection, regardless of the phosphorylation state of the site.

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TABLE OF CONTENTS

LIST OF TABLES. iiiv

LIST OF FIGURES ix

KEY TO SYMBOLS AND ABBREVIATIONS x

CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW 1
The herpes Viruses 1
HSV-1 Virion Structure 3
HSV-1 Infection 6
VP16 in HSV-1 infection 11
Transcriptional Activation by VP16 19
Virion Host Shutoff 24
VP16 in Egress and cellular localization 25
VP16 homologs 27
Regulation of VP16 29

CHAPTER TWO
VP16 IS PHOSPHORYLATED IN THE INFECTED CELL AND IN VIRION 31

Introduction 31
Search for phosphorylation sites *in vivo* 32

Results 33
Computer-aided prediction of phosphorylation sites in VP16 33
Confirmation of VP16 phosphorylation in infected cells and in virion 36
Peptide mapping of *in vivo* [^{32}P]-labelled VP16 isolated late in infection using lysylendopeptidase C 38
Peptide mapping of *in vivo* [^{32}P]-labelled VP16 isolated late in infection using trypsin 40
Phosphorylation of VP16 truncation mutants *in vivo* 42
Peptide mapping of the phosphorylation sites of VP16 truncation mutants using lysylendopeptidase C 45
Peptide mapping of the phosphorylation sites of VP16 truncation mutants using trypsin 50
Phosphoaminoacid analysis of VP16 from late in infection 51
Mass spectrometry of VP16 isolated late in infection 54
In vitro phosphorylation of the 375 CK2 site 59

Materials and Methods 64

Discussion 78

CHAPTER THREE
MUTATIONAL ANALYSIS OF THE POTENTIAL PHOSPHORYLATION SITES IN HERPES SIMPLEX INFECTION AT SERINE 375 AND SERINE 411 93
Introduction 93
The function of neither Ser375 nor Ser411 has been adequately described in the context of viral infection. 93
Determining the effect of substitution of Ser375 and Ser411 on the proliferation and transcriptional phenotype in infection 95

Results 95
Function of Ser375 *in vivo* 95
Phosphorylation of the EKC fragment of SJO2 99
Phosphoaminoacid analysis of Ser375Ala 100
Growth kinetics of SJO2 102
IE gene expression assay 104
Transcription of IE genes by VP16-Ser375Ala requires protein translation 107
Phosphorylation and function of Ser411 110
Generation of viral strains carrying point mutations at Ser411 110
Phosphorylation of VP16 lacking Ser411 115
Phosphoaminoacid complement in SO11 116
Growth kinetics of SO11, SO12 and SO13 117
Transcriptional activation of the IE genes by SO11, SO12, SO13 122

Materials and Methods 126

Discussion 131

CHAPTER FOUR
DISCUSSION AND FUTURE DIRECTIONS 143
Hypotheses 143
Future research: Addressing the hypotheses experimentally 147

LIST OF REFERENCES 150

LIST OF TABLES

Table 1: Phosphorylated and non-phosphorylated peptides detected by MS/MS covering Ser18, Ser353, Ser375 and Ser452 57

Table 2: Primers used for generating VP16 point mutations 71

LIST OF FIGURES

Figure 1. VP16 is a likely phosphorylation target 34

Figure 2. VP16 is labeled in the carboxyterminal 80 amino acids 37

Figure 3. Viral strains 44

Figure 4. VP16 is labeled outside of the activation domain 47

Figure 5. VP16 is labeled on serines 53

Figure 6. Peptide sequences identified by LC-MS/MS 56

Figure 7. *In vitro* phosphorylation of the VP16 core domain 62

Figure 8. Phosphorylation site conservation in herpes homologs 85

Figure 9. Alignment of VP16 and the Luman family 86

Figure 10. Loss of Ser375 does not affect the phosphorylation of VP16 98

Figure 11. Loss of Ser375 does not affect the proliferation of HSV-1 103

Figure 12. Loss of Ser375 has marginal effect on activation of the IE genes 106

Figure 13. Loss of Ser375 severely diminishes IE gene activation in the absence of protein synthesis 108

Figure 14. VP16-Ser411Ala, Glu and Thr are expressed and packaged in virion 114

Figure 15. Loss of Ser411 does not affect the phosphorylation of VP16 119

Figure 16. Loss of Ser411 does not affect the proliferation of HSV-1 121

Figure 17. Loss of Ser411 does not affect the activation of IE genes 123

Figure 18. Loss of Ser411 does not affect the activation of IE genes in the absence of protein synthesis 125

Key to Symbols and Abbreviations

aa	Amino acid
AD	activation domain
CAT	chloramphenicol acetyltransferase
CK2	Casein kinase 2
CsTFA	Cesium trifluoroacetate
DBD	DNA binding domain
DE	Delayed early
DMEM	Dulbecco's modified essential medium
FCS	Fetal calf serum
HBM	HCF-1 binding motif
hpi	Hours post infection
HSV-1	herpes simplex, type 1
IE	Immediate early
KDa	kilodalton
LC-MS/MS, MS	In-line liquid chromatography dual mass spectrometry
moi	Multiplicity of infection
Vhs	Virion host shutoff factor
VIC	VP16 induced complex
VP16	virion protein 16

Chapter One

Introduction and literature review

Virion protein 16 (VP16) (vmw65, α-TIF) of herpes simplex type 1 (HSV-1) is a transcriptional activator. Its carboxyterminal activation domain is frequently used in studies of transcriptional activation and in *in vivo* protein interactions because it is active in a variety of cells types and species ranging from yeast to human. In contrast, other activities and domains of the protein have been less well studied. In particular, little is known about regulation the of VP16 function during infection with HSV-1. Phosphorylation of VP16 has been proposed to be part of a regulatory mechanism of VP16, but this has not been shown conclusively. In this thesis, I will examine the function of phosphorylation of VP16 during HSV-1 infection.

The herpes viruses

Herpes viruses are among the largest known viruses. They carry genomes of 150 to 220 kbp, expressing more than 70 proteins in a temporally regulated cascade (Roizman *et al.* 1993). In addition to the proteins encoded by their own genomes, the viruses use factors and organelles from the host cell to produce and package virions. The virion itself is a complex structure, comprising dozens of viral proteins, as well as a membrane that has been scavenged from the host cell. Herpesviruses also must evade the host organisms systemic and cellular immune defense. Herpesviruses have been shown to suppress the interferon response, (Leib 2002) intracellular transport of antigen, (Bauer *et al.* 2002)

mRNA processing (Sandri-Goldin 1994) and the expression of cytokines (Hukkanen *et al.* 2002) to avoid destruction by the host. Yet, in order for the virus to proliferate efficiently, the systems that govern gene expression, protein translation and modification and nuclear transport must be regulated. The complexity of the virus program has allowed the virus to adapt, making the herpesviruses robust viruses with a wide host range, both in terms of cell types affected and in terms of the number of organisms that are susceptible to one or more member of the herpes family (Roizman *et al.* 1993).

This complex interaction with the host cell makes these viruses an attractive research subject. Understanding the general processes of viral infection could lead to therapies against many different herpes-associated diseases. For instance, the human herpes viruses are involved in human diseases ranging from the benignly annoying, like the cold sores caused by herpes simplex type 1 and 2, (Roizman *et al.* 1993) to the deadly, like human herpesvirus 8, which is involved in the development of Kaposi's sarcoma (Gandhi *et al.* 2002). The benefits would not only apply to human diseases. Some herpes viruses can lead to severe losses in agriculture. Marek's disease virus causes $1 billion of losses in poultry farms worldwide, (Suszkiw 2001) due to death of animals or failure to thrive (Payne *et al.* 2000). Similarly, the pseudorabies virus infects and kills pigs, causing equally large losses (Mettenleiter 2000). Understanding the weaknesses in the immune defense that the viruses use to evade detection and destruction could also help in finding therapies against other pathogens.

In addition to understanding the diseases related to herpes infection, the study of cellular responses to infection can help us understand the basic principles of the processes

of the host cell. By understanding how the virus uses these processes, we can also better our understanding of how the host cell normally works, in the absence of infection.

Herpes simplex type I (HSV-1) is often used as a model for herpes viruses. The simplex viruses (HSV-1 and HSV-2) are the most widespread herpes viruses among humans, with most studies finding approximately 70% of US citizens testing positive for antigens against one or the other (Smith *et al.* 2002). The most common effect of HSV-1 infection is cold sores, which are annoying but hardly a major health threat (Roizman *et al.* 1993). But the virus can also give rise to other, more serious and potentially lethal diseases, both in normal and immunosuppressed individuals. Recurring ocular herpes infections cause scarring of the cornea and are the most common cause of corneal blindness worldwide (Liesegang 2001). Genital herpes is most often associated with HSV-2, but can also be caused by HSV-1. Vaginal herpes can affect fertility and can be transferred to infants *in utero* or during birth (Liesegang 2001). Congenital herpes infections can cause severe birth defects such as micro-, hydro- or hydranencephaly, blindness and premature birth (Liesegang 2001). Finally, and quite rarely, herpes can cause herpes encephalitis (Kennedy *et al.* 2002). These infections are difficult to treat and very frequently lethal.

HSV-1 virion structure

Like all herpes viruses, HSV-1 is a double stranded DNA virus. It is classified as a member of the *alphaherpesvirinae* subfamily, based on the structure of its genome. HSV-1 expresses approximately 70 open reading frames from a 152 kbp genome. The viral genome is packaged in the virion particle as linear double stranded DNA. It is

complexed with the polyamine spermine, which neutralizes the charge of the polynucleotide-phosphate backbone and facilitates compact packaging of the DNA within the capsid (Roizman *et al.* 1993). The viral DNA within the capsid is organized in a structure resembling a spool with DNA spaced at 26 Å (Zhou *et al.* 1999; Bhella *et al.* 2000). DNA is packaged into a preformed pro-capsid by the virally encoded factors U_L6, U_L15, U_L17, U_L25, U_L28, U_L32, and U_L33 (White *et al.* 2003), some of which remain associated with the mature virion.

The mature capsid is an 1,250 Å eicosahedral protein structure, comprising VP5, VP26, VP23 and VP19C. The primary structural capsid component is VP5. VP5 forms structures known as pentons and hexons. These are five- and six sided multimers of VP5, which form the superstructure of the capsid. VP5 protrudes outwards and interacts with the tegument (Zhou *et al.* 1999; Bowman *et al.* 2003). VP5 is involved in capsid maturation (Warner *et al.* 2000; Desai *et al.* 2003). VP23 and VP19C form structural parts of the eicosadedron and are essential for capsid assembly. VP5 interacts directly with the VP26 protein (Desai *et al.* 2003). This interaction is ATP-dependent *in vitro* (Chi *et al.* 2000). The function of VP26 is unknown and it is dispensable for virus proliferation (Desai *et al.* 1998). The structure and assembly of the capsid is reviewed in (Homa *et al.* 1997) and in (Chiu *et al.* 2002).

The tegument is the compartment between the capsid and the envelope. The mature virion carries a number of viral proteins in this space. These proteins are delivered to the host cells during initial infection. The tegument does not appear to be organized, except for a limited interaction with VP5 of the capsid. The major proteins of the tegument are VP16, VP22, VP13-14, VP1-2, the product of the US11 gene and the

virion host shut-off factor (vhs). These factors are all involved in events occurring during the initial stages of infection. Between 500 and 1000 copies of VP16 are present in the tegument (Heine *et al.* 1974). The roles of VP16 in HSV-1 infection, including those associated with the formation of the virion will be addressed later in this chapter. Vhs is involved in degradation of mRNA early in infection. It has been suggested that interaction of vhs with VP16 inhibits the activity of vhs later in infection (Knez *et al.* 2003). This interaction will be also addressed later in this chapter. The role of VP13/14 is less well understood. It is dispensable for growth but may be involved in activation of the IE genes (Preston *et al.* 1984; McKnight *et al.* 1987b; Zhang *et al.* 1991). A spontaneous viral mutant, in which the genes for VP16 and VP13/14 have fused into one open reading frame appears to have no growth defect (McKnight *et al.* 1994) VP1-2 is an essential protein, with a role in virion maturation and egress (Desai 2000) and may interact with the capsid protein VP5 (McNabb *et al.* 1992) US11 is an RNA binding protein (Schaerer-Uthurralt *et al.* 1998) that is associated with the 60S proteasome particle(Roller *et al.* 1996). and is involved in the viral repression of cellular response to dsRNA (Poppers *et al.* 2000). VP22 is also an RNA binding molecule. It interacts directly with VP16 (Elliott *et al.* 1995) and is capable of migrating between cells (Elliott *et al.* 1997). It may also be involved in capsid maturation .

Recent reports have suggested that mRNA is also packaged in the virion of HSV-1 as well as other herpes viruses (Bresnahan *et al.* 2000; Greijer *et al.* 2000; Sciortino *et al.* 2001; Sciortino *et al.* 2002). The transport of these RNA molecules may occur through the RNA binding proteins in the capsid. It can be demonstrated that the mRNAs

that are transported become expressed in the host cell, but the function of the mRNAs remain unclear.

The virion is enveloped by a lipid bilayer membrane. The lipid composition of this envelope is consistent with that of membranes of the ER, the Golgi apparatus or another cytoplasmic membrane (van Genderen *et al.* 1994). In the mature virion, it contains a number of viral glycoproteins, including gD which has been shown to be required for in cell surface binding and entry of the virion into the host cell. The entry into the cell is mediated by the HveA/HVEM receptor. The envelope is shed during entry at the plasma membrane (Roizman *et al.* 1993). Three other glycoproteins, gB, gH, gL are essential for membrane fusion during infection, although the mechanism is not understood. Mutations in a fifth glycoprotein, gK, causes the formation of syncytial infection. During these infections, infected cells fuse with adjacent cells, forming large syncytia (Hutchinson *et al.* 1993; Dolter *et al.* 1994).

HSV-1 infection

HSV-1 infects most epithelial cells and neuronal cells in its natural host. Infections in epithelial cells typically result in a lytic infection, leading to generation of progeny virus and to the destruction of the infected cell. Viruses infecting neuronal cells typically remain latent until reactivated by a trigger event (Roizman *et al.* 1993).

Lytic infection

The lytic pathway follows a typical viral gene expression cascade. The genes required for the lytic pathway of the virus are expressed sequentially in three major

categories defined as the immediate early (IE) genes, the delayed early (DE) genes and the late (L) genes. The expression of each set of genes is activated by viral transcription factors and is regulated according to specific checkpoint events (Roizman *et al.* 1993).

The key events in the IE stage are the entry of the virus into the host cell and the activation of the IE genes. During entry, the tegument proteins are released into the cytosol and the capsid migrates to a nuclear pore complex and deposits the viral genome into the nucleus. Injection of DNA from the capsid into the nucleus early in infection is thought to occur through the nuclear pore complex in a manner requiring GTP (Sodeik *et al.* 1997; Ojala *et al.* 2000). VP16 is the primary activator of the IE genes during lytic infection (Campbell *et al.* 1984; McKnight *et al.* 1987a; Triezenberg *et al.* 1988b). The immediate early proteins are ICP0, ICP4, ICP22, ICP27 and ICP47. These proteins are part of the initial steps of infection and they mount a very intricate and very efficient subversion of the host cell defense system.

ICP0 is a transcriptional activator which has been shown to activate the IE genes, although no canonical response element has been identified. It is a RING-finger protein and the RING-finger is required for transcriptional activation. In the absence of VP16 activation, ICP0 is required for expression of the IE genes (Mossman *et al.* 1999). This has led to the suggestion that ICP0 belongs in a temporal class of its own, that might be called pre-IE (Mossman *et al.* 1999). It is also involved in the dispersion of certain nuclear structures know as ND10s or PML nuclear bodies (Maul *et al.* 1993; Everett *et al.* 1994) and can act as an E3 ubiquitin ligase (Van Sant *et al.* 2001). ICP0 is capable of ubiquitinylating p53 (Boutell *et al.* 2003). One hypothesis proposes that the deletion of ICP0 reduces the infectivity of HSV-1 by lowering the probability of initiation of lytic

infection. In the absence of ICP0, the infecting virus is far more likely to enter a latent state than in the presence (Everett 2000). This phenotype can be overcome by infection at a high MOI or by expressing wild type ICP0 from a separate promoter.

ICP4 is also a transcriptional activator, required for the expression of genes of all three classes. Initially in infection, ICP4 is diffusely distributed throughout the nucleus, but later in infection, it is found in nuclear structures adjacent to ICP0-containing structures (Everett *et al.* 2003). ICP4 acts as a transcriptional repressor on a few promoters, including its own and that of ICP0 (Lium *et al.* 1996). ICP4 is an essential gene.

ICP22 is not an essential protein in infection of cultured cells (Ogle *et al.* 1999). Some evidence suggests that ICP22 represses the expression of the IE genes, (Prod'hon *et al.* 1996) but the most significant effect of deleting the ICP22 gene is the loss of expression of DE and a subset of the L genes (Sears *et al.* 1985; Purves *et al.* 1993). ICP22 appears to affect transcription by changing the phosphorylation pattern of the CTD of RNAPII. The virally encoded kinase U_L13 is also involved in this function (Rice *et al.* 1995; Long *et al.* 1999).

ICP27 is involved in the nuclear export of the intron-less HSV-1 mRNAs, using the TAP export pathway (Chen *et al.* 2002). ICP27 inhibits host cell mRNA splicing, thereby preventing expression of the bulk of host mRNA (Sciabica *et al.* 2003). It is also involved in expression of certain L genes. ICP27 is required for efficient viral DNA replication (McMahan *et al.* 1990). It is an essential protein.

ICP47 is involved in avoidance of the host organisms immune response. It prevents T-cell antigen transport and thereby prevents triggering the immune response (Bauer *et al.* 2002).

The replication of the viral genome begins during the DE stage of infection. The genes required for the replication, including the viral DNA polymerase, belong to the DE group of genes. Although the recruitment of the VP16 activation domain to replication origins in heterologous systems, such as polyoma, papilloma and baculovirus (Baru *et al.* 1991; Bennett-Cook *et al.* 1991; Li *et al.* 1993; Pathakamuri *et al.* 2002) can facilitate replication, loss of VP16 binding sites at the HSV-1 origin of replication does not affect HSV-1 replication (Nguyen-Huynh *et al.* 1998).

The L genes are expressed after the replication of the viral genome has begun. This group of genes is sometimes divided into two sub-groups termed leaky late and true late (Roizman *et al.* 1993). The leaky late genes are expressed regardless of whether viral DNA synthesis takes place. The true late genes do not get expressed unless the viral genome is replicated. These genes include the structural genes of the capsid and the glycoproteins of the membrane. They also include the tegument proteins, including VP16, VP22 and vhs.

Expression of a number of host genes have been shown to increase during infection with both wild-type HSV-1 and with vhs, icp0 and VP16 mutants. The expression of these genes may be the result of the cellular stress response. In the absence of protein expression during infection, a group of interferon-responsive genes are turned on (Mossman *et al.* 2001). Similar genes are also expressed in infection with virus strains lacking VP16 and ICP0. The expression of these genes is part of a host defense

and would lead to destruction of both the virus and the cells that have been infected. How the wild type virus inhibits the expression of those genes and prevents apoptosis of the host cell remains to be determined (Khodarev *et al.* 1999; Taddeo *et al.* 2002).

Latency

HSV-1 that infects neuronal cells enters latency. HSV-1 typically infects the trigeminal ganglia, but is also found in other sensory ganglionic neurons (Roizman *et al.* 1993; Liesegang 2001). During latency, the viral genome exists as a nuclear episome (Su *et al.* 2002) but the transcription of viral genes is largely repressed. The only HSV-1 RNA that is expressed from the latent HSV-1 genome is the latency associated transcript (LAT) (Roizman *et al.* 1993). The LAT transcript is partially complementary to the ICP0 gene and was initially thought not to be translated. This prompted the notion that the LAT was an antisense repressor of ICP0, but the hypothesis was recently refuted (Burton *et al.* 2003). Other data suggest that the LAT transcript is translated, and that the gene products are present in the latently infected cell (Thomas *et al.* 1999; Thomas *et al.* 2002). They accumulate in the periphery of HSV-1 replication compartment and may be important for both the establishment of and the emergence from latency.

HSV-1 emerges from latency as a result of extracellular stress. Stressors include heat shock and UV irradiation (Roizman *et al.* 1993). Luman, a cellular protein that interacts with HCF-1 in a manner similar to VP16, seems to enhance the establishment of latency, possibly by sequestering HCF-1 in the cytoplasm or by activating the LAT gene (Lu *et al.* 2000a). The protein is also able to activate ICP0 expression. This suggests that Luman is also involved in the emergence from latency (Lu *et al.* 2000a). Also, deletion

of VP16 itself does not seem to affect establishment of latency or reactivation (Steiner *et al.* 1990). However, the exogenous expression of not only VP16, but also of ICP0 or ICP4 will activate a latent virus in trigeminal ganglia (Halford *et al.* 2001).

The HSC-1 genome may exist in several forms in the infected cell. Some reports suggest that it exists as naked DNA, others that it is packaged as nucleosomes. Micrococcal nuclease digestion of viral DNA from latent infection suggest that it is predominantly nucleosomal (Deshmane *et al.* 1989). In contrast, micrococcal nuclease digestion of lytic infection has shown that the viral genome is predominantly non-nucleosomal (Leinbach *et al.* 1980; Lentine *et al.* 1990).

VP16 in HSV-1 infection

VP16-mediated activation of the IE genes. The IE genes all have three upstream regulatory elements in common, TAATGARAT elements, SP1 elements and GA elements (Kristie *et al.* 1984; Jones *et al.* 1985; Kristie *et al.* 1986; Triezenberg *et al.* 1988a). The TAATGARAT element (R signifies a purine) is capable of regulating the IE genes (Gaffney *et al.* 1985). It is also capable of binding VP16 in the context of Oct-1 and HCF-1 (Kristie *et al.* 1989; Stern *et al.* 1989). Reviewed in. (Herr 1998; Wysocka *et al.* 2003b).

In addition to the TAATGARAT, the IE promoters have at least two more *cis* regulatory elements in common, the GA-box and the Sp1 binding site. Several published results hint to a function of VP16 through these elements. The GA-element is involved in VP16-mediated activation (Triezenberg *et al.* 1988b) as well as interact with HCF-1 in the cell (Vogel *et al.* 2000). Sp1 also interacts with HCF-1 *in vitro*, (Gunther *et al.* 2000)

and the deletion of Sp1 sites from the IE promoters affect expression (Jones *et al.* 1985; Gu *et al.* 1993).

VP16

The factor responsible for the transcriptional activation of the IE genes during lytic infection is VP16. VP16 is a 490 aa protein. VP16 belongs to the leaky late group of HSV-1 genes. It is expressed late in infection, but does not require the replication of the viral genome to be expressed. While VP16 is not strictly an essential gene, deletion of the VP16 locus in the HSV-1 strain 8MA does cause a severe reduction in proliferation. Curiously, the strain does not appear to have any difficulties in replicating its genome, (Weinheimer *et al.* 1992) suggesting that the strain is capable of expressing viral genes required for replication. The phenotype of the null mutant is attributed to defects in making mature virions. (Weinheimer *et al.* 1992; Mossman *et al.* 2000). In the absence of VP16, empty capsids accumulate within the perinuclear space. Transcriptional activation does not appear to be essential for viability, as viruses with mutations in VP16 that reduce activation of the IE genes are viable, albeit with reduced infectivity (Ace *et al.* 1989; Smiley *et al.* 1997; Tal-Singer *et al.* 1999).

The VP16 protein is typically divided into two major domains. The core domain lies between amino acids 49 and 410 (Greaves *et al.* 1990; Liu *et al.* 1999). The transcriptional activation domain AD resides at the carboxy terminus (aa 410-490) (Triezenberg *et al.* 1988a; Werstuck *et al.* 1989). Specific functions have been defined for each of these domains. VP16 does not have a traditional nuclear localization signal. It appears to be transported to the nucleus through its interaction with HCF-1 (La

Boissiere *et al.* 1999). The shuttling of this factor in and out of the nucleus by a factor called HPIP may be crucial for the transport of VP16 to the nucleus (Mahajan *et al.* 2002).

The VP16 induced complex

Because VP16 does not have a DNA-binding domain, VP16 relies on a complex comprising, in addition to VP16, the ubiquitous transcriptional activator Oct-1 (Stern *et al.* 1989) and HCF-1, a putative cell cycle regulator (Stern *et al.* 1989; Wilson *et al.* 1993; Wilson *et al.* 1995a). Oct-1, a member of the POU family of transcription factors, is a 100 KDa protein capable of activating both RNAPII and RNAPIII genes (Wysocka *et al.* 2003a). Oct-1 activates responsive genes through the regulatory element ATGCAAAT, also known as the octamer element.

Oct-1 interaction

The minimal domain of VP16 required for interaction with Oct-1 is aa 49-388 (Greaves *et al.* 1990). While the entire domain is required for the interaction, the interaction surface appears to be much smaller. Single aa substitutions at aa 331, 371, 373 and 375 abolish interaction between VP16 and Oct-1 but do not affect interaction with HCF-1 (Greaves *et al.* 1990; Lai *et al.* 1997). The Oct-1 POU homeo domain is sufficient for the interaction with VP16 (Kristie *et al.* 1989; Stern *et al.* 1989). Specific mutations of the homeo domain map the interaction surface of Oct-1 to the solvent exposed groove between helix 2 and helix 3 of the homeo domain (Lai *et al.* 1992; Pomerantz *et al.* 1992). A glutamic acid within helix 2 is critical for the interaction, as

the replacement of it abolishes interaction with VP16. This model is supported by the observation that VP16 does not bind Oct-2 (Arnosti *et al.* 1993) or Oct-1 from the mouse (Suzuki *et al.* 1993). Neither of these homologs of Oct-1 have a glutamic acid at this critical position. The spacing of the hydrophobic residues suggests that aa 370-375 may form an amphipathic α-helix. The groove between these two helices is lined with hydrophobic residues that may interact with the hydrophobic stretch of VP16 between residue 375 and 385.

The interaction between Oct-1 and aa 370-375 of VP16 has been suggested to be similar to the one that occurs between the two POU proteins MATa1 and MATα2 from *S. cerevisieae* (Li *et al.* 1995; Lai *et al.* 1997). The crystal structure of this dimer bound to its canonical DNA binding element has been solved (Li *et al.* 1995; Li *et al.* 1998). It clearly reveals the formation of a distorted helix of MATα2 that interacts with the back side of the MATa1 POU homeodomain. Additionally, the segment of this helix of MATα2 can be replaced with the corresponding segment of VP16, suggesting that they work in the same manner (Stark *et al.* 1999). Indeed, an alignment of the two motifs reveal that the pattern of critical hydrophobic residues is conserved. A noticeable difference is that the MATα2 helix is initiated by a proline residue, a very common residue to occur at the N1-position of the helix. In this model, the proline residue aligns with Ser375 of VP16. Serines are also favored in the amino termini of helices, (Doig *et al.* 1995) particularly when followed by an acidic residue two or three positions carboxyterminal (Harper *et al.* 1993; Doig 2002). This motif, incidentally, forms a casein kinase 2 consensus site (S/TxxD/E). Additionally, the presence of a phosphoserine at the N-terminus of an α-helix favors the formation of the helix (Andrew

et al. 2002). This all favors the model that the Oct-1-VP16 interaction is similar to the interaction between Mata1 and Matα2. It also raises the possibility that the interaction can be regulated by phosphorylation of VP16.

HCF-1 interaction

The other interaction partner of the VIC complex is HCF-1 (also known as C1 or VCAF). HCF-1 is a very large protein of more than 2000 amino acid residues (Wilson *et al.* 1993). The protein undergoes proteolytic cleavage after translation to produce two non-covalently linked subunits (Wilson *et al.* 1993; Wilson *et al.* 1995b). These subunits remain tightly bound, despite the cleavage. The N-terminus of the protein (aa 1-380) consists of a number of kelch or WD40 repeats. These repeats form a structure known as a β-propeller (Adams *et al.* 2000). Initially, HCF-1 was identified as a necessary cellular function for the interaction between VP16, Oct-1 and DNA (Katan *et al.* 1990; Kristie *et al.* 1990; Xiao *et al.* 1990; Stern *et al.* 1991). This function was mediated by a series of related peptides, all expressed from one gene (Wilson *et al.* 1993).

HCF-1 was identified independently in a screen that selected for temperature sensitive cell cycle defects in BHK cells (Goto *et al.* 1997). A serine to proline mutation at residue 134 (Ser134Pro) of HCF (tsBN67) caused the cell to enter cell cycle arrest at the non-permissive temperature (39.5°C). The HCF-1-VP16 interaction was found to be sensitive to the Ser134Pro mutation and did not allow formation of the VIC complex (Goto *et al.* 1997; Wilson *et al.* 1997). Ser134 lies within the kelch repeats and is conserved between the repeats. In the proposed β-propeller, this residue falls within a solvent exposed loop between two β-strands. The interaction surface of VP16 for HCF-1

was mapped by peptide competition and by mutagenesis (Shaw *et al.* 1995; Lai *et al.* 1997; Simmen *et al.* 1997). Two small motifs were identified, comprising aa 360-366 (REHAYS) and aa 385-387 (DDD). Mutations in these sites abolished VIC formation by disrupting the interaction between VP16 and HCF-1 specifically but it did not appear to affect the Oct-1 interaction. The cellular proteins Luman, Zhangfei (Zf) and HPIP were later found to interact with HCF-1 through a similar motif (Lu *et al.* 1997; Lu *et al.* 1998; Lu *et al.* 2000b; Mahajan *et al.* 2002). Luman and Zf are members of the basic leucine zipper family, although their activities in the cell are not known. HPIP is a nuclear shuttle factor that has a nuclear export signal and appears to shuttle HCF-1 between the nucleus and the cytoplasm. The minimal consensus for the interaction between Luman, Zf or HPIP and HCF-1 was determined to be E/DHxY. This was termed the HCF-1 binding motif (HBM). Luman, Zf and HPIP also interact with HCF-1 in a manner that is sensitive to mutations at residue 134. The three aspartates at aa 385-387 of VP16 are not conserved in the Luman, Zf and HPIP. Other mutations in HCF-1 allow the protein to maintain interaction with VP16 and Luman homologs, yet still cause cell cycle arrest (Mahajan *et al.* 2000). This suggests that the loss of interaction between HCF-1 and Luman is not required for cell cycle control. HCF-1 is present in most cell types with the exception of brain tissue (Wilson *et al.* 1995a). In cells that express HCF-1, it is mostly found to be nuclear with a notable exception of cells from trigeminal ganglia (Kristie *et al.* 1999). When these cells are subjected to stress, similar to that which can re-activate latent herpes genomes, HCF-1 rapidly changes cellular localization from the cytoplasm to the nucleus. This suggests that the nuclear localization of HCF-1 could be part of the triggering mechanism for reactivation. Dawn Greensides has shown that VP16

localization in the tsBN67 cell line is dependent on the localization of HCF-1 (Greensides 2002). At permissive temperatures, when HCF-1 is in the nucleus, VP16 is also found in the nucleus. At non-permissive temperatures for tsBN67, both VP16 and HCF-1 were found in the cytoplasm.

The VP16 Activation Domain

The activation domain of VP16 belongs to a group of activation domains known as acidic activation domains (Hope *et al.* 1988). In VP16, the carboxyterminal activation domain (aa 410-490) can be further divided into two subdomains known as the N-subdomain (410-454) and the C-subdomain (455-490) (Regier *et al.* 1993). The VP16 activation domains were found to be very potent activators when fused to a heterologous DNA binding domain (DBD) (Sadowski *et al.* 1988). Each of the two subdomains of this activation domain is centered on a cluster of hydrophobic residues, one or more of which are critical for transcriptional activation mediated by that domain (Cress *et al.* 1991; Sullivan *et al.* 1998).

In addition to the carboxy terminal activation domain, some evidence suggests the presence of a small activation domain in the very amino terminus of the protein. This was discovered by sequence alignment to VP16 homologues from varicella zoster virus, (Moriuchi *et al.* 1995) another herpes virus. Domain swapping experiments were conducted in yeast and this domain, while fully competent to function in the context of a DNA-binding domain, is not as potent as the carboxyterminal domain of VP16. It can be removed from VP16, without affecting the activation potential of VP16 in transfection assays (Triezenberg *et al.* 1988a). Yet, it is quite well conserved in the VP16 family and

whether it is a significant part of the VP16 activation function during any aspect of the HSV-1 lifecycle remains to be seen.

VP16 protein structure

The structure of the core domain of VP16 (aa 49-410) was determined by x-ray crystallography (Liu *et al.* 1999). This structure reveals a protein centered on a scaffold made by two extended α-helices, forming a large X. The remainder of the structural elements, mainly shorter α-helices are arranged around this scaffolding in a structure that resembles a chair. A stretch of DNA, adjacent to the response element, is proposed to lie across the seat of this chair, with the other members of the VIC complex attached to the side. Significantly, the domain of VP16 that contact both Oct-1 and HCF-1 is unstructured in the crystal. Small angle X-ray scattering (SAXS) studies of full length VP16 shows agreement with the crystal structure (Grossmann *et al.* 2001). It shows a large central, kidney shaped molecule, with a wing on each side,. The dimensions of the structure is in agreement with the crystal structure. The wings could correspond to the activation domain and the VIC interaction domain, respectively.

The activation domain of VP16 was found to exist as a random coil in solution (O'Hare *et al.* 1992). There was evidence that the domain did adopt some degree of helicity when interacting with $TAF_{II}32$ (Shen *et al.* 1996a; Shen *et al.* 1996b; Uesugi *et al.* 1997). This method of interaction led to a model termed the "induced fit". In this model, the VP16 activation domain adopts a structure only when interacting with its target. The hypothetical strength of this mode of interaction is the ability to use a single module to interact with a wide variety of targets.

Transcriptional Activation by VP16

The prevalent model for the function of transcriptional activators like VP16 is that they recognize a promoter element and then recruit other transcription factors to the promoter. These factors in turn bring activities to the promoter that may be directly involved in the generation of the RNA transcript or may facilitate formation of a transcription complex by altering the promoter structure and environment. VP16 has been shown to interact with factors from several of classes of transcription factors. These can be broadly defined as general transcription factors, covalent histone modification factors, such as histone acetyl transferases, and nucleosome remodeling complexes. In addition to these interactions, there is emerging evidence for involvement of parts of the proteasome and the ubiquitinylation pathway.

The general transcription factors act directly on the RNA polymerase, by facilitating formation of the pre-initiation complex, initiation, promoter clearance or elongation. In short, they are part of the physical mechanism of transcription. They are required for accurate initiation by RNAP II *in vitro* (Orphanides *et al.* 1996). They may act as recruitment devices or nucleation devices for the RNA-polymerase holoenzyme. For a review of the activities of the general transcription factors in transcriptional activation, see (Woychik *et al.* 2002).

Both the covalent histone modifiers and the nucleosome remodeling systems work on the nucleosomal template. The covalent histone modifiers add or remove substituents from histones. The initially discovered mechanism was acetylation of the histones, but also phosphorylation, methylation and ubiquitinylation are part of the modifications that take on histones. See (Berger 2002) for a review. Modification of histones affect gene

expression differentially, depending on the promoter context, even identical modifications can have starkly different effects on different genes. The variety of modifications that may occur at any given histone, as well as the gene specific effect of a particular modification has led to the model of the histone code, where it is not the individual modification *per se*, that regulates a promoter, but the combinatorial effect of multiple modifications. This model seeks to explain how all genes in an organism can respond differently in response to stimuli, even though only a handful of modifications takes place.

The last group of factors are the nucleosome remodeling complexes. These ATP-dependent complexes are capable of rearranging the local nucleosome environment on a given promoter in response to a transcription factor. The mechanism of the molecular machines are not fully understood, although it has been suggested that they have a helicase activity. For a review of the mechanisms of these factors, see (Narlikar *et al.* 2002).

It is clear that the activation of a particular gene does not solely rely on activation through one of these mechanisms. In fact, all three groups may be recruited to an activated promoter. The sequence by which this recruitment occurs appears to differ between different promoters and is obscured by the fact that some complexes have factors from more than one group, An example is the TAFs that are present in both the TFIID as well as in HAT-containing complexes, such as the SAGA complex (Grant *et al.* 1998). The ability of an activator to recruit factors from more than one of these groups of factors may be key to its strength.

VP16 has been shown to interact with several of the general transcription factors. These include TFIIA, TFIIB, TFIID and TFIIH. The prevalent model proposes that the recruitment on one or more of these factors by VP16 serves to nucleate the assembly of a transcriptional pre-initiation complex. This model is reviewed in (Woychik *et al.* 2002) and (Ptashne *et al.* 1997). An example of how this could occur is the interaction of VP16 with TBP, the central factor of TFIID, is well established (Shen *et al.* 1996b; Nedyalkov *et al.* 2003). This interaction appears to be mediated through a surface of TBP that is also required for TBP dimerization and TBP-DNA interaction (Nishikawa *et al.* 1997). This surface can also interact with $TAF_{II}250$ (Liu *et al.* 1998; Bagby *et al.* 2000). Several interesting models have been created to resolve the problem of having multiple partners interact through the same surface. Common to these models is that they suggests that the interactions occur sequentially. VP16 could act both by recruiting TBP/TFIID to the promoter and by disrupting either TBP-dimerization or TBP-$TAF_{II}250$ interaction, both of which inhibit TBP-DNA interaction. The interaction between VP16 and TBP is subsequently disrupted by the formation of a more favorable DNA-TBP complex, which in turn forms the basis for the formation of the preinitiation complex. The events that regulate the balance between binding affinities of the various interactions in TBP-recruitment are under investigation (Liu *et al.* 1998; Bagby *et al.* 2000; Pereira *et al.* 2001; Kobayashi *et al.* 2003). (See (Kobayashi *et al.* 2003) for an overview of several models of the mechanism of activation by VP16 through TBP/$TF_{II}D$.)

The VP16 activation domain is a strong activator in yeast (Sadowski *et al.* 1988). The activation domain has been found to rely on certain factors for activation. Early on it was found that VP16 was completely dependent on the ADA complex, some members of

which were Ada2, Ada3 and Gcn5 (Berger *et al.* 1992; Marcus *et al.* 1994; Barlev *et al.* 1995; Candau *et al.* 1997). It was clear that VP16 interacted directly with the complex, and furthermore, not all activators were dependent on it. The discovery that Gcn5 was a histone acetyl transferase (Brownell *et al.* 1996) added another layer of complexity to the model, but also provided the model with an activity that could physically facilitate the activation of transcription (Kuo *et al.* 1996; Kuo *et al.* 1998).

VP16 has also been found to recruit chromatin remodeling factors. This observation was initially considered a little curious, because the HSV-1 genome was reported not to be nucleosomal. It is possible that these factors have different functions on naked DNA, or it is possible that HSV- DNA is nucleosomal.

Phosphorylation is a common mechanism of regulation in transcription.

The regulation of a function by affecting interaction between factors required for a process is conceptionally quite simple. The function can be regulated by altering the strength of the interaction between the factors and this can be achieved by modifying the interaction surface in some manner. Covalent modification of one or both of the interaction surfaces can positively or negatively affect the interaction. There are many examples of this taking place in transcription. In the following examples, the modification is phosphorylation, but other modifications, such as acetylation or methylation, are also commonly found in transcriptional regulation.

An example of regulation of a transcription factor by phosphorylation is that of p53. Here, the phosphorylation serves to release the transcription factor from a inhibitor, MDM2. p53 is released from its inhibitor when p53 is phosphorylated at ser20. This

phosphorylation stabilizes the protein, allowing it to activate DNA damage response genes (Shieh *et al.* 1997; Ashcroft *et al.* 1999; Unger *et al.* 1999a; Unger *et al.* 1999b). In the case of NFκB, the transcription factor is released from its inhibitor by phosphorylation of the inhibitor, IκB. Here, the IκB-NFκB complex is located in the cytoplasm and not until IκB is phosphorylated does NFκB migrate to the nucleus (Jans *et al.* 1996). These are both examples of how transcription factors can be activated when phosphorylation abolishes interaction with a inhibitory factor.

The opposite effect can be observed in the interaction between CREB and the CREB-binding protein (CBP). In this case, the phosphorylation of the kinase inducible domain of CREB facilitates the interaction with the KIX domain of CBP. (Chrivia *et al.* 1993; Parker *et al.* 1996). The phosphorylation occurs directly in the interaction surface between CREB and CBP, making the interaction favorable. The increased affinity allows the CREB to recruit the CBP to the promoter. CBP in turn activates transcription through its HAT activity.

It is also possible that phosphorylation can act as a signal for subsequent modification. For instance, acetylation of p53 at Lys382 is stimulated by phosphorylation at Ser46. The acetylation of the lysine then blocks the ubiquitinylation of the same residue, which would otherwise mark p53 for proteasome-mediated degradation.

Ubiquitinylation and the proteasome in transcription.

Recently, another aspect of transcriptional activation has emerged. Tansey and colleagues noticed that activation domains typically look like degrons, the motifs that

signal degradation by the ubiquitin mediated pathway. They then discovered that Met30, a yeast ubiquitin ligase, was required for activation by the VP16 activation domain in yeast (Salghetti *et al.* 2000; Salghetti *et al.* 2001). In the absence of Met30, not only was VP16 unable to activate, it was also more stable. Furthermore, the addition of an in-frame ubiquitin-moiety restored the activation by the VP16 activation domain in a Met30 null strain. Adding to this was the observation that parts of the 19S proteasome particle appeared to be required for activation by the yeast Gal4 activator (Ferdous *et al.* 2001; Gonzalez *et al.* 2002). The striking requirement for the involvement of parts of the ubiquitin and proteasome pathways for activation by certain activators in yeast may be reflected in the action of these and similar activators in mammalian cells.

Virion host shut-off

Another protein that interacts with VP16 *in vitro* is the virion host shut-off factor (Vhs), the product of the HSV-1 gene UL41 (Roizman *et al.* 1993). Vhs is not essential for viability (Fenwick *et al.* 1990). Vhs is an RNase that specifically degrades mRNAs (Zelus *et al.* 1996; Elgadi *et al.* 1999; Everly *et al.* 2002). It is packaged in the tegument of the virion and upon infection starts degrading both host and newly synthesized viral mRNA (Smiley *et al.* 2001). The purpose of this factor appears to remove host cell transcripts presumably to conserve cellular resources for the translation of viral proteins. It also serves to specifically circumvent the host cells transcriptional response to the pathogen (Matis *et al.* 2001). While vhs also degrades the viral mRNA, the expression level of the IE and DE genes is so high that sufficient mRNA survives to provide proper protein synthesis for the subsequent steps in the infection cascade. At late stages in

infection, however, the RNase activity in the cell is decreased (Lam *et al.* 1996). Vhs function may need to be regulated to prevent depletion of viral transcripts late in infection. Interestingly, the vhs activity in a virus deleted for VP16 is very robust even at late times, (Lam *et al.* 1996) resulting in decreased yield of infectious particles. A double mutant, where both VP16 and vhs are deleted has a less severe phenotype than either of the deletion mutants by themselves. A model where VP16 plays a role in controlling vhs could explain this double phenotype. The model that VP16 is involved in the control of vhs is further supported by *in vitro* results that VP16 (1-344) is capable of interacting with a small domain of vhs (aa 310-330) (Smibert *et al.* 1994; Schmelter *et al.* 1996; Knez *et al.* 2003). Two point mutations have been identified in VP16 that abolish the interaction between VP16 and vhs. These lie at lysine 343 and leucine344 (Knez *et al.* 2003). Vhs is a competitor for the formation of the VIC complex (Smibert *et al.* 1994). While Oct-1 and vhs have distinct interaction surfaces on VP16, they appear to overlap. This fits with a model that suggests that VP16 interacts with Oct-1 during IE to facilitate IE gene expression. At this time, it has no effect on vhs function. Conversely, at late time, VP16 no longer interacts with Oct-1, but it does interact with vhs and subsequently inhibits its RNase activity. The determinants that alter the function of VP16 are not known.

VP16 in egress and cellular localization:

There is some evidence that VP16 also affects the packaging and egress of the maturing virion. The interaction with another tegument protein, VP22, may be required for this function. Tegument body formation and cellular co-localization of VP16 and

VP22 in infection relies on hydrophobic residues in the carboxyterminal AD of VP16 that are also critical for the activation potential of VP16. The interaction also relies on determinants in the putative amino terminal AD of VP16 (Elliott *et al.* 1995). VP16 is found associated with the maturing capsid in the rat dorsal ganglion. (Miranda-Saksena *et al.* 2002). This is not unexpected, as VP16 eventually will become packaged in the mature virion. There is also evidence that VP16 is functionally required for egress. Mossman and colleagues show that HSV-1 fails to package DNA in a VP16 deletion strain. They also show that the phenotype is partially complemented by the deletion of the vhs gene (Mossman *et al.* 2000). But in either case, immature virions accumulate in and around the perinuclear space. Mature virion or even partially formed virion particles do not appear in the cytoplasm *per se.* This suggests that VP16 is required for the maturing virion to traverse the nuclear membrane, but the mechanism has not been established.

Cellular localization and function:

Like other viral factors, VP16 occupy distinct domains within the infected cell. These domains change as the cell goes through the infectious cycle. Initially, newly synthesized VP16 accumulate in distinct spots in the nucleus. VP16 then appears along the nuclear periphery. The intranuclear spots grow bigger and merge as the infection progresses. Late in infection, VP16 is spread through the nucleus as well as throughout the cytoplasm (Greensides 2002). An interesting observation by O'Hare and colleagues is that the localization of nuclear lamin B and the lamin B receptor changes dramatically during infection (Scott *et al.* 2001). They argue that the lamin network, which serves as

a rigid nuclear scaffolding, is too finely meshed to allow the passage of newly assembled, equally rigid viral capsids. The pattern of the redistributed lamins is surprisingly similar to the distribution of VP16 for an extensive period of the infection, suggesting that VP16 could actively be involved in the egress of the capsids.

VP16 homologs

The core domain of VP16 is generally conserved between other herpesvirus homologs. This corresponds to aa 50-410 of HSV-1 VP16. The carboxy terminal activation domain is partially conserved in some virus species, like pseudorabies virus, equid herpes virus 1 and bovine herpes virus 1. In other species, like the closely related varicella zoster virus, the activation domain is entirely absent. Other domains that are less conserved is the amino terminus and, surprisingly, small domains flanking the residues required for interaction with Oct-1 and HCF-1. Even so, the VIC interaction domains are strictly conserved in closely related species.

Mutant viral strains

While the mutagenesis of VP16 in the context of expression plasmids allows focus on the transcriptional function of VP16, it excludes the effect that mutants may have on the viral life cycle. A number of mutant HSV-1 strains have been described and their behavior in infection may help us understand the complex function of VP16 in infection.

The VP16 deletion mutant, 8MA was mentioned earlier. It has had the entire VP16 ORF removed and replaced with a *LacZ* gene. This viral mutant can only

proliferate at a high moi and in the presence of the differentiating agent hexamethylenebisacetamide (HMBA) (Weinheimer *et al.* 1992). It does appear that the virus is fully capable of replicating its genome. How HMBA facilitates the proliferation of 8MA is not understood.

Truncations of the activation domain of VP16 affect the activation of the IE genes (Smiley *et al.* 1997; Tal-Singer *et al.* 1999). Both the RP5 and the V422 strains exhibit high particle to pfu ratios. This indicates that the viruses are fully capable of generating progeny virion, but that the virion do not easily give rise to a productive infection. Furthermore, both RP5 and V422 are capable of delivering their genomes to the nucleus of the host cell, indicating that the proliferation deficiency lies in the initiation of the viral gene expression cascade. Like 8MA, V422 proliferation improves in the presence of HMBA. IE gene expression is also partially complemented (Smiley *et al.* 1997). The In1814 strain contains an insertion of four amino acids at aa 379. This short insert prevents VP16 from forming the VIC and there was a significant reduction in IE gene expression during infection. The strain is also unable to generate plaques, indicating that little if any progeny is generated (Ace *et al.* 1989). Curiously, the defects of in1814 could be partially overcome by infection at a high moi, by adding HMBA to the medium or by overexpressing ICP0, (Jamieson *et al.* 1995) one of the IE gene products. The activity of the HMBA could be repressed by other cytodifferentiating agents (McFarlane *et al.* 1992; Preston *et al.* 1998). RP3, a strain that had the carboxy terminal subdomain (Δ454-490) deleted was only mildly affected. The internal deletion of the amino terminal subdomain of the activation domain (Δ412-453) had a an intermediate phenotype, both in terms of growth and IE gene activation (Tal-Singer *et al.* 1999).

Regulation of VP16:

Throughout infection with HSV-1, VP16 participates in different processes. Activation of the IE genes, vhs interaction and possible inhibition, packaging and nuclear egress of the maturing virion, possibly through interaction with VP22. In addition, a population of VP16 molecules must be dissociated from all activities in the cell and become packaged into the virion. There is evidence that these activities can be mutually exclusive. VP16 can not bind both vhs and Oct-1, and the virus must be programmed to ensure that separate populations of VP16 are partitioned for those two functions. That partition must be regulated through the life cycle of the virus. In a similar manner, because VP22 interact with the AD of VP16, it is possible that VP22 can interfere with the activation of VP16 responsive genes. That raises the question of whether regulation of the interaction between VP16 and VP22 is necessary, and consequently of whether phosphorylation could be involved in such a regulatory step.

Phosphorylation of VP16.

A way for the virus to regulate the function of VP16 is through phosphorylation. VP16 was shown to be phosphorylated early on (Gibson *et al.* 1974; Lemaster *et al.* 1980; Meredith *et al.* 1991). These studies found that many HSV-1 proteins were phosphorylated, including VP16. VP16 was found to be phosphorylated both in the virion and in infected cells. The next investigation was done in the laboratory of Peter O'Hare. They expressed VP16 in HeLa cells, in the presence of [^{32}P]-orthophosphate and confirmed that VP16 became phosphorylated (O'Reilly *et al.* 1997). They then proceeded to investigate the kinases for which bacterially expressed VP16 could serve as

a substrate. They focused on the aa 1-410 fragment of VP16. VP16 was discovered to be a substrate for protein kinase A (PKA) and casein kinase 2 (CK2). A nuclear extract from HeLa cells could also phosphorylate VP16. Lysylendopeptidase digestion suggested that one phosphorylation site fell within the carboxyterminal 120 aa. In an attempt to identify a phosphorylation site, they mutated a prominent CK2 site within this region, Ser375. The alanine substitution completely abolished VIC formation and IE gene activation by VP16. No reduction in the phosphorylation level of VP16 was observed. It was concluded that if Ser375 was a phosphorylation site, there had to be additional sites, which would explain why the loss of the serine did not abolish phosphorylation. They surmised from a small change in migration of the mutated protein as compared to the wild type, that the Ser375 site was in fact a phosphorylation site. Meredith and colleagues isolated VP16 from virion and found it to be a substrate for PKA, but not for CK2 (Morrison *et al.* 1998). They also saw that VP16 became phosphorylated early in infection, when the cells were infected at a very high moi.

In this study the patterns of phosphorylation of the herpes simplex 1 (HSV 1) virion protein 16 (VP16) during infection were examined. It was described how it differs from studies of VP16 in transfected cells. The significance of the discrepancy between the two and the ramifications it has on the way we look at HSV-1 infection in particular and transcriptional regulation in general were addressed.

Chapter Two

VP16 is Phosphorylated in the Infected Cell and in Virion

Introduction

The purpose of this project was to study the phosphorylation of VP16 in infection. The goal was to identify any regulatory role the phosphorylation might serve in infection by HSV-1. To examine the role of phosphorylation, it was necessary to determine if the phosphorylation pattern of VP16 in infected cells was similar to the pattern previously shown for VP16 expressed from a transfected plasmid (O'Reilly *et al.* 1997)

Phospho-VP16 can be detected both in infected cells and in purified virion (Gibson *et al.* 1974; Lemaster *et al.* 1980; Wilcox *et al.* 1980; Morrison *et al.* 1998). O'Hare and colleagues had identified one phosphorylation site, Ser375, in VP16 that was expressed from a transfected plasmid in HeLa cells (O'Reilly *et al.* 1997). They also reported that Ser375 was phosphorylatable *in vitro* by casein kinase 2 (CK2). Substituting the serine at this site with alanine led to a loss of transcriptional activation by VP16 and to loss of the ability to interact with the canonical response element *in vitro*. Both of these effects were attributed to the loss of VP16 interaction with Oct-1 (Lai *et al.* 1997). The interaction with HCF-1 seemed to be maintained, albeit at a much reduced affinity(Greaves *et al.* 1990; Lai *et al.* 1997). Meredith and colleagues had examined the gross phosphorylation of a number of HSV-1 virion components, both in infected cell extracts and in purified virions and had verified that VP16 is phosphorylated. They demonstrated that detergent-purified heat-inactivated tegument-derived VP16 was readily phosphorylated with PKA *in vitro* (Morrison *et al.* 1998). In contrast to O'Hare and

colleagues, Meredith and colleagues saw no phosphorylation of VP16 by CK2 *in vitro*. VP16 has several good consensus CK2 phosphorylation sites, but the lack of detectable phosphorylation in the *in vitro* CK2 phosphorylation assay was not necessarily contrary to the observations by the O'Hare group. If virion borne VP16 was already quantitatively phosphorylated at the CK2 phosphorylation site, labeling of VP16 purified from virion would be blocked. This complication would not have arisen in the studies by O'Hare and colleagues where bacterially expressed VP16 was used in the CK2 assay.

The search for phosphorylation sites *in vivo*. Before addressing the regulatory aspect of phosphorylation, it was first necessary to identify the phosphorylation sites during infection, which may or may not be similar to that of VP16 expressed from a transfected expression vector. The phosphorylation pattern of VP16 might be significantly different in the context of an HSV-1 infection than when expressed from a plasmid, due to the presence of regulatory factors and kinases in the virus. The cellular response to the infection could also cause VP16 to be modified differently.

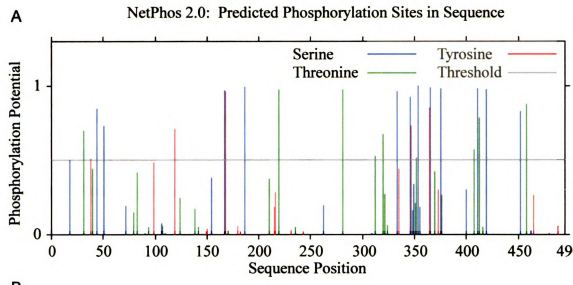
In order to examine the phosphorylation of VP16, VP16 was purified from infected cells and from virion. The phosphorylation sites were examined using phosphopeptide mapping, phosphoaminoacid analysis and mass spectrometry. Isolated VP16 from wild type as well as mutant HSV-1 strains was used in the experiment. These mutants had either partially or completely truncated activation domains. Phosphorylation was found at Ser18, Ser353 and Ser452. The likely phosphorylation of Ser411 was also inferred. There was no evidence that Ser375 was phosphorylated. Furthermore, Ser375 was found not to be required for the *in vitro* phosphorylation of VP16 by CK2.

Results

Computer-aided prediction of phosphorylation sites in VP16. The first step in identifying the sites of phosphorylation of VP16 was to see whether there were any theoretically strong consensus sites of phosphorylation in the protein. The amino acid sequence of VP16 was scanned for potential phosphorylation sites using a web-based prediction server (Blom *et al.* 1999). The Phosphobase phosphorylation prediction server identifies consensus phosphorylation sites in a given polypeptide, based on known kinase consensus sites. It also uses a database of previously characterized phosphoproteins to generate an algorithm that compares the local primary structure of all serines threonines and tyrosines in the query protein to the local primary structure of the phosphorylated serines, threonines and tyrosines of the phosphoproteins in the database. The algorithm then assigns each residue in the analyzed sequence a value between 0 and 1 according to their predicted phosphorylation potential. A low number indicates a poor substrate, a high number a good substrate.

The results of the prediction for VP16 suggested that a significant number of the serines, threonines and tyrosines in the sequence might serve as phosphorylation sites in VP16 (Figure 1). Panel A shows a graphical representation of the results of the prediction. The sequence positions of the residues in the query protein are plotted on the X-axis. The phosphorylation potential of each residue is indicated by the length of the bar at that position. Serine residues are indicated by a blue bar, threonines by a green and tyrosines by a red bar. The grey line indicates an arbitrary prediction cut-off. Residues that fall below this line are dissimilar to the phosphoproteins in the database and are

A



B

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  1 MDLLVDELFA DMNADGASPP PPRPAGGPKN TPAAPPLYAT GRLSQAQLMP
 51 SPPMPVPPAA LFNRLLDDLG FSAGPALCTM LDTWNEDLFS ALPTNADLYR
101 ECKFLSTLPS DVVEWGDAYV PERTQIDIRA HGDVAFPTLP ATRDGLGLYY
151 EALSRFFHAE LRAREESYRT VLANFCSALY RYLRASVRQL HRQAHMRGRD
201 RDLGEMLRAT IADRYYRETA RLARVLFLHL YLFLTREILW AAYAEQMMRP
251 DLFDCLCCDL ESWRQLAGLF QPFMFVNGAL TVRGVPIEAR RLRELNHIRE
301 HLNLPLVRSA ATEEPGAPLT TPPTLHGNQA RASGYFMVLI RAKLDSYSSF
351 TTSPSEAVMR EHAYSRARTK NNYGSTIEGL LDLPDDDAPE EAGLAAPRLS
401 FLPAGHTRRL STAPPTDVSL GDELHLDGED VAMAHADALD DFDLDMLGDG
451 DSPGPGFTPH DSAPYGALDM ADFEFEQMFT DALGIDEYGG
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Figure 1: VP16 is a likely phosphorylation target. The VP16 protein sequence was examined using NetPhos, a web based phosphorylation site prediction server. A: The predicted phosphorylation potential of serines, threonines and tyrosines of the VP16 protein are plotted as bars as a function of their position in the VP16 protein. The potential is reported as a number between 0 and 1. A number above 0.5 (indicated by the horizontal grey line) reflects a likely phosphorylation site. B: The sequence of the VP16 protein. The sites that were predicted by the NetPhos server to be likely phosphorylation substrates are indicated in bold.

rarely found to be phosphorylated *in vivo*. Residues with values at or above 0.5 are predicted to be structurally similar to the phosphorylation sites in the database. As the similarity increases the probability that the site in the query protein is phosphorylated increases and the residue is given a higher score.

Panel B shows the sequence of VP16 where the sites that were predicted to be potential phosphorylation sites are indicated in bold face type. 27 residues received a score of 0.5 or better. Several sites were given scores very near 1.0, indicating a very high likelihood of these sites being good kinase substrates. The predicted phosphorylation sites included threonines, serines and tyrosines. While all of these sites were theoretically likely phosphorylation sites, the likelihood that all of these sites were in fact phosphorylated during infection was much lower. For instance, some of the sites that had received a high score were not found to be phosphorylated by O'Hare and colleagues. None of the predicted threonines and tyrosines were found to be phosphorylated, nor were the two large endolysylpeptidase C fragments spanning residue 29-103 and 104-343. The server predicted eight sites in those two fragments alone. Ser375 received a high score, but it was not the only potential site in the 120 aa carboxyterminal fragment. There are six residues with scores above 0.5, two of which, Ser411 and Ser419, have scores higher than Ser375. Subsequently, it was to be expected that most of the predicted sites in VP16 in infection would remain unphosphorylated. The prediction did show that there were many potential sites of phosphorylation in VP16 and that a *de novo* search for phosphorylation during infection might reveal phosphorylation events specific to infection.

Confirmation of VP16 phosphorylation in infected cells and in virion. In order to start the identification of the phosphorylation sites of VP16 in infection, it was necessary to confirm that the previously published experiments could be repeated with the reagents available. It had been previously observed that VP16 isolated from virions was phosphorylated and that phosphorylation of incoming VP16 occurred within 30 minutes of infection (Morrison *et al.* 1998). It was also found to be among the major phosphoproteins expressed late in infection (Gibson *et al.* 1974).

To confirm that VP16 was phosphorylated in infection and in the virion, [^{32}P]-labeled VP16 was immunoprecipitated from infected HeLa cell lysates and from lysates of purified virions. HeLa cells were infected with wild type HSV-1 (KOS) at a multiplicity of infection (moi) of 5. [^{32}P]-orthophosphate was added at 1.5 hours post-infection (hpi). Either the infected cells were lysed at 8 hpi or virion were harvested from the cells at 14 hpi. VP16 was isolated from either sample by immunoprecipitation with the monoclonal antibody LP1 (McLean *et al.* 1982). The antibody recognizes an epitope within the amino terminal end of VP16. Proteins were eluted from the immunoprecipitation pellet at pH 12.5 and separated on a 10% SDS-PAGE gel. The contents of the gel were transferred to a nitrocellulose membrane. The ^{32}P-labelled proteins were visualized by autoradiography. The results are shown in figure 2. In panel A, a radiolabeled band of the expected mobility (apparent molecular weight 65 KDa) was observed in the immunoprecipitation pellet both at 8 hours (8h) and in the virion. The band is not observed in immunoprecipitation of lysate of non-infected cells from a parallel ^{32}P-labelling (mock). The radiolabeled species co-migrates with VP16, as

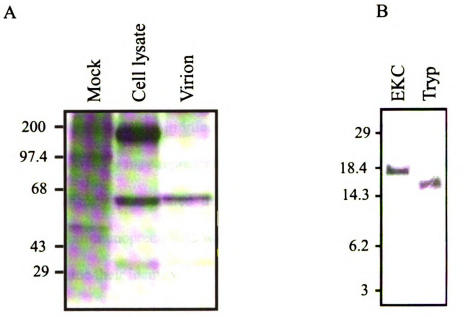


Figure 2: VP16 is labeled in the carboxy terminal 80 amino acids. HeLa cells were infected with KOS in the presence of [32P]-orthophosphate. Cell lysates were prepared at 8 hpi and virion were isolated and lysed at 14 hpi. VP16 was immunoprecipitated from either lysate with the monoclonal antibody LP1. A: The immunoprecipitated material was separated on an 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and visualized by autoradiography. A labelled band was observed at 65 KDa in both samples but not in a sample prepared from a mock infected lysate. Bands observed at 145 and 32 KDa were not identified. B: An aliquot of the immunoprecipitated VP16 from cell lysates were digested with lysylendopeptidase C (EKC) and trypsin (Tryp). The digestion products were separated on a 16% tris/tricine gel, then transferred to nitrocellulose membrane, The labeled bands were visualized by autoradiography. A band of 18 KDa was observed in the EKC sample and a band of 15 KDa was observed in the Tryp sample. These bands correspond to the expected migration of the aa 371-490 and aa 410-490 carboxy terminal fragments of VP16, respectively.

determined by immunoblotting (See figure 4, panel A). These results confirmed that VP16 isolated from either virion or from late in infection was phosphorylated.

The identities of the bands at 145 and 32 KDa seen in the immunoprecipitation from the cell lysate are not known. These bands were also present in the virion sample, albeit at a lower level. These bands were frequently but not consistently observed in immunoprecipitates of VP16 in infected cell extracts. Because they were not present in the mock sample, they may represent cellular proteins or viral proteins expressed in the cell that specifically precipitated through interaction with VP16. But because their presence in the immunoprecipitate was inconsistent between experiments, no effort was made to determine their identity

There was a slight difference in the mobility of VP16 immunoprecipitated from the cell lysate and VP16 purified from virion. This difference in migration between the cellular and the virion-derived VP16 might have been caused by a difference in phosphorylation of VP16. But, as subsequent efforts were to be focused on the phosphorylation of VP16 at late times in infection and not in the virion, it was not determined whether differences in phosphorylation or other modifications were the cause of this difference in mobility.

Peptide mapping of *in vivo* ^{32}P-labelled VP16 isolated late in infection using lysylendopeptidase C. The immunoprecipitation of ^{32}P-labelled VP16 from infected cells demonstrated that VP16 was phosphorylated, in accordance with the Phosphobase predictions that indicate many potential sites of phosphorylation in VP16. The first approach was to identify regions of VP16 that were phosphorylated. O'Reilly *et al* used

lysylendopeptidase C to map Ser375 as a phosphorylation site in HeLa cells. This approach relied on the fact that VP16 when digested with lysylendopeptidase C generated two large and three minor fragments. Both the larger and at least one of the three minor fragments could be resolved by SDS-PAGE. (Figure 4A)

To provide material for this mapping approach, HeLa cells were infected with KOS at an MOI of 5-10. [^{32}P]-orthophosphate was added to the infected cells at 1.5 hpi. At 8 hpi, the cells were lysed and VP16 isolated by immunoprecipitation. A sample of the [^{32}P]-labeled protein eluted from the immunoprecipitation pellet was separated on a 10% SDS-PAGE gel. The position of the radiolabeled VP16 was determined by autoradiography of the wet gel and the radioactive band at 65 KDa was excised. The contents of the gel slice were digested with lysylendopeptidase C. The resulting products of the digestion were separated on a 16.5% polyacrylamide gel in a tris/tricine buffer system (Schagger *et al.* 1987). The separated peptides were transferred to nitrocellulose and the positions of the radiolabeled species were visualized by autoradiography. Figure 2B shows a representative result of this experiment. Digestion with lysylendopeptidase yields a single phosphorylated species migrating with an apparent molecular weight of 18 KDa with only faint evidence of additional radiolabeled species.

The four lysine residues in the VP16 protein sequence are located at aa 29, 103, 343 and 370. Complete digestion would result in five fragments of distinct sizes: 3.0 KDa (aa 1-29), 8.0 KDa (aa 30-103), 28 KDa (aa 104-343), 3.1 KDa (aa 344-370) and 13 KDa (aa371-490). Assuming complete digestion of the protein, the labeled species in the lysylendopetidase C digestion had to correspond to one of these fragments. While none of them are close to the apparent molecular weight of the species on the gel, the 13.2

KDa carboxyterminal fragment of VP16 is known to have lower mobility than predicted from its molecular weight (O'Reilly *et al.* 1997). The 18 KDa size of the labeled species detected in the lysylendopeptidase digestion was consistent with the previously described migration of the carboxyterminal fragment of VP16. The conclusion from the experiment was that the labeled fragment corresponded to the carboxyterminal 120 aa of VP16. This was also the conclusion drawn by O'Hare and colleagues when they mapped VP16 expressed from a plasmid in HeLa cells.

The two smallest products of the lysylendopeptidase C digestions were nominally larger than the smallest molecular weight marker which resolved on the gel. However, the mobility of small peptides can vary significantly and it was not certain that these fragments would have resolved on the gel. It was possible that the two smaller fragments had failed to resolve on the gel and migrated with the dye front.

Peptide mapping of *in vivo* [^{32}P]-labeled VP16 isolated late in infection using trypsin. Because the major labeled lysylendopeptidase C fragment contains multiple putative phosphorylation sites (see figure 1) further efforts were needed to precisely identify which of these residues were sites of phosphorylation. Using trypsin, it was possible to digest the protein at the carboxyterminal side of both arginine and lysine residues. If VP16 was digested with trypsin, most of the protein (aa1-410) would be reduced to fragments smaller than 36 aa. However, the carboxyterminal 120 aa of the protein (371-490), which correspond to the proposed labeled lysylendopeptidase C fragment, would yield only three fragments of 29aa, 10 aa and 80 aa, respectively. Of

these three fragments, the 80 aa fragment would be expected to resolve on a 16% tris/tricine gel.

To determine whether the carboxyterminal 80 aa fragment of VP16 was phosphorylated late in infection, HeLa cells were infected with KOS at an MOI of 5-10. At 1.5 hpi, ^{32}P-orthophosphate was added to the infected cells and at 8hpi the cells were lysed. VP16 was isolated from the lysate by immunoprecipitation. Protein was eluted from the immunoprecipitation pellet and was separated on a 10% SDS-PAGE gel. The position of the radiolabeled VP16 was determined by autoradiography of the wet gel. The radioactive band at 65 KDa was excised and digested with trypsin. The products of the digestion were separated on a 16% polyacrylamide gel in a tris/tricine buffer system. The separated peptides were transferred to nitrocellulose and the positions of the radiolabeled species were visualized by autoradiography.

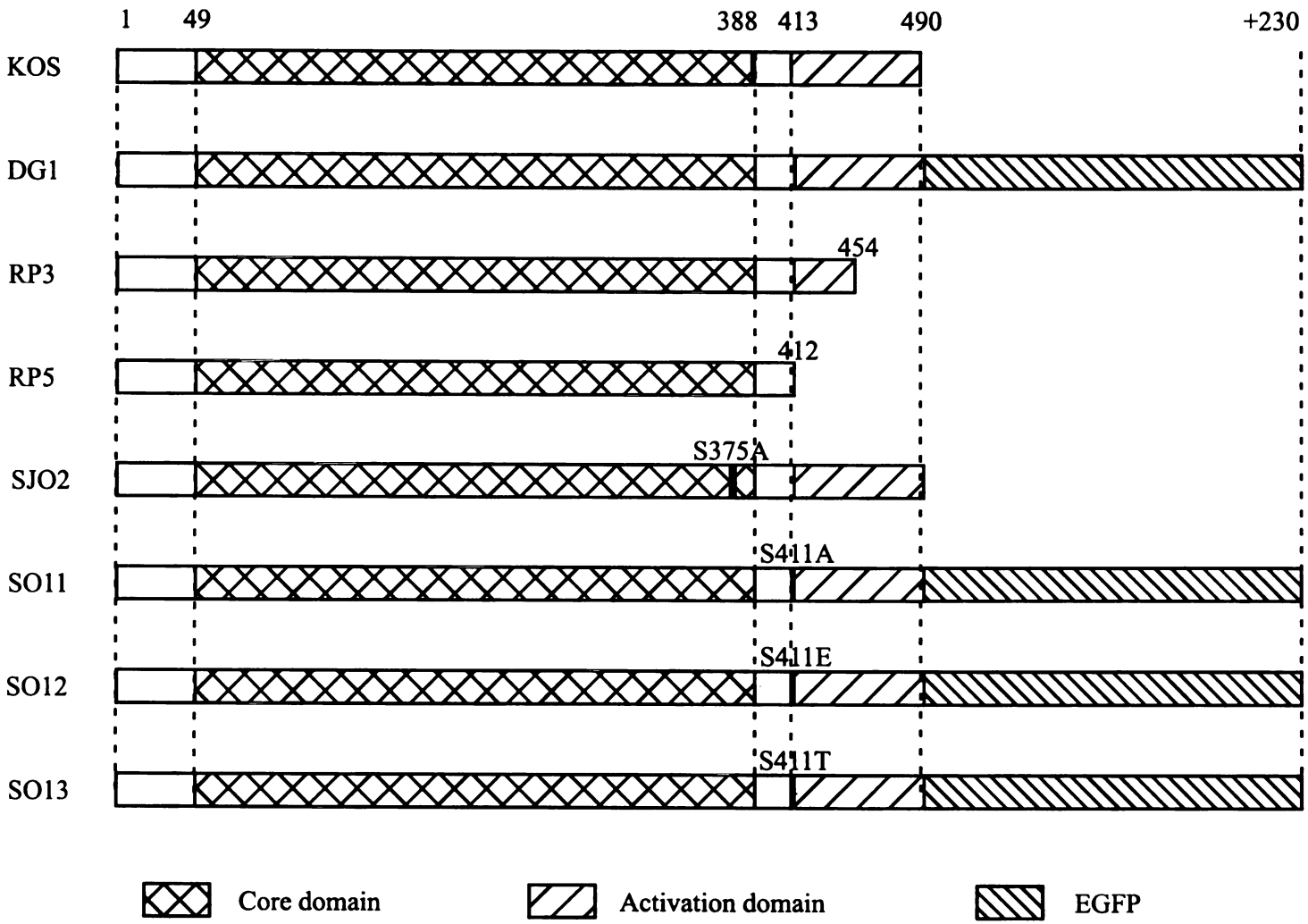
The digestion of ^{32}P-labelled VP16 isolated from HeLa cell infected with KOS gave rise to a single phosphorylated species (Figure 2B). This peptide migrates at 15 KDa. While the mobility of this fragment is lower than any of the expected products of a complete digestion of VP16 with trypsin, the carboxyterminal fragment was expected to migrate slower than expected from the molecular weight. The conclusion drawn from this experiment was that the 410-490 fragment of VP16 was phosphorylated. It also confirmed the identification of the labeled fragment from the lysylendopeptidase digestion as being aa371-490.

The smaller fragments of the digestions may have been lost during transfer of the peptides to the membrane. To address this issue, an alternative procedure of detecting the labeled bands was used. After electrophoresis of the digestion products, the glycerol was

soaked out of the tris/tricine gel and the gel was then dried onto Whatman 3MM paper. The dried gel was subjected to autoradiography. Again, the labeled species of higher apparent molecular weight were detected in both digestions but there was still no evidence of labeled species in the 3 KDa range. (Data not shown) However, even though the 3 KDa marker resolved and was clearly discernible in the dried gel, there was a concern that small fragments of the digestion might have been lost when glycerol was soaked out of the gel. It was not possible to skip this step as the glycerol impeded the drying of the gel and the presence of water in the gel would quench the emission of radioactivity from any weakly labeled peptides.

Phosphorylation of VP16 truncation mutants *in vivo*. The availability of a set of truncation mutants of VP16 in the virus made it possible to further map the location of the phosphorylation site. Two strains in particular would allow for the determination of whether the activation domain was required for the phosphorylation of VP16. The previous mapping of the full length VP16 suggested that phosphorylation took place within the carboxyterminal 80 aa of the protein. If the activation domain was the predominant site of phosphorylation, deletion of the activation domain would diminish or abolish the phosphorylation of the protein. The two strains that would be useful for this approach were RP3 and RP5. These two HSV-1 strains contain truncations within the VP16 ORF (Figure 3). The carboxyterminal activation sub-domain of VP16 is deleted in RP3 (Δ455-490) and the entire activation domain is deleted in RP5 (Δ413-490). Both of these strains are viable without complementation, but RP5 is known to have a reduced proliferation. An aspect of this phenotype is a 100 fold increased particle to pfu ratio.

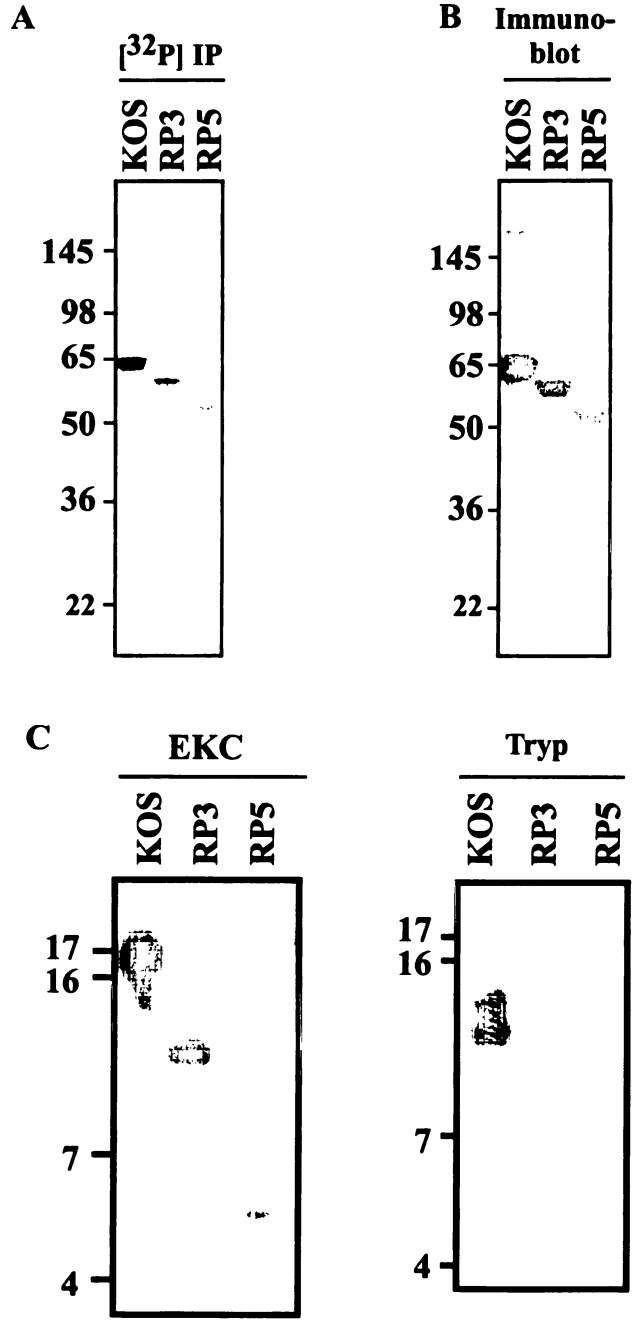
Figure 3: Viral strains. KOS is a wild type strain and the background for the remainder of the strains used in this study. The RP3 and RP5 strains carry carboxyterminal deletions of VP16, Δ454-490 and Δ413-490, respectively. DG1 carries wild type VP16 with a carboxyterminal EGFP-tag. SJO2, SO11, SO12 and SO13 were created for this study. SJO2 carries an alanine substitution at Ser375. SO11, SO12 and SO13 carry carboxyterminal EGFP-tags and alanine, glutamate and threonine substitutions at Ser 411, respectively.


1
49
388
413
490
+230
KOS
DG1
RP3
454
RP5
412
SJO2
S375A
SO11
S411A
SO12
S411E
SO13
S411T
Core domain
Activation domain
EGFP

HeLa cells were infected with KOS and RP3 at an MOI of 5, and with RP5 at an MOI of 0.1. At 1.5 hpi, ^{32}P-orthophospate was added and the infected cells were lysed at 8 hpi. Immunoprecipitation was performed as previously with the monoclonal antibody LP1 and the precipitated proteins were eluted from the immunoprecipitation pellet at pH 12.5. The eluate was separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The radiolabeled proteins were visualized by autoradiography. Figure 4A shows a representative autoradiogram. All three versions of VP16 give rise to a phosphorylated species, corresponding to the expected apparent molecular weight of the wild type or truncated proteins (65 KDa, 58 KDa and 52 KDa respectively.) Panel 3B shows an immunoblot of the same membrane, in which the VP16 specific antiserum C8 was used to verify the position of the wild type and truncated VP16. The immunoblot confirms that the radiolabeled species observed in the first panel correspond to VP16. These results show that truncated VP16 from both RP3 and RP5 was phosphorylated. Therefore phosphorylation sites must be present within aa 1-412. Moreover, the activation domain of VP16 is not necessary for phosphorylation of VP16. It could not be determined whether the specific labeling differed between the three versions of VP16. Therefore, the possibility that one or more phosphorylation sites of the full length protein were removed in the truncation of RP3 or RP5 could not be excluded.

Peptide mapping of the phosphorylation sites of VP16 truncation mutants using lysylendopeptidase C. The previous experiments indicated that at least one site was phosphorylated within the 80 carboxyterminal aa of VP16, but did not resolve whether any of the sites between aa 1-28 and 344-410 were phosphorylated. This

Figure 4: VP16 is labeled outside of the activation domain. HeLa cells were infected with KOS, RP3 and RP5 at 5-10 moi in the presence of [32P]-orthophosphate. Cell lysates were prepared from each infection at 8 hpi. VP16 was immunoprecipitated from each lysate with the monoclonal antibody LP1. A: The immunoprecipitated material was separated on an 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and visualized by autoradiography. A labeled band was observed at 65 KDa in the KOS sample, at 60 KDa in the RP3 sample and at 53 KDa in the RP5 sample. These bands corresponded to the expected migration of the VP16 from each sample. Additional labeled bands were similar in all three lanes. B: The identities of the labeled bands were verified by immunoblotting. The nitrocellulose membrane was probed with the polyclonal antiserum C8. In all three samples, the immunoblot identified species corresponding to the migration of the labeled samples in the autoradiogram. A minor band was observed at 150 KDa in both KOS and RP3. These bands did not comigrate with a [^{32}P]-labeled species. C: An aliquot of the immunoprecipitated VP16 from cell lysates were digested with lysylendopeptidase C (EKC). The digestion products were separated on a 16% tris/tricine gel, then transferred to nitrocellulose membrane, The labeled bands were visualized by autoradiography. A band of 18 KDa was observed in the EKC sample and a band of 15 KDa was observed in the Tryp sample. These bands correspond to the expected migration of the aa 371-490 and aa 410-490 carboxy terminal fragments of VP16, respectively.


A
[32P] IP
KOS
RP3
RP5
145
98
65
50
36
22
B
Immuno-blot
KOS
RP3
RP5
145
98
65
50
36
22
C
EKC
KOS
RP3
RP5
17
16
7
4
Tryp
KOS
RP3
RP5
17
16
7
4

question was most easily approached by mapping the sites of phosphorylation within VP16 derived from RP3 and RP5 by proteolysis. Similarly to full length VP16, digestion of either truncated proteins with lysylendopeptidase C would yield five fragments of VP16. The four aminoterminal fragments would be the same in for all three versions of VP16. The only fragment that would vary between the three samples would be the carboxyterminal fragment. In full length VP16, this fragment would be 120 aa and was seen in the previous experiments to migrate with the apparent molecular weight of 18 KDa. In the RP3 sample, the protein was truncated to aa 454. Correspondingly, the expected carboxyterminal fragment of the digestion would be 84 amino acids. The mobility of this fragment had not been determined previously, but was expected to migrate faster than the 120 aa fragment from the KOS sample. In RP5, VP16 is truncated at aa 412. Subsequently, the carboxyterminal fragment resulting from the lysylendopeptidase C digestion would only be 44 aa. This fragment was expected to resolve on the gel, yet migrate faster than both the corresponding fragment from the KOS and the RP3 sample. Phosphorylation between aa 412 and aa 454 would give rise to a labeled band in the RP3 sample, but not in the RP5 sample. Phosphorylation between 371 and 412 would result in labeled bands in both RP3 and RP5. In this case, additional phosphorylation between aa 412-454 could not be excluded. If no phosphorylation occurred outside of aa 455-490, there would be no labeled band in either sample. Regardless of whether any phosphorylation was detected in the mutants, phosphorylation between aa 455-490 could not be excluded by this method.

HeLa cells were infected with KOS or RP3 at an MOI of 5, or with RP5 at an MOI of 0.1. At 1.5 hpi, [^{32}P]-orthophosphate was added and the infected cells were lysed

at 8 hpi. Immunoprecipitation was performed as previously with LP1 and the precipitated protein was eluted from the immunoprecipitation pellet at high pH. An aliquot of the eluate from the immunoprecipitation pellet was resolved on a 10% SDS-PAGE gel and the position of the labeled species was determined by autoradiography of the wet gel. The radioactive bands corresponding to VP16 in the KOS, RP3 and RP5 samples were excised as described above and the labeled protein was subjected to proteolysis in the gel by endolysylpeptidase C. The fragments resulting from the digestion were separated on a 16.5% tris/tricine gel and visualized by autoradiography (figure 4C).

As seen previously, digestion of the full length protein gave rise to a single radiolabeled band of 17 KDa. The truncated VP16 from RP3 also gave rise to a single radioactive band that migrated at 11 KDa. RP5 gave rise to two bands, migrating at 6 and 5 KDa. The significance of the double band is unclear, although a band corresponding in mobility to the upper band was also present in the KOS sample but not in the RP3 sample. The lower band was unique to the RP5 sample. The predominant band in both the RP3 and the RP5 samples migrated as would be predicted for the carboxyterminal fragment of the lysylendopeptidase C digestion. This observation was in agreement with the previous identification of the labeled peptide in the digestion of the full length protein as the one spanning aa 410-490. Because both the carboxyterminal fragments of RP3 and RP5 were labeled, I conclude that VP16 was phosphorylated between aa 371-412. These results do not rule out additional phosphorylation sites between 413-490.

Peptide mapping of the phosphorylation sites of VP16 truncation mutants using trypsin. To determine whether any phosphorylation sites fall between aa 410-454, the mapping of the phosphorylation sites of VP16 by trypsin digestion was repeated on VP16 derived from RP3 and RP5 infection of HeLa cells. Like full length VP16, digestion of RP3-derived VP16 would render the majority of the protein too small to be detected on the gel. But like the full length protein, digestion of the carboxyterminal end of VP16 would yield a peptide of 44 aa (aa 411-454). It was reasonable to expect this peptide to resolve on the gel. A similar digestion of RP5 was not expected to yield any fragment of detectable size. If the RP3 peptide was labeled, it would indicate a phosphorylation site between aa 411-454. If it was not, it would indicate a phosphorylation site between 455-490.

HeLa cells were infected with KOS or RP3 at an MOI of 5, or with RP5 at an MOI of 0.1. At 1.5 hpi, ^{32}P-orthophosphate was added and the infected cells were lysed at 8 hpi. Immunoprecipitation was performed as previously with LP1 and the precipitated protein was eluted from the immunoprecipitation pellet at high pH. An aliquot of the eluate from the immunoprecipitation pellet was resolved on a 10% SDS-PAGE gel and the position of the labeled species was determined by autoradiography of the wet gel. The radioactive bands corresponding to VP16 in the KOS, RP3 and RP5 samples were excised as described above and the labeled protein was subjected to proteolysis in the gel by trypsin. A representative experiment is shown in figure 4C.

The digestion of full length VP16 once again gave rise to the expected 12 KDa fragment. In this particular experiment a digestion product migrating at 14 KDa was observed, suggesting incomplete digestion. This band most likely corresponds to the aa

400-490 fragment of VP16. The RP5 digestion gave little evidence of any unique bands. The bands that were seen were common to either all three or to at least two of the samples (5KDa band is present in both KOS and RP5.) The RP3 sample gave rise to a band migrating at 11KDa. This is likely to be a partial digestion product, similar in migration to the carboxyterminal lysylendopeptidase C product of RP3 (aa 370-454). There is also a band that migrates slightly faster than the 5KDa band common to the KOS and RP5 samples.

The migration of the major species in the KOS sample as well as the absence of bands in the RP5 sample were expected. The result of the RP3 experiment is not as easy to interpret. If the 4.5 KDa species did in fact correspond to the aa 410-453 peptide, the labeling of both the aa 371-454 and the aa 371-412 fragments could be attributed to phosphorylation at Ser411. Unfortunately, it is difficult to ignore the presence of bands in both the KOS and RP5 samples which have similar migration. If one argues that the 4.5 band is not unique to the RP3 sample, the conclusion must be that there is no phosphorylation of RP3 between aa 411-454. This means that the phosphorylation of the carboxyterminal lysylendopeptidase fragments of both RP3 and RP5 is phosphorylated aminoterminal to aa410 and that the carboxyterminal trypsin fragment of the wild type would be phosphorylated carboxyterminal to aa454. Unfortunately, the data does not favor one interpretation over the other.

Phosphoaminoacid analysis of VP16 from late in infection. The phosphoamino acid composition of VP16 expressed from a plasmid in HeLa cells had been shown only to consist of phosphoserine (O'Reilly *et al.* 1997). Given that the

regulatory controls of VP16 function imposed by the HSV-1 gene expression cascade would be different in infection, resulting in different phosphorylation patterns of VP16, it would stand to reason that the type of phosphorylation site needed not be conserved, either. It was possible that the labeled peptides discovered in the mapping attempts of VP16 derived from infected cells represented phosphorylation of any of the three major types of phosphorylation sites, namely serine, threonine or tyrosine. Excluding one or more of these phosphoamino acids would narrow the range of possible phosphorylation sites.

To identify the type of residues that are phosphorylated during infection, a phosphoamino acid analysis was performed. An aliquot of each of the [^{32}P]-labeled proteins immunoprecipitated from HeLa cells infected with KOS, RP3 and RP5 were separated on a 10% SDS-PAGE gel and transferred to PVDF membrane. The position of the radioactive band was determined by autoradiography and the bands corresponding to the three VP16 species were excised from the membrane. The proteins on the membrane were hydrolyzed in 6N hydrochloric acid at 100°C. Phosphoserine, phosphothreonine and phosphotyrosine standards were added to the sample and the resulting amino acid mixture was separated by one-dimensional thin layer electrophoresis at pH=2.5. The radioactive amino acids were visualized by autoradiography (Figure 5) and the phosphoamino acid standards were stained with ninhydrin. A single radioactive spot was observed in each sample. In all three cases, this spot co-migrated with the phosphoserine standard. The conclusion from the experiment was that serine was the only residue phosphorylated in VP16 derived from late stages of infection of HeLa cells.

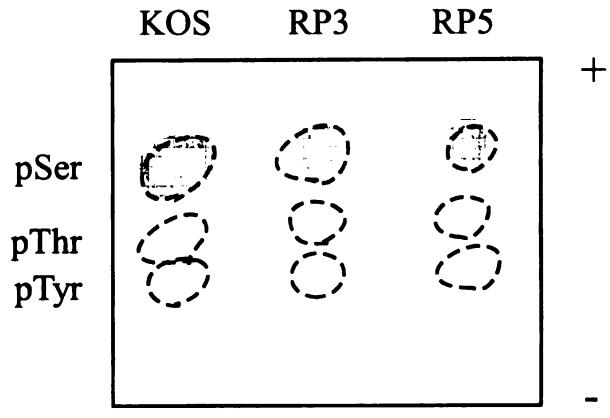


Figure 5: VP16 is labelled on serines. HeLa cells were infected with KOS, RP3 or RP5 in the presence of [^{32}P]-orthophosphate. VP16 was immunoprecipitated with the monoclonal antibody LP1. The immunoprecipitated material was separated on an SDS-PAGE gel, transferred to a PVDF membrane and the labeled band at 65 KDA, corresponding to VP16, was excised from the membrane. The protein was hydrolyzed in 6N HCl for 1 hour at 100^{o}C. The samples were mixed with phosphoserine, -threonine and -tyrosine standard and separated by thin layer electrophoresis on a cellulose plate at pH 2.5. The phosphoamino acid standards were visualized by staining with ninhydrin (indicated by a stippled outline) and the [^{32}P]-labeled species were identified by autoradiography. The only labeled species in each sample comigrated with the phosphoserine standard. + and - indicate the electrophoretic poles.

The experiment reduced the number of possible sites within the carboxyterminal tryptic fragment of full length VP16 to only 4: Ser411, Ser419, Ser452 and Ser462. At least one of these sites must be phosphorylated to give rise to the labeled tryptic fragment in the peptide mapping of the full length protein. Phosphorylation at Thr412, Thr416, Thr480, Tyr465 and Tyr488 can be excluded. In the potentially labeled but possibly not detected minor fragments of either lysylendopeptidase C or trypsin digestion, phosphorylation at Thr351, Thr352, Tyr364, Thr369, Thr376 or Thr407 was excluded. It did not completely exclude phosphorylation of any of these fragments, as each encompasses at least one serine. The potential phosphorylation sites that remain within these fragments are Ser18, Ser346, Ser348, Ser349, Ser353, Ser355, Ser365, Ser375 and Ser400.

Mass spectrometry of VP16 isolated late in infection. While the phosphopeptide mapping and the phosphoamino acid analysis had excluded a number of potential phosphorylation sites, thirteen serines remained as possible sites of phosphorylation. Of these thirteen, phosphorylation had to occur at at least one site among the sites at Ser 411, Ser419, Ser453 and Ser462. Whether any of the remaining were phosphorylated was not known, but none could be rigorously excluded. To further refine the mapping of the phosphorylation of VP16, a different approach was chosen. Subjecting the protein to mass spectrometry would not only allow the detection of phosphorylated fragments of a digestion, it would also allow positive identification of the phosphorylation sites within the fragments.

Non-radiolabeled VP16 was purified from 10^9 HeLa cells infected with KOS by immunoprecipitation. The protein was eluted from the immunoprecipitation pellet at high pH and the resulting eluate was separated on a 10% SDS-PAGE gel. The gel was stained with coomassie blue, and the gel was divided in two by cutting the lane of the immunoprecipitated protein lengthwise into two unequal parts. Five prominent bands migrating between 60 and 70 KDa were excised from both parts and the slices from the smaller part (~1/5 of the loaded sample) were loaded back on a 10% SDS PAGE gel. This gel was transferred to nitrocellulose and the slice corresponding to VP16 was identified by immunoblotting with the polyclonal rabbit serum LA2-3. The slices from the other part of the gel (~4/5 of the sample) were washed in acetonitrile, dried and frozen at -80°C. After determination of the band corresponding to VP16, this band was submitted to the Harvard Microchemistry Facility for protease digestion and subsequent LC-MS/MS. Separate samples were digested with trypsin and with aspartyl-endopeptidase N to facilitate broader coverage of the protein.

A summary of the results from both digestions is shown in figure 6 and table 1. The MS/MS returned data for 79% of the protein. The MS/MS positively demonstrated phosphorylation at Ser18, Ser353 and Ser452. For these three sites, spectra were observed that were consistent with the presence of both the phosphorylated and the unphosphorylated species. Six serine residues were not covered by the MS. Of these six, four fell in regions of the protein that were covered by the peptide map, but did not appear to be phosphorylated (Ser72, Ser177, Ser186 and Ser262). The remaining two, Ser 411 and Ser419 both fell within the [^{32}P]-labeled carboxyterminal trypsin fragment (aa411-490). Their phosphorylation state could not be determined by either the peptide

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  1 MDLLVDELFA DMDADGASPP PPRPAGGPKN TPAAPPLYAT GRLSQAQLMP
 51 SPPMPVPPAA LFNRLLDDLG FSAGPALCTM LDTWNEDLFS ALPTNADLYR
101 ECKFLSTLPS DVVEWGDAYV PERAQIDIRA HGDVAFPTLP ATRDGLGLYY
151 EALSRFFHAE LRAREESYRT VLANFCSALY RYLRASVRQL HRQAHMRGRD
201 RDLGEMLRAT IADRYYRETA RLARVLFLHL YLFLTREILW AAYAEQMMRP
251 DLFDCLCCDL ESWRQLAGLF QPFMFVNGAL TVRGVPIEAR RLRELNHIRE
301 HLNLPLVRSA ATEEPGAPLT TPPTLHGNQA RASGYFMVLI RAKLDSYSSF
351 TTSPSEAVMR EHAYSRARTK NNYGSTIEGL LDLPDDDAPE EAGLAAPRLS
401 FLPAGHTRRL STAPPTDVSL GDELHLDGED VAMAHADALD DFDLDMLGDG
451 DSPGPGFTPH DSAPYGALDM ADFEFEQMFT DALGIDEYGG
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Figure 6: Peptide sequences identified by LC-MS/MS of VP16 isolated late in infection from HeLa cells infected with HSV-1. The isolated protein was digested with either endolysylpeptidase C or endoaspartylpeptidase C. Dark typeface indicates sequences of peptides identified. Light typeface sequences were not observed.

TABLE 1. Phosphorylated and non-phosphorylated peptides detected by MS/MS covering Ser18, Ser353, Ser375 and Ser452

Residue	Coverage	Representative spectra (peptide)[a,b]
Serine 18	4-29	LVDELFADMDADGA**S**PPPPRPAGGPK
	5-29	VDELFADMDADGASPPPPRPAGGPK
	5-29	VDELFADMDADGA**S**PPPPRPAGGPK
	6-29	DELFADMDADGA**S**PPPPRPAGGPK
	8-29	LFADMDADGA**S**PPPPRPAGGPK
	9-29	FADMDADGA**S**PPPPRPAGGPK
	10-29	ADMDADGA**S**PPPPRPAGGPK
Serine 353	342-360	AKLDSYSSFTTSPSEAVMR
	344-360	LDSYSSFTTSPSEAVMR
	344-360	LDSYSSFTT**S**PSEAVMR
	345-360	DSYSSFTTSPSEAVMR
	348-360	SSFTTSPSEAVMR
	351-359	TTSPSEAVM
	351-360	TTSPSEAVMR
	351-360	TT**S**PSEAVMR
	351-362	TTSPSEAVMREH
	351-364	TTSPSEAVMREHAY
	353-360	SPSEAVMR
Serine 375	369-398	TKNNYGSTIEGLLDLPDDDAPEEAGLAAPR
	369-401	TKNNYGSTIEGLLDLPDDDAPEEAGLAAPRLSF
	371-398	NNYGSTIEGLLDLPDDDAPEEAGLAAPR
	372-398	NYGSTIEGLLDLPDDDAPEEAGLAAPR
	374-401	GSTIEGLLDLPDDDAPEEAGLAAPRLSF
Serine 452	445-460	DMLGDGD**S**PGPGFTPH
	449-460	DGDSPGPGFTPH
	449-460	DGD**S**PGPGFTPH

[a]Bold-face S indicates phosphorylation of the serine. [b]Additional spectra were observed representing peptides of the same length as those shown here, but carrying methioninesulfoxide, an artifact of sample treatment.

mapping, the MS or the combination of the two. Ser44, Ser51, Ser90, Ser106, Ser110, Se154, Ser167, Ser309, and Ser333 were found not to be phosphorylated by both the MS and the peptide mapping. Ser346, Ser348, Ser349, Ser353 and Ser355 were covered by the MS but not by the peptide mapping. The corresponding peptide (Ser344-Ser370) was most likely too small to resolve on the tris/tricine gel. Of these, only Ser353 was found to be phosphorylated.

The MS results were consistent with the mapping of the aa29-103 and the 104-343 fragments of the lysylendopeptidase experiment. No spectra in the MS suggested any phosphorylation of any sites within these two peptides. Furthermore, the MS did not find evidence of phosphorylation of any threonine or tyrosine residues, in agreement with the phosphoaminoacids analysis.

The detection of phosphorylation of Ser18 and Ser353 by mass spectrometry was a strong indication that neither of the two smaller lysylendopeptidase C fragments resolved on the tris/tricine gel. If either of the phosphorylation events at Ser18 and 353 represented major sites of phosphorylation it would have given rise to a signal, had the corresponding fragment resolved on the gel. Subsequently, it was important to ascertain whether any of the remaining sites within these fragments were in fact phosphorylated. The experiment also invoked concerns about the detection of the 371-398 fragment of the trypsin digestion.

No evidence of phosphorylation at Ser375 was found. All of the multiple spectra covering this serine show it to be unphosphorylated. To ascertain with the highest degree of confidence that this was the correct result targeted ion mass spectrometry (TIMM) was done. The TIMM approach is a modification of the standard method of LC-

MS/MS, where peptides corresponding to the expected phosphorylated and non-phosphorylated masses of a particular digestion are selected in the first dimension MS. Only these fragments are subjected to fragmentation and sequencing by the second dimension MS. Because longer time is spent analyzing these peptides, the likelihood of detecting a low-abundance species is greatly increased. This more sensitive technique did not detect any phosphorylated fragments corresponding to phosphorylation at Ser375.

Acetylation of VP16. VP16, like a number of transcription factors, interact with histone acetyl transferases (HATs) when activating its target genes. The HATs were initially identified as factors capable of acetylating histones. Recently, a number of these have been shown to acetylate transcription factors as well. It was possible that VP16 could also serve as an acetylase substrate. To test whether VP16 isolated from infected cells was acetylated, the MS/MS data set was scrutinized for evidence of acetylation. VP16 encode four lysines, all of which were detected only as unmodified peptides. No spectra were observed that would indicate that any of these sites were acetylated (Data not shown). The conclusion from the experiment is that late in infection, VP16 was not acetylated.

***In vitro* phosphorylation of the 375 CK2 site.** Both the MS and the peptide mapping show Ser375 of VP16 to be unphosphorylated late in infection. As mentioned previously, the O'Hare lab found that residue to be phosphorylated (O'Reilly *et al.* 1997). They also reported that bacterially expressed VP16 was a substrate for CK2 *in vitro*. Because Ser375 is a CK2 consensus site, they tested whether mutations at Ser375

affected phosphorylation of VP16 *in vitro*. They found the Ser375Ala mutation to abolish phosphorylation of VP16 *in vitro* and surmised that Ser375 was the primary site of phosphorylation by CK2. In contrast, Meredith *et al.* unsuccessfully attempted to phosphorylate VP16 purified from virion with CK2 *in vitro*. It is possible that the inability of virion-derived to act as a CK2 substrate reflected a block of the CK2 sites of VP16 in the virion, either by modification of the serine itself or of the consensus site. In both instances it is peculiar that VP16 could not serve as a substrate. In addition to Ser375, one other serine and five threonines of the VP16 core domain conform to the CK2 consensus sequence (Ser353, Thr79, Thr83, Thr94, Thr124 and Thr210). It is a little surprising that in either *in vitro* assay, whether Ser375 is mutated or not, none of the remaining four consensus sites get phosphorylated. Especially when taking into account that at least one of them (Ser353) falls in an unstructured domain that is surface-exposed (Liu *et al.* 1999). To test whether Ser375 is the only kinase accessible CK2 site in VP16, an *in vitro* kinase assay was done, using bacterially expressed VP16.

A GST-tagged version of the VP16 core domain (aa 49-410) was expressed in E. coli and affinity-purified using glutathione-sepharose. The core domain of VP16 was eluted from the affinity column by cleaving the thrombin site located between the GST-tag and VP16. The purity of the sample was examined by SDS-PAGE, followed by Coomassie brilliant blue staining of the gel (Data not shown). Commercially available CK2 was used to phosphorylate VP16 in the presence of γ-[^{32}P]-ATP. Labeling of VP16 was visualized by SDS-PAGE of the reaction mixture. An aliquot of each sample were run on each of two separate gels. One gel was dried and subjected to autoradiography, the other transferred to nitrocellulose and the presence of the VP16 core domain was

verified by immunoblotting with the polyclonal serum SO1-2. To verify that the VP16 was a substrate, the experiment was done with and without VP16. To verify that CK2 was the kinase, the experiment was done with and without CK2.

Figure 7A shows a representative result from these experiments. The two left panels show the autoradiograms of the labeling reaction and the two right panels represent the corresponding immunoblots. In the first panel, signals were detected in both lanes. The band at 18 KDa as well as the fainter band at 40 KDa represent autophosphorylation of the α and β subunits of the CK2 kinase. The sample in the first contained both the kinase and VP16. In this lane, two unique labeled bands are present at 41 KDa and 12 KDa. These bands were surmised to represent the bacterially expressed VP16. In the second panel, where no CK2 was added, none of the bands were detected. This showed that CK2 was required for the phosphorylation of VP16. The immunoblot of the samples in the two panels at the right verified that the 40 KDa band does in fact correspond to VP16. The band reacted with the C8 antibody in the samples where VP16 was added, regardless of whether CK2 was present. The 12 KDa band did not react with the antiserum and may be a degradation product or an abortive translation product of VP16 that does not carry the predominant epitopes for the C8 antibody or it could be a minor species from the bacterial extract that co-purified with VP16 and has a CK2 consensus signal. The conclusion of the experiment was that the VP16 core domain can in fact serve as a CK2 substrate *in vitro*.

To determine whether Ser 375 was the major site of CK2 phosphorylation *in vitro*, a number of mutations were introduced at this site. The site was altered to alanine, proline or threonine. If the site was the major site of phosphorylation, the alanine and

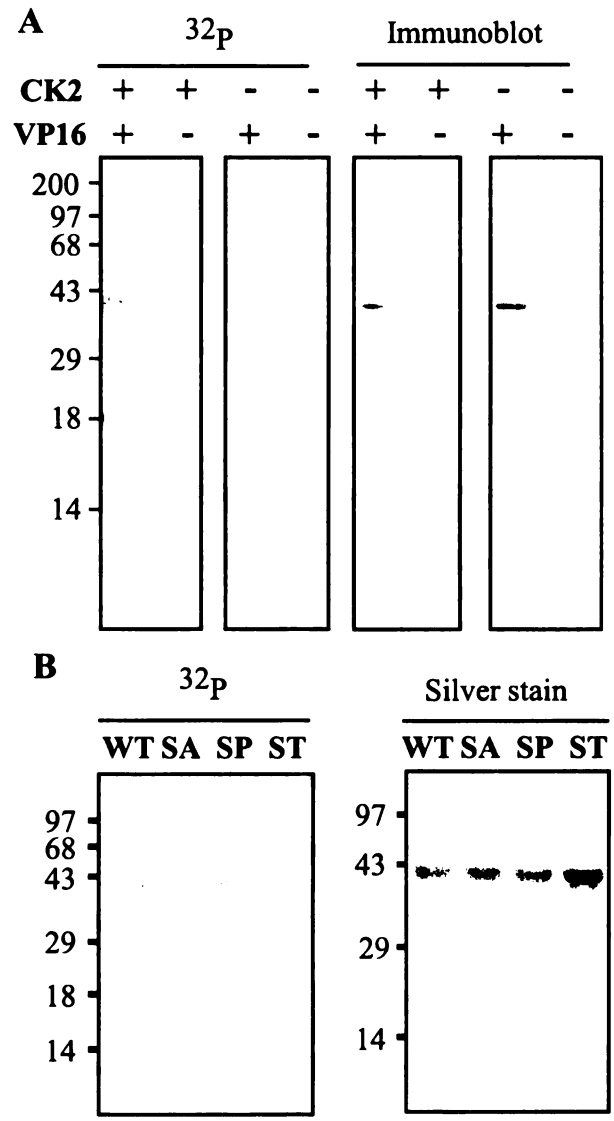


Figure 7: In vitro phosphorylation of the VP16 core domain by CK2 is independent of Ser375. Wild type (wt) and Ser375 mutant (SA, SP, ST) of the VP16 core domains (49-410) was expressed in E. coli. The purity of the protein was examined (Panel B. Silver stain). The peptides were phosphorylated *in vitro* with CK2 in the presence of γ-[^{32}P]-ATP. The labelled proteins were separated on a 10% SDS-PAGE gel and transferred to nitro cellulose. A: The VP16 core domain is phosphorylated by CK2. The appearance of the 40 KDa radiolabelled species, corresponding to VP16, is dependent on CK2. The identity of the 41 KDa species was verified by immunoblotting with the polyclonal anti-serum SO1-2. The bands at 40 and 18 KDa correspond to autophosphorylation of the CK2 α and β subunits. B: The substitution of Ser375 with alanine, proline or threonine does not abolish labeling of VP16. All of the Ser375 mutants gave rise to a labelled band corresponding to VP16. The variation in the intensity of the labeling was not reproducible between experiments.

proline substitutions would be expected to abolish phosphorylation of VP16 in the *in vitro* experiment. The threonine would most likely allow the kinase to phosphorylate VP16.

The mutations were introduced into VP16 in the GST-core domain fusion by PCR based site directed mutagenesis. The mutations were verified by sequencing and the protein was expressed in E. coli and purified on a GST-sepharose matrix. The protein was eluted by cleavage of the thrombin site that had been engineered between the GST-tag and the VP16 protein. The expression of the mutant protein was verified by coomassie brilliant blue staining. The mutant proteins were tested in the CK2 assay as described above. The wild type and mutant proteins were subjected to phosphorylation by CK2 *in vitro* in the presence of γ-[^{32}P]-ATP. Aliquots of each reaction were separated by SDS-PAGE and one gel was dried and subjected to autoradiography, one gel was stained with silver and one gel was transferred to nitrocellulose and VP16 was visualized by immunoblotting with the polyclonal serum C8 (Data not shown). A representative result of the autoradiogram and the silver staining is shown in figure 7B. In the autoradiogram depicted in the left panel, each of the four samples tested gave rise to a significant band migrating at the predicted size for the VP16 core domain. Additional labeled species were observed at 14 KDa, 16 KDa and 25 KDa. The 25 KDa species was an autophosphorylation product of the β subunit of CK2. The slower migration of the β-subunit migrated in this experiment compared to the previous may be attributed to either to differences in the commercial CK2 source, the molecular weight markers or in gel conditions. The fainter 41 KDa band that correspond to the α-subunit was occluded by the VP16 in this experiment. The other two bands were described previously and do not

affect the outcome of this experiment. The major labeled species comigrated with the purified VP16 shown in the silver stained gel. The variation in phosphorylation level was not reproducible. No gross difference in phosphorylation appeared between the wild type and the three mutants. The conclusion from this experiment was that because VP16 does not depend on Ser375 to act as a substrate for CK2 *in vitro*, another CK2 site can serve as a efficient acceptor for the CK2 kinase. .

Materials and methods

Cell lines and tissue culture

Four cell lines were used in this dissertation. HeLa and Vero cells were acquired from the American Type Culture Collection. Vero 16-8 was a generous gift of Steven Weinheimer. They were derived from Vero cells, into which a gene expressing VP16 (Weinheimer *et al.* 1992) has been stably transformed. BHK-MMTV-VP16, a Syrian hamster kidney cell-derived cell line, also stably transformed to express VP16 (Hippenmeyer *et al.* 1993). The cells were grown in Dulbecco's modified essential medium (DMEM) (Gibco) supplemented with 3.7 g/L sodium bicarbonate and 10% fetal calf serum (FCS) (Select fetal calf serum, Atlanta biologicals). Vero16-8 and BHK-VP16 were grown in the presence of geneticin at 0.9 or 0.5 μg/mL, respectively, to maintain the expression of the transgene. Cells were grown in a water-jacketed incubator at 37°C and 10% CO2. Passage was done by standard trypsinization methods.

In applications that required the seeding of a specific number of cells, the cells were counted after trypsinization and resuspension in fresh growth medium. A small

aliquot of the cell suspension was counted on a standard Haemocytometer with a chamber volume of $10^{-4}\mu L/mm^2$. The cell suspension was then diluted to the desired cell density and the diluted suspension was distributed to the required tissue culture vessels.

Viral strains

Nine viral strains were used in this dissertation (figure 3). KOS is the wild-type virus from which all of the other strains used were made. KOS was obtained from the American type culture collection. RP3 and RP5 contain truncations of the carboxyterminal activation domain (AD) after amino acid 454 and 413, respectively (Tal-Singer *et al.* 1999). 8MA carries an substitution of the VP16 ORF with the LacZ-gene (Weinheimer *et al.* 1992). In DG1, the endogenous VP16 gene has been replaced with a gene expressing a carboxyterminal EGFP-tagged wild type VP16 (Greensides 2002). The strains SJO2, SO11, SO12 and SO13 were created for this study. SJO2 carries a substitution of the codon for Ser375 with a codon for alanine. SO11, SO12 and SO13 are derived from DG1 and encode EGFP-tagged VP16. Each carries a substitution of the codon for Ser411 of VP16. SO11 carries an alanine substitution, SO12 carries a glutamate substitution and SO13 carries a threonine substitution of this codon.

Viral strains were propagated in Vero cells or Vero 16-8 cells. KOS, DG1, SJO2 and RP3 stocks were grown in Vero cells. RP5, SO11, SO12 and SO13 primary stocks were grown in Vero 16-8 cells to complement the mutation in the virus strain. RP5, SO11, SO12 and SO13 were grown in Vero cells when testing the phenotypes of these strains in transcriptional assays and in growth curves. 8MA does not proliferate without complementation and stocks were grown in Vero 16-8 cells.

To generate stocks of the viral strains, Vero or Vero 16-8 cells were seeded in 25 mL DMEM in T150 tissue culture flasks (Corning) at $5x10^6$ cells per flask as described above. After 24 hours, the cells were infected at a multiplicity of infection (moi) at 0.01. The growth medium was removed from the cells and the cell layer was washed with DMEM without serum. The viral inoculum was prepared by diluting an aliquot of viral stock, containing 5×10^8 plaque forming units. This aliquot was diluted to a total volume of 1.5 mL with DMEM. The inoculum was left on the cells for 1 hour at 37 °C. The flasks were rocked every 15 minutes to ensure equal distribution of the inoculum and to ensure that all cells remained immersed in the medium. After one hour, the inoculum was removed by aspiration and the cells were washed once with DMEM. The cells were then fed with 25 mL of DMEM supplemented with 2% FCS. The progression of the infection was monitored and the cells were harvested when 80-90% of the cells exhibited signs of cytopathic effect (CPE), such as balling-up and releasing from the tissue culture vessel. Typically, KOS, RP3, RP5, DG1, SJO2, SO11, SO12 and SO13 stocks were harvested after three days, RP5 stocks were harvested at 4-5 days. The infected cells were suspended in the growth medium and transferred to a 50 mL conical tube (Corning). The cells were pelleted gently at ambient temperatures for 5 minutes at 200×g in a Sorvall Techospin swinging bucket centrifuge (Sorvall) and approximately 15 mL of the supernatant was removed and aliquoted in 0.5 or 1 mL aliquots in cryovials (Laboratory Products Sale). This low titer stock was stored at -80 °C. The cell pellet was resuspended in the remaining supernatant (approximately 9 mL) and transferred to a 25 mL tissue culture flask. The sample was sonicated in a sonicator (Heat Systems Sonicator W-385) equipped with a cup horn to disrupt the cells and release cell-

associated virus. The sonication was done discontinuously in 1 second intervals, at 100% output and 100% load. The sonication was repeated 3 times for 30 seconds each time. Between each sonication, the sample was allowed at least one minute for the temperature to re-equilibrate. After sonication, the cellular debris was pelleted at 3000×g for 5 minutes in the Sorvall Techospin centrifuge at ambient temperature. This supernatant was termed high titer stock and transferred to cryovials in 0.25 or 0.5 mL aliquots. The stock was saved at -80 °C.

Plaque assays

Plaque assays were used to determine the titer of virus stocks and to determine the titer of individual samples from single stage growth curves. Plaque assays were done on Vero cells, except for RP5 and 8MA, where Vero 16-8 cells were used. To do the plaque assay, cells of the appropriate cell line were seeded in P60 tissue culture plates at 5x105 cells per plate in DMEM supplemented with 10% FCS and 0.9 μL/mL geneticin, if required. 24 hours after seeding, the growth medium was removed by aspiration and the cells were washed with 5 mL of DMEM. The virus samples were diluted in a tenfold dilution series in triplicate to generate inoculi that were expected to contain 10 pfu/100 μL, 100 pfu/100 μL and 1000 pfu/100 μL. 100 μL of each inoculum was added to a P60 and the infection was allowed to proceed for one hour at 37°C. The plates were rocked every 15 minutes to ensure even distribution of the inoculum. After one hour, the inoculum was removed by aspiration and the cells were washed once with 5 mL of DMEM. The cells were overlaid with DMEM supplemented with 5%FCS and melted 0.9% SeaPlaque agarose (FMC) and the SeaPlaque was allowed to congeal at ambient

temperature. The plates were incubated, until foci of infection (plaques) were clearly visible with the naked eye, typically three days, four days when RP5 was titered on Vero cells. The Seaplaque overlay was removed and the cells were stained with methylene blue (Sigma) in 70% isopropanol (J. T. Baker). The plaques were observed as well-defined, circular voids in the blue-stained cell layer. The plaques were counted and the titer of the original sample was calculated and recorded as pfu/mL. The standard deviation of the triplicate samples was calculated to assess the accuracy of the final titer.

SDS-PAGE

Protein samples from infected cell and virion lysates, *in vitro* kinase assays, immunoprecipitations, protease digestion and GST-purification of the bacterially expressed VP16 core domain were separated by SDS-PAGE. When examining infected cell and virion lysates, immunoprecipitated VP16, *in vitro* kinase assays and GST-purified VP16 core domain, the samples were separated on a 10% PAGE gel, using the laemmli buffer system (Gallagher 1999). Peptides derived from digestion of VP16 by trypsin and endolysylpeptidase C were analyzed on a 16.5% gel, using a Tris/tricine buffer system (Gallagher 1999).

Immunoblots

Immunoblots of full length wild type VP16, as well as point mutants and truncated versions of VP16 were done using the rabbit polyclonal antisera LA2-3, C8 or SO1-2. LA2-3 was raised against a GAL4(1-147)-VP16(410-490) fusion protein and recognizes the activation domain of VP16 (Sullivan *et al.* 1998) C8 is a rabbit polyclonal

antiserum. It was raised against full length VP16, derived from HSV-1 virion (Triezenberg *et al.* 1988a). SO1-2 was raised against a bacterially expressed VP16 core fragment (aa49-410) (Ottosen). The proteins were electrophorectically transferred to a 0.45 μm nitrocellulose membrane (Protran BA85, Schleicher and Schuell) in western transfer buffer (25mM Tris pH 8.3, 50 mM glycine, 20% methanol). The proteins were transferred for 48 minutes at 400 mA in a Hoefer Transphor electrophoresis unit at 4°C. After transfer, the transfer was assessed by reversible Ponceau S staining to verify even transfer of all samples. The membrane was briefly incubated with a 0.1% solution of Ponceau S in 5% acetic acid (BioRad) and the excess stain was washed out with deionized water. The membrane was blocked for one hour at ambient temperature under constant agitation with 10% non-fat dry milk in T-TBS (125 mM Tris pH 7.5, 1.5 M sodium chloride , 0.1% Tween 20) to prevent non-specific interaction between the antibody and the membrane. Approximately 1 mL of the blocking reagent and all subsequent T-TBS washes were used per cm^2 of the membrane. After removing the blocking reagent, the membrane was washed three times with T-TBS for five minutes each time with constant agitation to remove the excess blocking reagent. The primary antibody was diluted in T-TBS supplemented with 10% calf serum (Gibco). Approximately 0.2 mL of antibody solution was used per cm^2 of membrane and the antibody solution was left on the membrane for one hour under constant agitation. After incubation, the antibody solution and the membrane was washed three times for five minutes with T-TBS at ambient temperature under constant agitation. The secondary antibody was HRP-conjugated Goat-anti-rabbit IgG (BioRad, 172-1013). In each experiment, the secondary antibody was diluted at the same dilution as the primary

antibody in T-TBS supplemented with 10% calf serum. The membrane was incubated for one hour with the secondary antibody solution under constant agitation. After the incubation, the antibody solution was removed and the membrane was washed five times for five minutes each time with T-TBS. The immunoblot was visualized with the Lumi-Light chemiluminescence substrate (Roche). The chemiluminescence signal was recorded on Kodak X-Omat AR film. Multiple exposures were done with each blot, ranging from 5 seconds to 5 minutes. Holes were made through the edge of the membrane into the film to align the film after development with the prestained molecular weight markers on the membrane. The film was developed in an automated x-ray film developer under standard conditions.

Mutagenesis

PCR-mediated site directed mutagenesis was used to generate all mutations in the study. Complementing primers corresponding to the mutated codon and 9-12 flanking nucleotides on each side were purchased (Table 2). PCR reactions were set up with 125 ng of each primer, 250 ng of parent plasmid, 1×PfuTurbo PCR-buffer (Stratagene), 100 mM dNTP, 10% DMSO and 2.5 U of PfuTurbo DNA polymerase (Stratagene) in 100 µL. Thermocycling was typically done with 1 minute at 94°C, 1 minute at 55°C and 8 minutes at 74°C. After 15 rounds of thermocycling, 1 U of Dpn1 restriction enzyme (New England Biolabs) was added to the reaction to remove the parental template DNA. The resulting DNA was precipitated with 70% 2-propanol and redissolved in 10 µL of Tris·HCl (pH=8.5). 5 µL of the sample was electroporated into DH5α cells, plated on LB-plates, supplemented with 100 µg/mL ampicillin. Resistant clones were picked, re-

TABLE 2. Primers used for generation of point mutations in VP16

Mutation	Primers
Ser375Ala	5'-CCCTCGATGGTAGCCCCGTAATT-3'
	5'-AATTACGGGGCTACCATCGAGGG-3'
Ser375Pro	5'-GCCCTCGATGGTAGGCCCGTAATT-3'
	5'-AATTACGGGCCTACCATCGAGGGC-3'
Ser375Thr	5'-GCCCTCGATGGTAGTCCCGTAATT-3'
	5'-AATTACGGGACTACCATCGAGGGC-3'
Ser411Ala	5'-CGCAGACTGGCCACGGCCCCC-3'
	5'-GGGGGCCGTGGCCAGTCTGCG-3'
Ser411Glu	5'-CGCAGACTGGAGACGGCCCCC-3'
	5'-GGGGGCCGTCTCCAGTCTGCG-3'
Ser411Thr	5'-CGCAGACTGACGACGGCCCCC-3'
	5'-GGGGGCCGTCGTCAGTCTCGC-3'

streaked and re-picked. The insertion of the correct mutation was verified by automated sequencing. Ser375Ala were introduced into the VP16 gene of pKOS-VP16.2 (Tal-Singer *et al.* 1999). Ser375Ala, Ser375Pro and Ser375Thr mutations were introduced into the pGST-VP16 bacterial expression vector pGST-VP16 (49-412) (Liu *et al.* 1999). Ser411Ala, Ser411Glu and Ser411Thr mutations were introduced into the pDRG29-1 plasmid (Greensides 2002).

GST-protein purification

The proteins were expressed in the E. coli strain BL21 DE3 Codon Plus. One liter of LB was inoculated with the bacteria. The expression of the protein was induced with 0.5 μM IPTG for 3 hours at 30°C. The cells were harvested by centrifugation at 5000×g for 15 minutes in a Sorvall Superspeed with the SA rotor at 4°C.. The cell pellet was resuspended on ice in 10 mL HEMGT-250 buffer (25 mM HEPES pH7.9, 0.1 mM EDTA, 12.5 mM magnesium chloride, 10% glycerol, 0.1% Tween-20, 250 mM potassium chloride, 1 mM DTT, 1×Complete Protease Inhibitor cocktail (Roche)) . The cells were lysed in a french press. The lysate was cleared at 10,000×g in a SS32 rotor in a Sorvall superspeed centrifuge at 4°C for 10 minutes. The GST-protein was bound to 1 mL of a 50% slurry of GSH-sepharose beads (Roche) for three hours at 4°C under constant agitation. The beads were washed three times for 15 minutes each time in HEMGT-250, two times in HEMGT-100 (as HEMGT-250, except only 100 mM potassium chloride) and resuspended in 1 mL of thrombin cleavage buffer (20 mM Tris·HCl, pH=8.5, 150 mM sodium chloride, 25 mM calcium chloride). The proteins were eluted from the beads by proteolysis. 5U of biotinylated thrombin were added to

the slurry and the mixture was digested for 16 hours at 20°C. After digestion, 50 μL of a 50% slurry of streptavidin-sepharose beads were added to the slurry to remove the thrombin. The sepharose beads were pelleted by centrifugation at 5000×g in an Eppendorf tabletop centrifuge. The supernatant was recovered, the protein concentration was determined by Bradford analysis and stored at -80°C. The purity of the protein was evaluated by SDS-PAGE followed by Coomassie staining.

Coomassie and silver staining.

Coomassie staining was done by fixing the protein gel in 40% methanol and 10% acetic acid for 15 minutes. The fixing solution was replaced with the staining solution (40% methanol, 10% acetic acid and 0.05% Coomassie brilliant blue R250 (Pierce)). The gel was stained for 1 hour, then the stain was removed and the excess dye was washed out with fixing solution, until background staining was no-longer discernible. Gels were dried between two layers of cellophane, except when preparing LC-MS/MS samples. Gels that were to be silver stained were also fixed in fixing solution. They were then treated with 175 μM DTT for 15 minutes and stained with 0.1% silver nitrate. The stain was developed with 3% sodium carbonate/ 0.05% formaldehyde until the bands had reached desired intensity. The reaction was stopped with fixing solution and the gel was dried between sheets of cellophane.

Casein kinase 2 assay

200 ng of purified wild type or mutant VP16 core domain was incubated with 5 units of CK2 (New England Biolabs) for 30 minutes at 30°C in 1×CK2 reaction buffer

(20 mM Tris·HCl (pH 7.5), 50 mM potassium chloride, 10 mM magnesium chloride, 200 μM ATP and 1 μCi of γ-[^{32}P]-ATP). The labeled protein was separated on a 10% SDS-PAGE gel. The gel was either transferred to nitrocellulose or coomassie stained and dried between two pieces of cellophane. The [^{32}P]-label was detected by autoradiography.

Immunoprecipitation

[^{32}P]-labeled VP16 was isolated from infected cells and from virion by immunoprecipitation. 1×10^7 HeLa cells were infected with KOS, RP3 or RP5 as described above. Infections were typically done at an moi of 5-10, with the exception of RP5, where infections were done at an moi of 0.1. After 1 hour, the DMEM was replaced with phosphate free DMEM supplemented with 2% dialyzed FCS. After another 30 minutes, the medium was replaced with fresh phosphate free DMEM supplemented with 2% FCS. Then, 1 mCi of [^{32}P]-orthophosphate was added per 1×10^7 cells. The infection was allowed to proceed for 8 hours, if isolating VP16 late in infection or 14 hours, if isolating VP16 from virions. After 8 hours, the infected cells were washed with PBSa, dislodged from the culture dish with a scraper, resuspended in 5 mL of PBSa and transferred to a 15 mL conical tube. The cells were pelleted gently at ambient temperatures for 5 minutes at 200×g in a Sorvall Techospin swinging bucket centrifuge (Sorvall). The supernatant was removed and the cells were disrupted by addition of 500 μL of IP lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1.0% sodium deoxycholate, 1.0% Triton X-100, 2 mM PMSF and 1xComplete (Roche)). The cells were left on ice for 30 minutes and the tubes were then sonicated

directly in the cup horn to ensure complete disruption of the cells. The sonication was done discontinuously in 1 second intervals, at 100% output and 100% load. The sonication was repeated 3 times for 30 seconds each time. Between each sonication, the sample was left on ice for at least one minute for the temperature to re-equilibrate. The sample was transferred to a clean 1.5 mL eppendorf tube and 100 μL of a 10% suspension of heat-killed, formalin-fixed staphylococcus aureus (SAC) was added to remove factors that would bind non-specifically to the SAC during the IP. The sample was incubated 3 hours at 4°C with constant agitation. The SAC was pelleted for 10 minutes at 16000×g at 4°C in an eppendorf microcentrifuge and the supernatant was transferred to a new tube. 5 μL of the monoclonal antibody LP1 was added to the supernatant and the sample was incubated for 1 hour at 4°C with constant agitation. 50 μL of SAC was then added and the incubation continued for another 2 hours. The SAC was then pelleted at 200×g for 2 minutes in the microcentrifuge, the supernatant was removed and the pellet resuspended in lysis buffer. This washing step was repeated for a total of 3 times. After the last wash, the SAC were pelleted again and briefly washed in 1 mM phosphate buffer at pH 8. The SAC were pelleted again and 50 μL of 10 mM phosphate buffer at pH 12.5 was added to elute the bound VP16. The sample was incubated for 30 minutes, the SAC was pelleted and the supernatant was transferred to a fresh tube, containing 20 μL of 100 mM phosphate buffer, pH 6.8, to neutralize the pH of the sample.

Peptide mapping

In gel digestion: Immunoprecipitated [^{32}P]-labeled VP16 isolated infected HeLa cells 8 hpi was separated on a 10% SDS-PAGE gel. The migration of the labeled VP16 species was determined by autoradiography of the wet gel. The band was excised from the gel, then washed twice in 0.1 M ammonium carbonate, 50% acetonitrile for 50 minutes at 30°C. The wash was removed and the gel slice was dried under a N_2-stream. The gel was rehydrated with 4 μL of 0.1 M ammonium carbonate, pH 7.5, 0.02% Tween 20. 1 μL of digestion buffer with either 2 μg of trypsin or endolysylpeptidase C was added immediately. The sample was digested overnight at 30°C. The protein was eluted from the gel slice by gently sonicating the sample in a sonicating waterbath for five minutes. The supernatant was recovered from the mixture and another 5 μL aliquot of digestion buffer was added. The sonication was repeated and the supernatant pooled with the first aliquot. The sample was separated on a 16.5% tris/tricine gel and then either transferred to nitro cellulose or soaked in several washes of 10% methanol/7% acetic acid, then dried onto Whatman 3MM paper. The labeled protein on either the membrane or the dried gel was visualized by autoradiography.

Phosphoaminoacid analysis

Immunoprecipitated [^{32}P]-labeled VP16 isolated infected HeLa cells 8 hpi was separated on a 10% SDS-PAGE gel. The gel was transferred to a PVDF membrane and the labeled species corresponding to VP16 was identified by autoradiography. The band was excised from the membrane and washed in distilled water. 6N hydrochloric acid was added to cover the membrane and the sample was digested for 1 hour at 100°C. The hydrochloric acid was removed from the sample by evaporation under vacuum. The

dried samples were mixed with phosphoamino acid standards (2 μg each in 5 μL), then spotted on a cellulose TLC plate. The samples were resolved by thin layer electrophoresis was run at 1000V for 50 minutes at 0°C at pH=2.5 (2:1 mixture of pH 3.5 electrophoresis buffer (5% glacial acetic acid, 0.5% pyridine in 0.5 mM EDTA) and pH 1.9 electrophoresis buffer (2.2% formic acid, 7.8% glacial acetic acid). The plate was dried and sprayed with ninhydrin (0.5% in acetone), then developed at 70°C, until the standards were clearly visible. The radiolabeled amino acids were visualized by autoradiography (Vacratsis *et al.* 2002).

Discussion

Identification of phosphorylation sites in VP16 during late stages of infection

The goal of this thesis was to examine the function of phosphorylation of VP16 in viral infection. An intermediate goal was to identify the phosphorylation sites of VP16 in infected cells and compare that to what is known about the phosphorylation of VP16 expressed from a plasmid. I positively identified three phosphorylation sites by MS/MS. These sites were Ser18, Ser353 and Ser452. I also inferred phosphorylation of Ser411, based on the peptide mapping and the absence of phosphorylation at Ser375 and Ser400, detected by MS/MS. The *in vitro* phosphorylation of the VP16 core domain by CK2 was examined. Both wild type VP16 and VP16 with mutations at Ser375 were tested. Both the wild-type and the mutant VP16s were found to be a substrate for CK2. MS/MS did not detect acetylation of VP16 late in infection.

VP16 in phosphorylated at several sites late in infection

Addressing the phosphorylation by MS/MS demonstrated that at least three sites were phosphorylated. These sites were Ser18, Ser 353 and Ser452. The presence of a site at Ser452 could explain the phosphorylation of the aa 371-490, aa 371-454 and aa 410-490 fragments, but could not account for the phosphorylation of the 371-412 fragment observed for RP5 infection. Thus, at least one additional site must be present in this fragment. There was MS data to show unphosphorylated species derived from Ser 375 and Ser 400. There was no data for the site at Ser411. Phosphorylation of Ser 411 would be consistent with the data from both the MS and the peptide mapping. There were no contradictions between the mass spectrometry data and the peptide mapping.

The observations from both the MS and the peptide mapping must be understood to only represent the majority or at least significant minority populations of the VP16 late in infection. It is possible that minority populations have gone undetected. It is possible, maybe even likely that the phosphorylation during other stages of infection is very different from what is presented here. It must be taken into account that for at least one of these serines (Ser375), the function that it has been implicated in takes place early in infection. Maybe only the minority of protein required for this activity is modified and subsequently not detected by these methods.

In vitro phosphorylation of the core domain of VP16 was shown not to depend on Ser375. It was concluded that there is another CK2 site within VP16. While the *in vivo* phosphorylation site at Ser353 does conform to the CK2 consensus, no tests were performed to show this to be the *in vivo* phosphorylation site. The *in vitro* kinase data are in disagreement with the published findings of O'Reilly *et al* (O'Reilly *et al.* 1997). They present data that shows VP16 to be dependent on Ser375 for *in vitro* phosphorylation. Here I show that the substitution of Ser375 with Ala or Pro does not abolish the *in vitro* phosphorylation of VP16 by CK2. These results only show that Ser375 is not the sole possible phosphorylation site in VP16 *in vitro*. To determine whether Ser375 is a CK2 site *in vitro*, it would be necessary to map the sites of phosphorylation of VP16 subjected to CK2 phosphorylation, either by peptide mapping or by MS. At the very least, it would be necessary to quantify the specific labeling of the wild type and the mutants in the *in vitro* labeling assay.

Correlation between Phosphobase and the *in vivo* phosphorylation site mapping/redundancy of determination

Of the four identified phosphorylation sites, all were predicted to be phosphorylation sites by the phosphobase prediction server. All of the 13 serines predicted not to be phosphorylated by Phosphobase were determined not to be phosphorylated *in vivo*. Of the 13 serines predicted to be phosphorylated, four were, eight were not. The phosphorylation state of Ser419 could not be determined by either peptide mapping or MS/MS . None of the threonines and tyrosines that were predicted to be phosphorylated were phosphorylated. The summary of these results suggests that the prediction server was quite reliable in predicting unphosphorylated sites under the conditions tested. While most of the predicted phosphorylation sites were not found to be phosphorylated under these conditions, it is possible that they are phosphorylated under different conditions, such as earlier in infection or in different cell types.

Resolution of the minor peptides

VP16 derived from HeLa cells infected with the truncation mutants RP3 and RP5 were also phosphorylated, implying that at least one site of VP16 phosphorylation lies between aa 1-412. Analysis of the phosphoamino acid complement of VP16 from either of these three strains of HSV-1 clearly demonstrated that the only targets of phosphorylation were serines. Also, peptide mapping of these strains clearly demonstrated that no significant phosphorylation of VP16 occurred between aa 29-344. The peptide mapping yielded evidence that phosphorylation took place in fragments representing aa 371-490, aa 371-453, aa 371-412 and aa 410-490. Some evidence also

suggested that the 410-454 fragment was phosphorylated. These experiments did not determine whether this phosphorylation was the result of one or more phosphorylation events, nor whether the 1-28 fragment was phosphorylated. The labeled fragment detected in the trypsin digestion does not encompass the serine 375 described by O'Hare and colleagues (O'Reilly *et al.* 1997). The fragment containing Ser375 (aa371-399) would migrate at 3 KDa. No labeled species were seen at this molecular weight. But as mentioned previously, it was not clear whether fragments of this size would resolve on the gel. The issue of whether the small fragments of either trypsin or lysylendopeptidase C digestions resolved on the gel was crucial for conclusions concerning phosphorylation at several sites. The most critical fragments were the 1-28 and 344-370 fragments of the lysylendopeptidase C digestion and the 371-399 fragment of the trypsin digestion. The 400-408 fragment of the trypsin digestion did encompass putative phosphorylation sites, but it was clear that a fragment as small as this would not resolve. Furthermore, the trypsin digestion would not give any information about the sites corresponding to the 1-28 and the 344-370 fragments of the lysylendopeptidase C digestion. These fragments would be further digested and would clearly not be detectable on the gel. The molecular weight marker expected to migrate at an apparent molecular weight of 2.9 KDa did resolve on the Tris/tricine gel suggesting that the fragments of interest might resolve. But as mentioned, small fragments are more likely to migrate aberrantly and it was possible that the mobility of the marker was reduced or that one or more of the minor fragments would migrate abnormally fast.

It was necessary to consider the ambiguous conclusions about the phosphorylation status of the minor peptides in the lysylendopeptidase and trypsin before trying to draw

additional conclusions about specific phosphorylation sites. If it was hypothesized that none of these minor peptides were in fact phosphorylated, the result of the immunoprecipitation of the truncated VP16 suggested that all significant phosphorylation sites of the 410-490 tryptic fragment of full length VP16 were present in a motif common to the wild type and the two truncation mutants. The only common segment was aa 410-412 ($LST_{(410-412)}$). Two of these were potential phosphorylation sites. This would not exclude the possibility of additional sites in the activation domain, either in wild type VP16 or in the truncated VP16 from RP3.

Another possibility was that phosphorylation of either of the two minor peptides (aa 371-399 and aa 400-410) of the trypsin digestion was not detected. Phosphorylation of any site within those peptides would be common to all three version of VP16. It could not account for the phosphorylation of the carboxyterminal tryptic fragment of full length VP16, but it would raise the possibility that RP5 was labeled between 371 and 410. In that case, it would not be clear whether VP16 was phosphorylated between 410 and 412.

Finally, it was apparent that phosphorylation of either of the two minor peptides (aa 1-29 and aa 344-370) resulting from the lysylendopeptidase C digestion went undetected. The bulk of the phosphorylation of all versions of VP16 could be attributed to phosphorylation in these peptides. The phosphorylation within the carboxyterminal regions of VP16 might represent a small fraction of the overall phosphorylation of VP16.

Homologs and structural function of the identified phosphorylation sites

In order to gain insight in the function of these phosphorylation sites, one can align the phosphorylated domains with the amino acid sequences of homologs of VP16

from α-herpesviruses (Figure 8). This sequence alignment offers a different view of these serines. While Ser375 is highly conserved throughout this group of proteins, Ser18, Ser353 and Ser411 are conserved only to a limited degree. Ser18 is conserved only in between HSV-1 and HSV-2, which already has an overall 90 % conservation. Serine 353 of HSV-1 aligns with threonines in EHV-1 and -4. But despite being flanked by highly conserved tracts, the putative phosphorylatable residue falls in a very poorly conserved motif, even when compared with HSV-2. And although EHV-1 and -4 both have a conservation of the S/T-P motif, they are otherwise quite dissimilar. Serine 411 also only has limited homologies in the related proteins. In this case, only HSV-2 and PRV have conserved acceptors, although in these, the local environment is more conserved than in the previous examples and may constitute a canonical phosphorylation site. Ser452 falls in the carboxyterminal activation domain which is poorly conserved between the homologs.

Interestingly, Ser353 of VP16 aligns well with serines in both Zhangfei and Luman, if the HCF-1 binding motif (HBM) is used as a landmark (figure 9). The sequence of the canonical HBM motif is E/D-H-x-Y and is conserved in both Luman and Zhangfei. When aligned with the HBM of VP16, a conserved motif appears 7-8 aa aminoterminal to the first residue of the HBM. The sequence of this motif in VP16 is PheThrThrSer and can be generalized from the alignment to Φ-S/T-S/T-S where the last S is invariant and Φ denotes a hydrophobic residue. This motif is conserved between mouse, rat and cow homologs of Zhangfei and Luman and with the HBM, the entire motif becomes Φ-S/T-S/T-S-x_{7-8}-E/D-H-x-Y-s. The E/D-H-x-Y part of this motif has been shown to be critical for interaction with HCF but the amino terminal residues of the

Figure 8: Phosphorylation site conservation in herpes homologs. A-D: Alignment of identified phosphorylation sites in VP16 with VP16 homologs from other herpes viruses. The phosphorylation sites of VP16 are indicated by an asterisk. VP16-2: HSV-2, VZV: verocella zoster virus, PRV: pseudo rabies virus, EHV-1: equiid herpes virus 1, BHV-1: bovine herpes virus 1, MDV: Marek's disease virus.

```
A:                                          *
VP16        4   E L F A D M N A D G A S P P P P R P A G G P
VP16-2      4   D L F A D - - A D G V S P P P P R P A G G P
VZV         1   - - M E C N L G T E H P S T D T W N R S - -
PRV         1   - - - - - - - - - - - - - - - - - - - - - -
BHV-1       4   R I K T A G R A L A S Q C G G A A A A T - -
EHV-1      16   W Y F D A R P A A S I V M F A A A E E N D D
MDV         4   M S F E N D Y Y S P I Q L F A E I E A Y A N


B:                                          *
VP16      339   A K L D S Y S S F T T S P S - E A V M R E H
VP16-2    337   A K L D S Y S S V A T S E G - E S V M R E H
VZV       345   R K L D A Y A V K H P Q E P - R H V R A D H
PRV       301   I K M E A Y S R E Y - - - - - - - - R D H
BHV-1     344   A K M G A P A E A G G R - - - L A P E R E H
EHV-1     376   T K M E A Y S D A H P A T P - L F P L A E H
MDV       361   S K L E V Y S L S H P P N P Q L H V H K E H


C:                                          *
VP16      400   L P A G - - H T R R L S - T A P P T D V S L
VP16-2    399   L S A G Q - R P R R L S T T A P I T D V S L
VZV       393   L P G D P P R P P T C G F L T R
PRV       343   L P C D P T P P P R V S - A A P L I T T V T
BHV-1     391   L P C D P A P A A T V R V A S P A T H L A Q
EHV-1     426   L P G D P V P P L T V G V R Q T A A T L A I
MDV       413   S P G D P - - - - - V A T T I S T L


D:                                      *
VP16      441   L D M L G D G D S P G P G F T P H D S A P Y
VP16-2    442   L E M L G D V E S P S P G M T - H D P V S Y
VZV         -   - - - - - - - - - - - - - - - - - - - - - -
PRV       380   A V A T A T V A S P G P A - T - H A Y H L I
BHV-1     474   A V A - - - G P S P A N P F G G T Y D A L L
EHV-1     434   T L A - - - I P S N L T L Q S M E T D G L D
MDV         -   - - - - - - - - - - - - - - - - - - - - - -


E:                                          *
VP16      362   S R A R T K N - N Y G S T I E G L L D L P D
VP16-2    360   S R G R T R N - N Y G S T I E G L L D L P D
VZV       366   A K V V E N R - N Y G S S I E A M I L A P P
PRV       322   C R P P S P V A S Y G S T A E A L L P - P P
BHV-1     367   A R P R G A I - N Y G T T P E A M L R - P P
EHV-1     399   S K R I G G R L S Y G T T T E A M M D - P P
MDV       384   V Q K L E S P P N Y G T T V E A L L M D S S
```

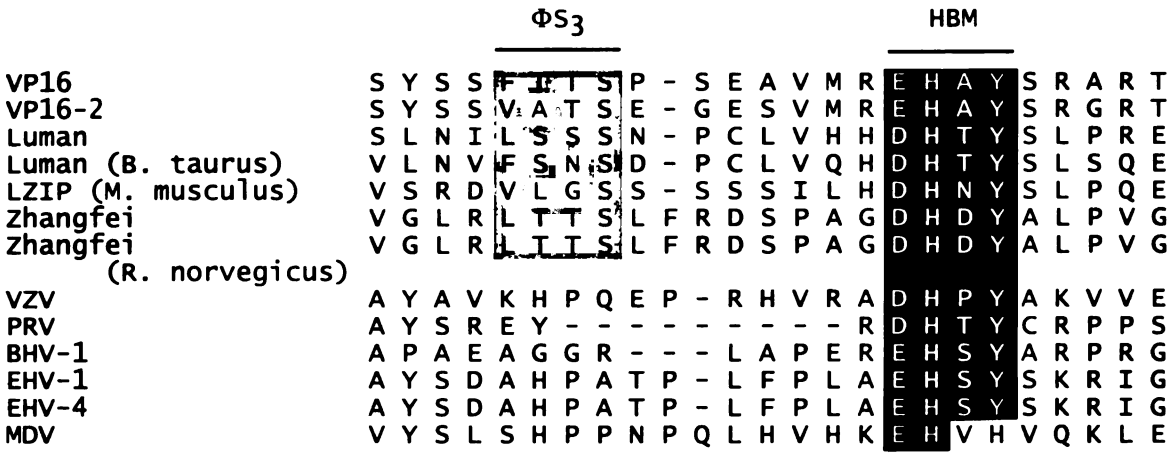


Figure 9: Alignment of VP16 and the Luman family. A small motif aminoterminal to the HBM is conserved between VP16 and Luman and its homologs, but not with the viral homologs of VP16. The HBM consensus motif is E/DHxY. It is indicated with a black box. The putative conserved aminoterminal motif has the consensus Φ–S/TS/T-S where Φ indicates a hydrophobic residue. The motif is indicated with a grey box and labelled ΦS_3.

putative motif have never been tested for their role in HCF-1 interaction and whether this motif is necessary for function remains to be shown.

Function of the identified phosphorylation sites.

None of the phosphorylation sites that were identified have been implicated in any function of VP16. None of them are highly conserved within the herpes homologs and none have been seen in functional mutagenic screens. But the sites may still offer clues to their function.

Ser18 lies in a putative AD in the amino terminus of VP16. The domain is conserved in the VZV homolog of VP16, Orf10. This protein does not have the carboxy terminal activation domain and relies solely on the amino terminal AD (Moriuchi *et al.* 1995). The aminoterminal activation domain of VZV Orf10 can be functionally replaced with the aminoterminal 25 aa of VP16. In HSV-1, this domain is not required for activation of IE promoters in cultured cells (Triezenberg *et al.* 1988a). The domain is also dispensable for the formation of the VIC. However, the functional conservation of the domain suggests that this domain might be involved in transcriptional activation under different conditions. In this case, the phosphorylation at Ser18 could be involved in the regulation of that function.

Ser353 lies adjacent to a region of VP16 required for the interaction of VP16 with the Oct-1 and HCF-1. These proteins are required for the formation of the VIC, the DNA binding complex of VP16. None of the surveys of the VIC interaction domains has tested mutations at Ser353 (Greaves *et al.* 1990; Lai *et al.* 1997). It is possible that the phosphorylation of Ser353 affects the formation of the VIC.

The site is also adjacent to a region required for the interaction with the vhs factor. The nearby Lys344 is a key determinant for interaction with vhs, but deletion mutagenesis of the region clearly shows that Ser353 is dispensable for the interaction. (Smibert *et al.* 1994; Smiley *et al.* 2001; Knez *et al.* 2003). Vhs is involved in the degradation of mRNA early in infection and the interaction with VP16 may be involved in the repression of the mRNase activity late in infection (Matis *et al.* 2001; Smiley *et al.* 2001; Taddeo *et al.* 2002). Because vhs and Oct-1 are competitors for the interaction with VP16, it is possible that the phosphorylation event at Ser353 regulates both interaction by blocking the interaction of one of these factors, thereby favoring the other interaction.

Ser411 falls in the disordered C-terminus of the peptide used in the crystal structure (Liu *et al.* 1999). It may simply have been disordered due to the proximity to the carboxy terminus. Phosphorylation of this site is close to both the Oct-1 and HCF-1 interaction sites and to the activation domain. This site could be involved in regulation of either. The site marks the amino terminal boundary of what is considered the canonical bipartite activation domain of VP16. The prevailing model proposes this segment of VP16 to be an inert linker between the DNA binding core domain and the activation domain of VP16. However, no thorough survey has been done on this putative linker and again, the possibility remains that although not directly involved in the mechanism of transcriptional activation, the site serves as a regulatory switch.

Ser452 lies between the two subdomains of the carboxyterminal activation domain of VP16. Both of these subdomains have been examined in great detail (Triezenberg *et al.* 1988a; Cress *et al.* 1991; Regier *et al.* 1993; Shen *et al.* 1996a;

Sullivan *et al.* 1998) but Ser452 lies in a small motif between them that has not been investigated. It is possible that the site is involved in regulation of the transcriptional activation by these domains.

Ser18, Ser353 and Ser452 are all followed by a proline. The SerPro (SP) motif is a target site for a group of kinases typified by the Jun kinase (JNK). The function of the phosphorylation of SP sites has been associated with the binding of Pin1 prolylisomerases, which in turn can cause conformational changes in the protein and subsequently may serve as a regulatory event.

Reports of phosphorylation of VP16.

The identification of phosphorylation sites in VP16 during infection and in the virion is consistent with observations by several groups. The literature on the phosphorylation of VP16 has shown that VP16 is phosphorylated in infection (Gibson *et al.* 1974; Wilcox *et al.* 1980; Morrison *et al.* 1998). Morrison *et al* immunoprecipitated VP16 from virion preparations and demonstrated that VP16 was phosphorylated (Morrison *et al.* 1998). I also found phosphorylated VP16 in the virion. They also saw phosphorylation of VP16 at IE stages of infection. I was unable to see detect phosphorylated VP16 this early in infection. However, they infected the cells at an MOI of 50, providing five to ten fold higher amounts of VP16 as an immunoprecipitation target than was used in my experiment.

The literature is in disagreement about the *in vitro* phosphorylation of VP16 by CK2. There is evidence that bacterially derived VP16 is a CK2 substrate *in vitro* (O'Reilly *et al.* 1997). This is countered by evidence that VP16 derived from virion is

not a CK2 substrate (Morrison *et al.* 1998). One might argue that this difference could be explained by the prior modification of the CK2 sites of the virion-derived VP16. Both of these studies find that VP16 is a PKA substrate. O'Reilly *et al* further report that the loss of the phosphorylation site at Ser375 abolishes phosphorylation by CK2 and that the site must be the only *in vitro* CK2 site in VP16. In my hands, I confirm that bacterially expressed VP16 can serve as a substrate for CK2. However, in contrast to the findings of O'Reilly *et al*, (O'Reilly *et al.* 1997) the loss of Ser375 does not abolish phosphorylation of VP16 by CK2 and therefore can not be the sole CK2 site in the protein. These results are clearly contrasting and because of the similarity of the experimental procedures, they are hard to reconcile. I believe my argument is bolstered by the fact that there are several additional CK2 consensus sites in VP16. In the *in vitro* kinase assay, it is likely that at least one of these sites would serve as a substrate.

An attempt of identifying the phosphorylation sites of VP16 *in vivo* was done by O'Reilly *et al.* The primary difference between their approach and mine was the source of the material. I chose to examine VP16 derived from cells infected with HSV-1, while they looked at VP16 that had been expressed from a plasmid in HeLa cells. The results from both projects were to some extent similar, but there were also significant differences. Both approaches employed the lysylendopeptidase mapping of VP16 to identify the region of phosphorylation of VP16. In both cases, a single labeled fragment was recovered from the digestion, corresponding to the aa371-490 fragment. The phosphorylation of the 371-413 fragment of VP16 from infection with the RP5 strain in my experiment was mirrored by the observation of phosphorylation of the 371-411

fragment derived from a similar truncation mutant, expressed in HeLa cells. They also saw phosphoserine as the only phosphorylated residue in VP16.

O'Reilly *et al* did not test the effect of the Ser375Ala mutation on phosphorylation *in vivo*. Rather, they examined the phosphorylation of Ser375Ala mutants in *in vitro* kinase assays. They either used CK2 or HeLa nuclear extract as the source of the kinase. They did not see a major effect of the alanine substitution on the overall phosphorylation of VP16 by the nuclear extract. Furthermore, they also saw phosphorylation of the 371-411 by the nuclear extract fragment of endolysylpeptidase C digestion of both the wild type and the Ser375Ala mutation. Regardless, they took a small difference in migration between the two species to indicate that Ser375 was in fact a phosphorylation site. O'Reilly *et al* supported this by showing that VP16 can be phosphorylated by CK2, but VP16-Ser375Ala can not. They proposed that the phosphorylation of the fragment, despite the loss of Ser375, was due to additional phosphorylation, presumably at either Ser400 or Ser411.

It is my contention that the phosphorylation data presented by O'Hare *et al* can be interpreted in a manner not consistent with phosphorylation at Ser375. This interpretation is independent of the source of the material. Their conclusions rely on two results, the *in vitro* CK2 phosphorylation data that I have discussed above and the assertion that a small difference in migration between two the lysylendopeptidase fragments of VP16 can be attributed to the loss of a phosphorylation site. They make this conclusion without addressing the migration difference experimentally. While this difference in migration could be a phosphorylation effect, it could also simply be due to an alteration in gel retention, for instance due to the loss of the hydroxyl group of Ser375.

They report that the specific labeling of the phosphoamino acids of the mutant VP16 is reproducibly less than that of the wild type. This would be consistent with the loss of a phosphorylation site, but they do not rigorously address the quantification of this result. They also chose to downplay the major finding, that no significant loss of phosphorylation of the Ser375Ala mutants by the nuclear extract is observed.

Regardless of the phosphorylation data, the results of the O'Hare group and others (Greaves *et al.* 1990; Lai *et al.* 1997; O'Reilly *et al.* 1997) show clearly that Ser375 is required for the interaction between VP16 and Oct-1 and for the activation of VP16-responsive genes. Replacement of Ser375 with alanine, aspartate or glutamate (Greaves *et al.* 1990; Lai *et al.* 1997; O'Reilly *et al.* 1997) or with proline (Greaves *et al.* 1990) renders the protein unable to interact with Oct-1, unable to form the VIC and unable to activate the IE promoters. Replacement with threonine does not diminish this interaction and does not reduce the transcriptional potential of the protein (Greaves *et al.* 1990; O'Reilly *et al.* 1997). Whether phosphorylation of Ser375 is required for this activity is not clear. In fact, the formation of the VIC is possible with factors entirely expressed in E. coli and without the use of a eukaryotic nuclear extract (Babb *et al.* 2001). Assuming that the factors are not phosphorylated in the bacterial expression host, it is reasonable to conclude that phosphorylation of VP16 is not required for this interaction. Even so, the phosphorylation of VP16 at Ser375 could increase the affinity of VP16 for Oct-1 and support the formation of a stronger VIC.

Chapter Three

Mutational analysis of the potential phosphorylation sites in herpes simplex infection at serine 375 and serine 411.

Introduction

The function of neither Ser375 nor Ser411 has been adequately described in the context of viral infection. In the previous chapter, a number of phosphorylation sites were identified. None of the sites have been examined in infection previously. The function of these sites need to be determined. Ser375, which was expected to be phosphorylated, was found not to be. This residue has been shown previously to have an important role in the formation of the VIC complex *in vitro* and in transcription *in vivo*, when VP16 is expressed from a plasmid (Greaves *et al.* 1990; Lai *et al.* 1997). The function of the residue has not been examined in infection, but if the function of VP16 in infection mimics that of VP16 in transfection, it would be reasonable to suggest that Ser375 is essential for the expression of the IE genes. Because Ser375 has been shown to be important for interaction between Oct1 and VP16, and thus for the expression of the IE genes, we hypothesized that a virus with mutations at Ser375 would be deficient in IE gene activation and thus in growth. A reduction or loss of proliferation had been observed previously in other transcription-deficient versions of VP16. Both the RP5 strain used in this study and the in1814 strain, which contains a 4 amino acid insertion at aa379, are deficient in IE gene activation as well as in growth. In the case of RP5, the strain is viable, but the yield of infectious particles is reduced 100 fold, as compared to KOS (Tal-Singer *et al.* 1999). Outside of a complementing cell line, in1814 replicates

poorly, although the proliferation is improved if infections are done at a high moi (Ace *et al.* 1989). Based on the phenotypes of these two strains, one might expect that if a mutation at 375 affects IE gene transcription, it might also affect the growth of the virus.

The phosphorylation of Ser411 site has not been examined previously. But it is known that VP16 purified from virion is a PKA substrate *in vitro*. Ser411 lies in one of 6 serine consensus sites for PKA phosphorylation. But while the Phosphobase prediction did suggest that this site was a good target for phosphorylation, its position in the linker between the core domain and activation domain does not lend itself as a obvious site for regulation. Notably, both the core domain and the activation domain is capable of functioning without this particular domain. Just as Ser375, it is also most likely a surface exposed residue. In the crystal structure of the core domain, adjacent residues are located at the bottom of the "Chair", although Ser411 is not ordered in the structure and the exact position is not known (Liu *et al.* 1999). The site is poorly conserved among the herpes virus homologs (Figure 8). Any function of the region would therefore have to be host-specific, either because it is not required in other animal models or because the function is carried out in a different manner. Regardless of these facts, the site appeared to be robustly phosphorylated and was in proximity of both the activation domain and the VIC formation domain, both of which are regions that are putative targets of regulation. This site was identified using the peptide mapping approach, even though the MS failed to cover it. However, because the carboxyterminal lysylendopeptidase C fragment of VP16 from RP5 was labeled and the MS found no evidence of phosphorylation at the only other two serines in the fragment, it was most likely that Ser411 is a phosphorylation site.

The phosphorylation sites that were found at Ser18, Ser353 and Ser452 late in infection are obvious choices for a mutagenic study. However, these sites were discovered at such a late time in the course of this project that it was not feasible to generate and test mutants of these sites in the virus. These sites should be examined at a later date, as their phosphorylation may take part in regulating functions of VP16.

Determining the effect of substitution of Ser375 and Ser411 on the proliferation and transcriptional phenotype in infection.

To test the roles of Ser375 and Ser411 in viral infection, mutations of the corresponding codons were introduced into the viral genome. An alanine substitution of Ser375 had little or no effect on the phosphorylation level of VP16 and little or no effect on the growth of the virus in tissue culture. The mutation had a significant effect on the expression of the IE genes in infection when the transcriptional effect of VP16 was tested directly. When other factors of the viral expression cascade were allowed to act on the VP16-responsive genes, this defect was masked. To examine the function of phosphorylation of Ser411, a panel of three virus strains were generated carrying alanine, glutamate and threonine substitutions at Ser411, respectively. These mutations had no apparent effect on the proliferation of the virus nor on the activation of the IE genes.

Results:

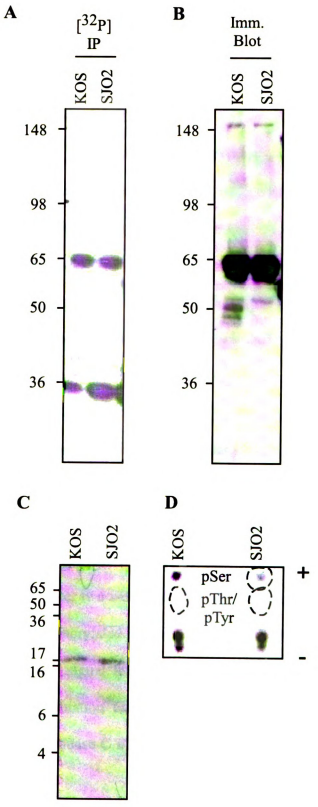
Function of Ser375 *in vivo*.

To determine whether Ser375 is involved in the function of VP16 during infection by HSV-1, a virus strain was generated in which Ser375 of VP16 was replaced with an

alanine. The strain was named SJO2 (Figure 3). The presence of the mutation in the recombinant viral genome was confirmed by sequencing a PCR product spanning the locus of interest amplified from the viral DNA. Southern blotting with a probe spanning the open reading frame and parts of the 3'- and 5'-UTR demonstrated that VP16 had been inserted into the VP16 locus of the VP16-null strain 8MA. The mutant protein was properly expressed in infected cells, and could be immunoprecipitated and detected by immunoblotting using an antibody directed against the activation domain (LA2) (See figure 10B).

To determine whether the mutation affected the phosphorylation of VP16, the gross level of phosphorylation of VP16 isolated from HeLa cells infected with SJO2 was compared to that of VP16 from KOS-infected cells. This determination was done similarly to the previous study of KOS, RP3 and RP5. VP16 was immunoprecipitated from HeLa cells infected with SJO2 or with KOS 8 hours post-infection in the presence of [^{32}P]-labeled orthophosphate. The monoclonal antibody LP1 and heat-killed S. aureus cells were used for the immunoprecipitation. The precipitated protein was eluted from the antibody matrix at pH 12.5 and separated on a 10% SDS-PAGE gel. The proteins were transferred to a nitrocellulose membrane and visualized by autoradiography. The identity of the precipitated proteins was verified by immunoblotting, using the rabbit polyclonal serum C8. The autoradiogram in figure 10A reveals phosphorylated bands at 65 KDa, corresponding to the expected size for VP16. This band corresponds to the bands detected in the immunoblot, which shows that the phosphorylated species is VP16. The experiment demonstrated that VP16 derived from SJO2 is phosphorylated. Furthermore, there was no apparent alteration in the level of phosphorylation of SJO2,

Figure 10: Loss of Ser375 does not affect the phosphorylation of VP16 late in infection. HeLa cells were infected with KOS and SJO2 at 5-10 moi in the presence of [32P]-orthophosphate. Cell lysates were prepared from each infection at 8 hpi. VP16 was immunoprecipitated from each lysate with the monoclonal antibody LP1. A: The immunoprecipitated material was separated on an 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and visualized by autoradiography. A labeled band was observed at 65 KDa in both the KOS and the SJO2 samples. These bands corresponded to the expected migration of full length VP16. The 36 KDA band was observed consistently, the identity of the band is not known. B: The identities of the labeled bands in panel A were verified by immunoblotting of the same membrane. The nitrocellulose membrane was probed with the polyclonal antiserum C8. In both samples, the immunoblot identified species corresponding to the migration of the labeled samples in the autoradiogram. Minor bands were observed at 150-160 KDa in both KOS and RP3. These bands did not comigrate with a [^{32}P]-labeled species. C: An aliquot of the immunoprecipitated VP16 from cell lysates were digested with lysylendopeptidase C (EKC). The digestion products were separated on a 16.5% tris/tricine gel, then transferred to nitrocellulose membrane, The labeled bands were visualized by autoradiography. A band of 18 KDa was observed in both the KOS and the SJO2 sample These bands correspond to the expected migration of the aa 371-490 carboxyterminal fragments of VP16 observed previously. D: A sample of the [^{32}P]-labeled immunoprecipitate was separated on a 10% SDS-PAGE gel and transferred to PVDF membrane. The labeled proteins were visualized by autoradiography and the 65KDa species was excised and the protein was hydrolyzed in 6N HCl for 1 hour at 100°C. The samples were mixed with phosphoserine, -threonine and -tyrosine standard and separated by thin layer chromatography on a cellulose plate at pH 2.5. The phosphoamino acid standards were visualized by staining with ninhydrin (indicated by a stippled outline) and the [32P]-labeled species were identified by autoradiography. The only labeled species in each sample co-migrated with the phosphoserine standard. Numbers to the left in panel A, B and C indicate the migration of a molecular weight marker.


A
[32P]
IP
KOS
SJO2
148
98
65
50
36
B
Imm.
Blot
KOS
SJO2
148
98
65
50
36
C
KOS
SJO2
65
50
36
17
16
6
4
D
KOS
SJO2
pSer
pThr/
pTyr
+
-

when compared to KOS. From this experiment we conclude that Ser375 is not necessary for phosphorylation of VP16 and Ser375 is not a major site of phosphorylation at late times in infection. This is in agreement with the findings in chapter 2, where no phosphorylation of Ser375 was found.

In this experiment, an additional phosphorylated species co-precipitated with VP16. This protein has an apparent molecular weights of 36 Kda. It most likely correspond to the 36 KDa band observed in the immunoprecipitation of VP16 in chapter 2. The 145 KDa band that was observed then was not observed in this experiment. The 36 KDa species was not detected in the immunoblot, indicating that the labeled species were not derived from VP16. It was not investigated further.

Phosphorylation of the EKC fragment of SJO2

The overall [^{32}P]-labeling of the intact, length protein isolated from SJO2 infected HeLa cells did not seem to be affected by the mutation. However, results described in chapter 2 demonstrated that some, but not all phosphorylation of VP16 occurred in the carboxyterminal lysylendopeptidase fragment of VP16. If the sites at Ser18 and Ser353 that were identified in chapter two were phosphorylated much more abundantly, the loss of a less abundantly phosphorylated site would not be detectable. Such a loss could possible be detected if the signal from the other sites were removed. To determine whether the Ser375Ala mutation caused a loss of phosphorylation in the 371-490 fragment, the lysylendopeptidase C digestion from chapter 2 was repeated on the SJO2 substrate.

As previously, HeLa cells were infected with KOS or SJO2 at an MOI of 5. The infection was done on the presence of [^{32}P]-orthophosphate and the infected cells were lysed at 8 hpi. VP16 or VP16-S375A was immunoprecipitated from the lysates using the monoclonal antibody LP1. An aliquot of the immunoprecipitated material was separated on a 10 % SDS-PAGE gel. The labeled band corresponding to the predicted migration of VP16 was located by autoradiography of the wet gel. These bands were excised and the protein within the slice was digested with lysylendopeptidase. The resulting fragments were separated on a 16.5% tris/tricine gel, then transferred to a nitrocellulose membrane and the radiolabeled peptides were visualized by autoradiography. The digestion of both the KOS and the SJO2 samples gave rise to a band of an apparent molecular weight of 18 KDa (Fig 10C). This band was identical to the one observed in the experiments in chapter 2. As in the labeling of the full length protein, the intensity of labeling of the peptide was similar between the two samples. This experiment showed that both wt and mutant VP16 recovered from late in infection are phosphorylated within the carboxyterminal 120 amino acids. Assuming that the mutation did not cause gross alteration in the pattern of phosphorylation, this implies that the phosphorylation sites in the carboxyterminal fragment is conserved, and thus that Ser375 is not a major site of phosphorylation late in infection of HeLa cells.

Phosphoaminoacid analysis of Ser375Ala.

It is possible that the loss of one phosphorylatable residue in a consensus phosphorylation site could be compensated for by another. This is particularly a concern with adjacent Ser/Ser or Ser/Thr pairs. Here, both Ser375 and Thr376 represent

consensus CK2 sites. One might imagine that even though the kinase has a higher affinity for Ser375, in the absence of serine at 375, it will phosphorylate Thr376.

To rule out the possibility that the kinase altered specificity to the adjacent threonine of the CK2 site, or indeed that the Ser375 mutation caused any other threonine or tyrosine to become phosphorylated, phosphoamino acid analysis was performed on the VP16 derived from the SJO2 infection. An aliquot of the [^{32}P]-labeled, immunoprecipitated VP16 from KOS infection and SJO2 infection described in the previous section was separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The radiolabeled band was visualized by autoradiography and then excised from the membrane. The protein was hydrolyzed in 6N HCl at 100°C, and phosphoserine, phosphothreonine and phosphotyrosine standards were added to each sample. The resulting mixtures of amino acids were separated on a cellulose plate by 1D-TLC at pH=2.5. The radioactive amino acids derived from the hydrolysis were visualized by autoradiography and the standards were stained with ninhydrin. As shown in figure 10D, only a single spot was detected in each sample. These spots corresponded to the migration of the phosphoserine standard indicating that in either strain, serine remains the only phosphorylated residue. The conclusion was that no compensatory phosphorylation is taking place at the threonine adjacent to Ser375 or at any other Threonine or Tyrosine.

These experiments confirm that the major phosphorylation site(s) of both KOS and SJO2 lie within the last 120 aa and that Ser375 is not a significant phosphorylation site of VP16 late in infection. These experiments do not entirely rule out the possibility

that in the KOS infection, VP16 phosphorylated at Ser375 is present, either at other times in infection or at low levels late in infection.

Growth kinetics of SJO2.

To examine the hypothesis that the loss of the phosphorylation site at Ser375 led to a loss of activation of the IE genes and thereby to a loss of viability of the virus, the single stage growth kinetics of SJO2 was examined.

The single stage growth curve measures the rate of proliferation and the peak yield of viral progeny in a single round of infection. Vero cells in p60 tissue culture plates were infected with KOS or SJO2 at an MOI of 10. In parallel, a set of cells were infected with RP5 at an MOI of 0.1. This was done to compensate for the 100 fold lower particle/pfu ratio in RP5 and ensured a similar particle/cell ratio in all three infections. Triplicate samples of infected plates were harvested at 4, 8, 12, 16, 20 and 24 hpi, respectively. The titer of each sample was determined and plotted as a function of time after infection. Figure 11 shows that the growth kinetics of SJO2 are similar to those of KOS and quite unlike those of RP5. At 4 hpi, infectious virus was present at comparable levels in all three infections (1×10^4-4×10^4pfu/mL). Some of these may represent inoculum virions that adhered to the cells but did not enter. The titers at 8 hpi are higher than at 4 hpi for all three strains. This shows that the time of initial emergence of the new virions is similar between the three strains. While the yield of infectious virus was somewhat higher in the SJO2 infection than in the KOS and RP5 infections, this difference is marginal and does not represent a significant trend. At 12, 16, 20 and 24 hpi, the amount of KOS and SJO2 progeny increased at similar rates, starting to taper off

at 5×10^7-1×10^8. The yield of virus from the RP5 infection at these time points was 1.5 to two orders of magnitude lower, reaching 10^6 pfu/mL at 24 hpi. The experiment shows that the rate of proliferation and the yield of progeny virus in SJO2 infection very closely resemble those of KOS. In contrast, RP5 proliferation and virus yields are both much lower. The overall conclusion from these results is that SJO2 proliferation in Vero cells is indistinguishable from KOS and clearly distinct from those of RP5, which is deficient in IE gene expression.

IE gene expression assay

The results of the growth curve seemed to contradict the expected phenotype of a virus carrying the Ser375Ala mutation in VP16. The loss of promoter binding *in vitro* and decrease in activation of the IE gene promoters by VP16-Ser375SA when expressed by transfection, is well documented (Greaves *et al.* 1990; Lai *et al.* 1997; O'Reilly *et al.* 1997). One possible explanation is that the Ser375Ala mutation does not disrupt IE gene expression during infection as it does when VP16 is expressed from a plasmid. Alternatively, it may be that the defects in the IE gene expression caused by this mutation affect viral growth differentially than the defects caused by deletion of the activation domain in RP5 or the insertion in the HCF-1 interaction motif in the in1814 strain. To resolve this conundrum, one of the basic assumption about VP16 action in early infection had to be rejected. For instance, the transcription of the IE genes might in fact be dispensable for viral proliferation. Alternatively, VP16 might be dispensable for IE gene expression. Thirdly, the IE genes might require VP16 to be expressed but could do so

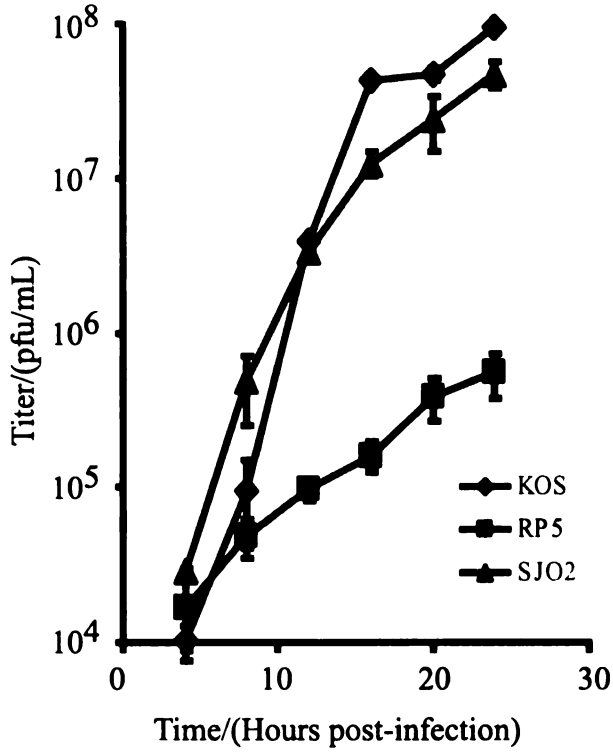


Figure 11: Loss of Ser375 does not affect the proliferation of HSV-1. Single stage growth curves were performed on KOS, RP5 and SJO2. HeLa cells were plated on p60 tissue culture plates (p60s) at 2x105 cells per p60. 24 hours after seeding the cells, triplicate samples for each time point were infected with KOS or SJO2 at an MOI of 10 or RP5 at an MOI of 0.1. Samples were harvested at 4 hour intervals for 24 hours. The titer was established for each sample and the titer plotted as a function of time after infection. The error bars represent the standard deviation of the triplicate samples.

independently of VIC formation. Finally, the VIC formation may be independent of Ser375 in infection, even though it is clearly dependent in *in vitro* experiments.

To begin to differentiate between these four possibilities, IE gene activation during infection with KOS, SJO2 and RP5 was tested using a CAT-reporter assay. In the assay, reporter plasmids were used that express a CAT gene product under the transcriptional control of the promoters from the HSV-1 IE genes ICP0, ICP4, ICP22/47 or ICP27. The activation of these reporters was tested in HeLa cells during infection with SJO2, KOS and RP5. These reporters were introduced individually into HeLa cells by liposome mediated transfection. After 24 hours cell transfected by a given reporter construct were pooled and plated in six-well plates. 48 hours after transfection, the cells were infected with KOS or SJO2 at an MOI of 5-10, or RP5 at an MOI of 0.05-0.1. Mock infection was done in parallel. The infected cells were harvested at 2 hpi and lysates were generated by disrupting the cells by multiple freeze/thaw cycles. The cell lysates were then tested for CAT activity.

A representative set of results are shown in figure 12. Each panel of the figure shows the CAT enzyme activity for one of the four reporters. As expected, the IE gene expression of the IE genes in the KOS infection was 2-7 fold above the mock infection in all four strains. Also as expected, infection with RP5 did not activate any of the reporter genes significantly above the mock infection. For SJO2, the activation varied between comparable to that of KOS and to 50% of that of KOS. In no instance did the activation of the IE genes fall to the level of RP5 and mock infected cells. These results lead to the conclusion that activation of the IE genes in SJO2 may be slightly reduced but remains significantly more robust than that of RP5. In other words, the unexpected expression of

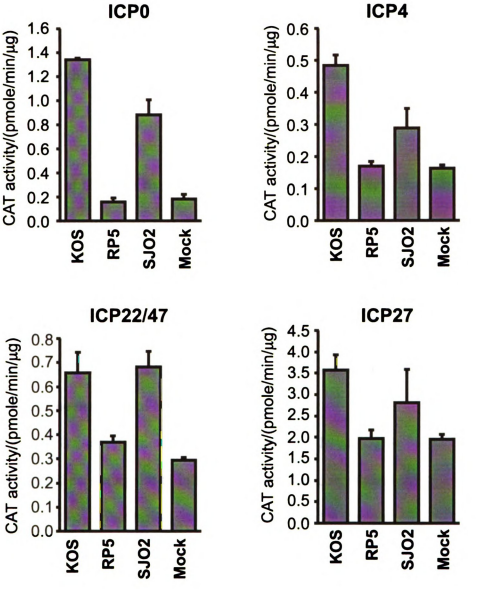


Figure 12: Loss of Ser375 has a marginal effect on the activation of the IE genes during infection. HeLa cells were transfected with pAN5, pAN6, pAN7 or pICP27-CAT. These reporters express the CAT-gene from the ICP0, ICP4, ICP22/47 or ICP27 promoters, respectively. The cells were super infected in triplicate with KOS, RP5 or SJO2 at an moi of 10 or mock infected. Extracts were made from the infected cells 2 hpi. The extract were tested for CAT activity, which is reported as pmole acetyl transferred per minute per µg of total protein. The error bars represents the standard deviation between the triplicates.

the IE genes in SJO2 would explain the wild type growth phenotype of the strain. But while the expression of the IE genes in SJO2 may explain the growth phenotype, it seems incompatible with the transcriptional phenotype of the Ser375Ala mutation in transfected VP16.

Transcription of IE genes by VP16-Ser375Ala requires protein translation.

To confirm that the expression of the IE-CAT reporters was in fact stimulated by the incoming VP16 and not by proteins expressed subsequent to virion entry, it was necessary to prevent the expression of proteins during infection. Other viral genes, primarily ICP0, are capable of partially complementing loss of IE gene expression when VP16 function is lost (Mossman *et al.* 1999). These secondary mechanisms may mask the effects of the mutation of SJO2.

To block the translation of the IE proteins cycloheximide was added to the growth medium of the host cells prior to infection. The IE gene expression assay was repeated in the presence of cycloheximide using the same reporter construct and the same three HSV-1 strains. To permit the translation of the CAT reporter protein, the cycloheximide-containing medium was removed after two hour of infection and replaced with medium containing the RNA synthesis inhibitor actinomycin D. This allowed for the translation of the accumulated reporter gene messages, while preventing any subsequent transcription of the IE reporters. Lysates were prepared from the infected cells after two hours incubation with actinomycin D. The lysates were assayed for CAT enzyme. Representative results for this experiment are shown in figure 13.

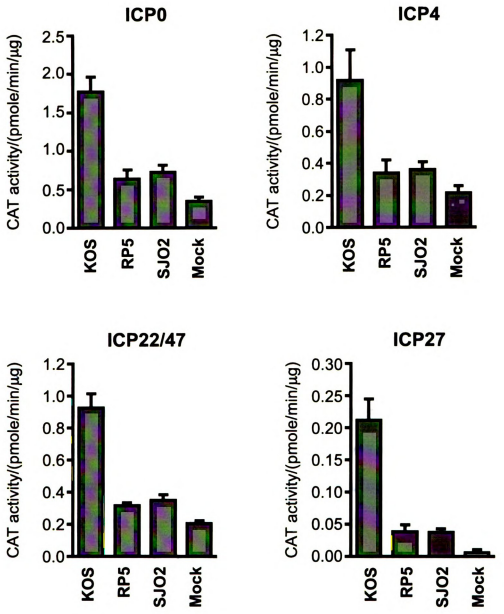


Figure 13: Loss of Ser375 severely diminishes IE gene activation in the absence of protein synthesis. HeLa cells were transfected with pAN5, pAN6, pAN7 or pICP27-CAT. These reporters express the CAT-gene from the ICP0, ICP4, ICP22/47 or ICP27 promoters, respectively. 48 hours after transfection, cycloheximide was added to the growth medium. After 30 minutes, the cells were superinfected in triplicate with KOS, RP5 or SJO2 or mock infected at 10 moi. 2 hpi, the cycloheximide was removed and replaced with actinomycin D. Extracts were made from the infected cells 4 hpi. The extract were tested for CAT activity, which is reported as pmole acetyl transferred per minute per µg of total protein. The error bars represents the standard deviation between the triplicates.

In this experiment, KOS activated the IE reporters 3-5 fold over mock infection. This is similar to what is observed in the absence of cycloheximide. Likewise, the activation domain truncation of RP5 renders that virus unable to activate any of the reporters here as it did in the absence of cycloheximide. But in contrast to the first set of experiments, SJO2 was unable to activate the reporters, leaving it as or almost as deficient as RP5 for all four reporters.

The conclusion from this experiment was that VP16-Ser375Ala by itself was unable to activate the IE genes. The combined conclusion from these two experiments was that protein synthesis is required for IE gene expression during infection with SJO2. This is most likely is due to stimulation of expression of the IE reporter constructs by the products of the viral IE genes themselves

In summary, the investigation of the phosphorylation and function of Ser375 of VP16 in infection with HSV-1 showed that loss of the serine at 375 did not affect the level of phosphorylation of VP16 late in infection. This led to the conclusion that Ser375 was not a significant site of phosphorylation when VP16 was examined late in infection. This is consistent with the findings in chapter 2 which found no evidence of phosphorylation of Ser375. The results also showed that the Ser375Ala mutation of VP16 did not affect the growth of the virus in Vero cells. Furthermore, the expression of the IE genes under standard conditions was largely unaffected. However, in the absence of protein synthesis, a significant defect in IE gene expression was revealed. The conclusions of these experiments are that the expression of the IE genes during infection with SJO2 was stimulated by a factor expressed subsequent to infection. These conclusions were in agreement with published findings that showed mutation of the

Ser375 led to a failure to form the VIC *in vitro* (Greaves *et al.* 1990; Lai *et al.* 1997; O'Reilly *et al.* 1997). It also demonstrated that during infection the IE genes can be activated by a mechanism that does not require this interaction.

Phosphorylation and function of Ser411.

Ser411 is one of the potential phosphorylation sites that were discovered in chapter 2. This site was found by peptide mapping and appears to contain a significant portion of the label. To test whether mutations of this site had any effect on VP16 function during infection a set of mutant viral strains were generated (Figure 3). These strains, SO11, SO12, SO13, carry alanine, glutamate or threonine substitutions, respectively, at Ser411. SO11 was examined to detect changes in phosphorylation levels and phosphorylation residue preference. All three strains were examined for their growth phenotypes and their ability to activate the IE genes during infection.

Generation of viral strains carrying point mutations at Ser411

The function of the potential phosphorylation site at Ser411 had not been addressed previously and no correlation had been found to demonstrate a connection between mutations in this region of VP16 and changes in the function of VP16. A set of mutant strains of HSV-1 were generated to determine whether substitution of Ser411 with alanine, glutamate or threonine would interfere with the function of VP16 during infection.

The viruses were to be generated by homologous recombination into 8MA, a strain lacking the VP16 gene. This strain is not viable in the absence of a complementing

cell line. In the previous section, the SJO2 strain was also generated from 8MA by recombination and candidates were selected by their ability to reproduce outside of a complementing cell line. This method had been successful in generating the recombinant virus but there was some concern about possible side effects of the selection method. It was possible that by only selecting viable strains, a selection pressure for complementing second site mutations was introduced. In effect, this selection would favor increased viability due to both the introduction of the recombinant VP16 gene as well as any other spurious mutation within the viral genome. The concern over the possibility of a second site mutation prompted a change in the strategy for generation of the these new viruses. In order to avoid the undesirable selection pressure, it was decided to screen all potential recombinants for the presence of a marker while complementing any mutant phenotypes caused by the mutation with wild type VP16. An enhanced green fluorescent protein (EGFP) tag was added to VP16 to serve this purpose. A virus strain (DG1) carrying wild type VP16 with a carboxyterminal EGFP-tag had been generated and characterized previously (Greensides 2002). The EGFP-tag had no negative effect on the growth of this virus. By introducing the mutations at Ser411 into a version of VP16 that carried the EGFP-tag, it would be possible to screen for recombination events in a complementing cell line. This would greatly reduce the probability of inadvertently picking recombinants with second site mutations in a viability screen.

The mutations at 411 were introduced into the VP16-EGFP plasmid construct using a PCR based mutagenesis method. The plasmid was sequenced to verify the presence of the mutations. The mutant gene along with flanking regions was excised from the plasmid and introduced along with purified viral genomic DNA from 8MA into

BHK-MMTV-VP16 by liposome mediated transfection. The cell line is a derivative of a baby hamster kidney cell line (BHK), constitutively expressing VP16. This cell line was chosen for the recombination because it is very amenable to liposome-mediated transfection. The supernatants of these transfections were harvested and used as inoculum for the infection of Vero 16-8 cells in agarose-containing growth medium. Because BHK cells do not give rise to well-defined plaques, Vero 16-8 cells were used for the infection. Infectious foci in Vero cells can be readily distinguished from non-infected cells. This reduces the probability of picking plaques that contain progeny from multiple, conjoined foci. Individual recombinant strains were recovered by screening for EGFP-fluorescence. The infected vero 16-8 cells were examined under a fluorescence microscope and solitary plaques that expressed the EGFP-protein were identified. Recombinant HSV-1 was recovered from these plaques and used to infect Vero 16-8 cells. Re-infection, identification and recovery of EGFP-expressing plaques was repeated sequentially on the same initial plaque to ensure that the resulting strain was derived from a single recombination event. After the purification, larger stocks of each recombinant virus were grown in Vero 16-8 cells.

To verify that the desired mutation was indeed present in each strain, viral DNA was purified from an aliquot of the virus stock. A fragment of the VP16 gene spanning the mutation site was amplified by PCR and the PCR product was sequenced. The expression of the VP16-EGFP fusion protein was verified by immunoblot. Aliquots of the viral stock were separated on a 10% SDS-PAGE gel and the proteins were transferred to a nitrocellulose membrane. The presence of VP16 was determined by immunoblot, using the rabbit polyclonal serum LA2-3. Aliquots of the wild type KOS, the EFGP-

tagged wild type DG1 and of 8MA were added as controls (figure 14). The KOS sample gave rise to the expected 65 KDa band, corresponding to full length VP16. DG1 gave rise to a 110 KDA band. While this band migrates slower than would be expected, based solely on the molecular weight of the protein, it was taken to correspond to full length, EGFP-tagged VP16. The SO11, SO12 and SO13 strains gave rise to both the 65 KDa band and the 110 KDa band. The presence of both bands was expected because these strains had been grown in the presence of wild type VP16 in Vero 16-8 cells. This resulted in the packaging of both versions of the protein. The VP16 null mutant, 8MA was also grown in 16-8 cell and therefore carried wt VP16 in the virion. As expected, the 65 KDa band was detected in this sample.

The virus stocks used for this experiment had dissimilar titers, giving rise to different levels of both wt and EGFP-tagged VP16. The SO13 sample does not appear to have any wild type VP16. This is most likely an artifact of the low titer of the SO13 stock used in conjunction with the unequal loading in the virions of VP16 expressed by the cell and the virus. The low titer of the SO13 stock observed here is aberrant; the strain ordinarily gives rise to titers similar to those of the SO11, SO12 strains. (Data not shown). Titers of all three stocks ranges from 5×10^8 to 2×10^9, both when generated in complementing cell lines and in Vero cells, similar to wild type titers. The experiment verifies that the recombinant virus strains express and package EGFP-tagged VP16.

It is significant that both tagged and untagged VP16 was detected in the virion of both SO11 and SO12. This shows that the Ser411Ala and the Ser411Glu mutations do not affect the packaging of VP16 into the virion. In fact, it appears that the virus preferentially packages the mutant VP16. This is most likely to reflect a difference in the

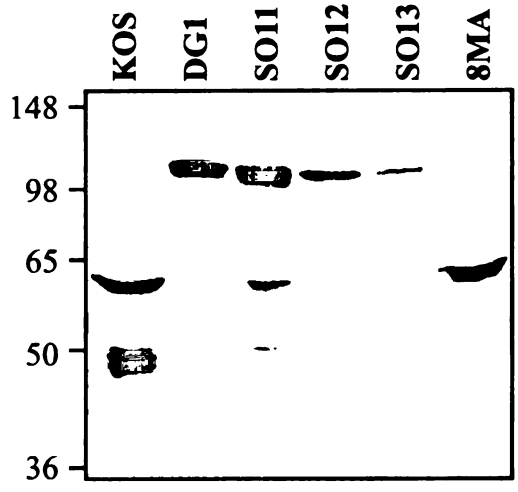


Figure 14. Aliquots of virus stock from KOS, DG1, SO11, SO12, SO13 and 8MA were separated on a 10% SDS-PAGE gel. SO11, SO12, SO13 and 8MA were derived from Vero 16-8, a complementing cell line. The proteins were transferred to nitrocellulose and the presence of VP16 proteins was detected by immunoblotting with the polyclonal rabbit anserum LA2-3. The migration of molecular weight markers is indicated in KDa in the left side of the figure

levels of the two proteins in the host cell. The expression of the wt VP16 gene from a gene in the infected cell is likely lower that that of the EGFP-tagged gene expressed from the VP16 locus in multiple copies of the viral genome.

Phosphorylation of VP16 lacking Ser411

To examine whether the Ser411Ala mutation in SO11 affected phosphorylation of VP16, HeLa cells were infected with KOS, SO11 and DG1 at an moi=5. [^{32}P]-orthophosphate was added at 1.5 hpi and the cells were lysed after 8 hours of infection. VP16 was immunoprecipitated from the lysates, using the LP1 antibody and eluted from the antibody at pH 12.5. The eluted protein was separated on a 10% SDS-PAGE gel and then transferred to nitrocellulose. The radiolabeled species were detected by autoradiography and their identities were verified by immunoblotting the membrane with the polyclonal rabbit serum C8.

As expected, a 65 KDa, [^{32}P]-labeled band was detected in the KOS sample. Similarly, as expected, a 110 KDa labeled band was detected in the DG1 sample. This demonstrated that the EGFP-tag did not abolish phosphorylation of VP16. The SO11 sample also contained a 110 KDa [^{32}P]-labeled species. This species was similar to the one observed in the DG1 sample. This showed that the Ser411Ala mutation did not result in the loss of phosphorylation of VP16. The immunoblot verified the identity of the protein in all three samples. (Fig 15A) It also showed that the protein levels of VP16 were similar in DG1 and SJO2. The lower level of VP16 recovered from in the DG1 and SO11 samples, as compared to the KOS sample could have been be due to a reduced efficiency of transfer of the protein to nitrocellulose. This may be a function of the larger

size of the protein or it may be that EGFP imparts a lower affinity of VP16 for the membrane. This experiment showed that the loss of Ser411 did not affect the gross level of phosphorylation.

Additional labeled bands, including those at 36 Kda and 140 KDA were conserved between the three samples but most are not detected in the immunoblot. One exception was a labeled band of 80Kda which appeared in the DG1 and SO11 samples. It also appeared in the immunoblot of those samples but did not appear in the KOS sample. This may have represented a premature termination of VP16 translation or a degradation product. A similar band was never observed in KOS and presumably represented an artifact caused by the EGFP-tag.

The conclusion from the experiment was that the level of phosphorylation of VP16-Ser411Ala derived from SO11 was no different that that of the wild type EGFP-tagged VP16. But as was discussed with SJO2, the possibility remained that the loss of Ser411 caused an alteration in the pattern of phosphorylation remained.

Phosphoamino acid complement in SO11

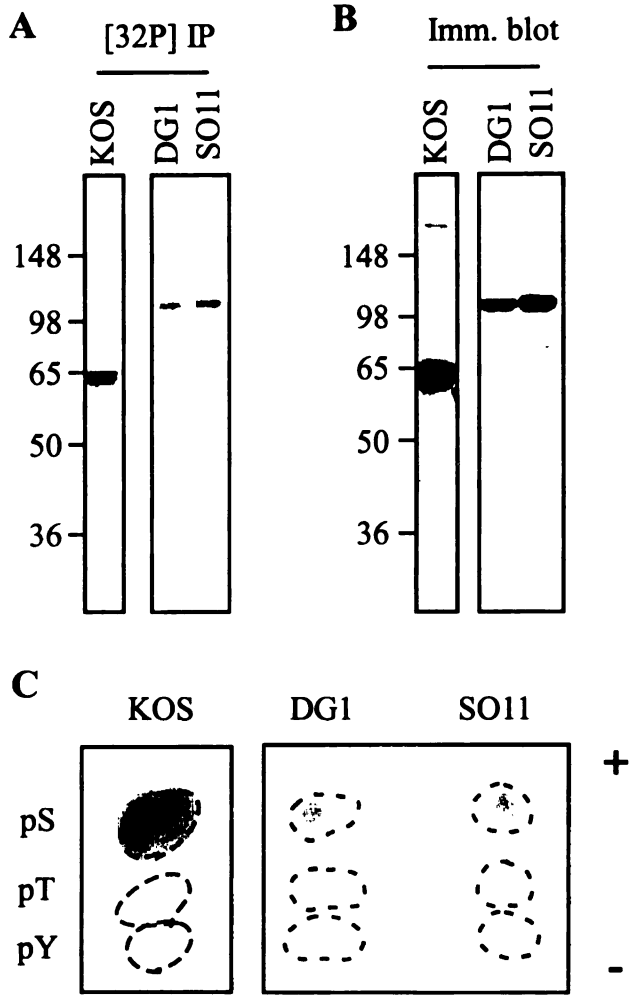
Like in the case of the Ser375 site, the conservation of the bulk label of the SO11, could be attributed to an alteration of kinase specificity. In the case of Ser411, the nearest neighbor, Thr412 might serve as an alternative acceptor of the phosphate group. To examine this possibility, the phosphoaminoacid complement in SO11 was examined. VP16 was immunoprecipitated from HeLa cells infected with KOS, SO11 and DG1, respectively, in the presence of ^{32}P-orthophosphate. The precipitated protein was separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The position

of the labeled bands were determined by autoradiography of the membrane and the bands were excised from the membrane. The protein in the band was hydrolyzed in 6N HCL at 100°C for 1 h. The resulting amino acid mixtures were spiked with phosphoserine, -threonine and –tyrosine standards and loaded on a cellulose plate. The samples were separated by 1D TLC at pH=2.5 and the radioactive residues were detected by autoradiography. The standards were detected by ninhydrin staining and used as a guide for the radioactive spots. Figure 15C shows that each sample only gave rise to a single radioactive spot. The outlines of the ninhydrin-stained standards are indicated by the stippled lines. In each sample [^{32}P]-label was detected at the phosphoserine position, but not at the phosphothreonine or –tyrosine. As in the immunoprecipitation, the relative levels of [^{32}P]-label in each sample may be due to the lower efficiency of transfer to the PVDF membrane. The experiment shows that the only phosphorylated amino acid in SO11 is serine. This rules out compensatory phosphorylation at Thr412, as well as at any other threonine or tyrosine site in VP16.

Growth kinetics of SO11, SO12 and SO13

Although the loss of the serine at 411 does not seem to affect the total level of phosphorylation of VP16 in infection, it was identified in chapter 2 as a site of phosphorylation. This phosphorylation may indicate that it is a site of regulation of VP16 function. Because no data existed to point to a specific function that this phosphorylation might regulate, the first step to find such a function was to determine whether the loss affected the viability of any of the mutants.

Figure 15: Loss of Ser411 of VP16 does not affect the overall phosphorylation of VP16, nor alter the phosphoamino acid preference. HeLa cells were infected with KOS, DG1 and SO11at 5-10 moi in the presence of [^{32}P]-orthophosphate. Cell lysates were prepared from each infection at 8 hpi. VP16 was immunoprecipitated from each lysate with the monoclonal antibody LP1. A: The immunoprecipitated material was separated on an 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and visualized by autoradiography. A labeled band was observed at 65 KDa in the KOS sample. A 100 KDa band was observed in both the DG1 and SO11 sample. These sizes correspond to the expected migration of full length VP16, without or with the EGFP-tag, respectively. Additional [^{32}P]-labeled bands in the DG1 and SO11 samples were also present in the KOS sample. B: The identities of the labeled bands in panel A were verified by immunoblotting the same membrane. The nitrocellulose membrane was probed with the polyclonal antiserum C8. In all samples, the immunoblot identified species corresponding to the migration of the labeled samples in the autoradiogram. C: Samples of the [^{32}P]-labeled immunoprecipitates was separated on a 10% SDS-PAGE gel and transferred to PVDF membrane. The labeled proteins were visualized by autoradiography, a 65KDa species was excised and the protein was hydrolyzed in 6N HCl for 1 hour at 100 C. The samples were mixed with phosphoserine, -threonine and -tyrosine standard and separated by thin layer chromatography on a cellulose plate at pH 2.5. The phosphoamino acid standards were visualized by staining with ninhydrin (indicated by a stippled outline) and the [^{32}P]-labeled species were identified by autoradiography. The only labeled species in each sample co-migrated with the phosphoserine standard. Numbers to the left in panel A, B indicate the migration of a molecular weight marker.


A
[32P] IP
KOS
DG1
SO11
148
98
65
50
36
B
Imm. blot
KOS
DG1
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148
98
65
50
36
C
KOS
DG1
SO11
+
pS
pT
pY
-

As described previously, the usual approach to study major growth defects in herpes is to examine the kinetics of proliferation. To this end, stocks of SO11, SO12 and SO13 were grown in non-complementing Vero cells. The titer of these stocks were determined and found to be similar to those of wild-type virus. Each of these stocks as well as wild type KOS were used to infect a set of p60 plates seeded with vero cells at an MOI of 5. A subset of plates of infected cells were lysed at 4, 8, 12, 18 and 24 hpi and the progeny virus was extracted from the cellular debris. The titer of each of these supernatants was determined by plaque assay and the results were plotted as a function of time (figure 16). The growth of the mutants mimicked that of the wild type closely. The infectious virus harvested at 4 hpi represented a mixture of remnants of the initial inoculi and newly synthesized virion. At 8 hpi, the amount of virion harvested from all strains had increased by an order of magnitude from that of 4 hpi. None of the three mutant strains lagged behind in initial emergence of virion. At 12, 18 and 24 hpi, the titer of the mutant strains remained very similar to that of KOS. This showed that the rate of virion synthesis was the same and it demonstrated a maximal yield of virion at 18 hpi. This shows that the mutations at Ser411 did not affect any vital function of VP16, required for infection of HeLa cells. These three mutant strains exhibit wild type growth and kinetics. They all differ from the activation domain deficient strain RP5. The conclusion was that any function that is associated with the putative phosphorylation site at Ser411 is not vital for the proliferation of HSV-1 in lytic infection of cultured HeLa cells.

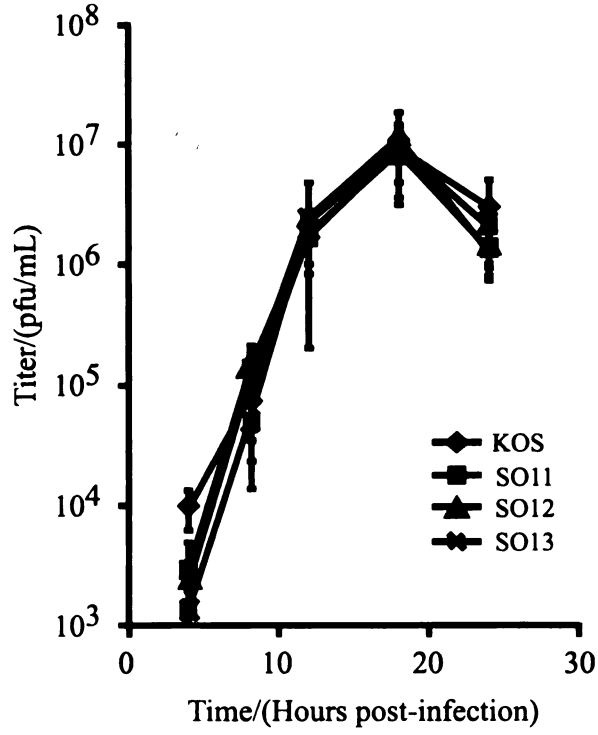

Figure 16: Loss of Ser411 does not affect the proliferation of HSV-1. Single stage growth curves were performed on KOS, SO11, SO12 and SO13. HeLa cells were plated on p60 tissue culture plates (p60s) at $2x10^5$ cells per p60. 24 hours after seeding the cells, triplicate samples for each time point were infected with KOS, SO11, SO12 or SO13 at an MOI of 10. Samples were harvested at 4 hour intervals for 24 hours. The titer was established for each sample and the titer plotted as a function of time after infection. The error bars represent the standard deviation of the triplicate samples.

Transcriptional activation of the IE genes by SO11, SO12, SO13

The growth curve did not reveal any loss of function of VP16, associated with Ser411. Consequently, the search for a function had to be approached in a different manner. Because the closest functional domains to Ser411 are involved in DNA binding and in transcriptional activation, it seemed reasonable to investigate whether there was any defect in IE gene activation. To this end, the CAT-reporter assays were repeated for the SO11, SO12 and SO13 in the presence and in the absence of cycloheximide. The use of cycloheximide would take address the possibility that if Ser411 was indeed involved in the IE gene activation by VP16, it might also be bypassed by the same unknown function that bypassed the Ser375Ala defect of SJO2 in infection. Again, reporter constructs expressing the CAT gene under the control of the IE-gene promoter from ICP0, ICP4, ICP22/47 and ICP27 were used. The reporters were transfected into HeLa cells. 24 hours after transfection, the transfected cells were plated in p60 plates. 48 hours after transfection, the cells were infected with KOS, SO11, SO12 or SO13 at an MOI of 5 or RP5 at an MOI of 0.05. As a negative control, a set of plates were treated with a mock inoculum. When testing expression of the CAT reporters in the absence of cycloheximide, the cells were harvested and lysed at 2 hour post infection and the cell lysates were tested for CAT activity. Figure 17 show a representative result of this experiment. As expected RP5 was unable to activate the reporter genes above mock (background) levels in any of the constructs. Activation of ICP0 and ICP4 in KOS infection was 3-5 fold over that of mock and RP5. In the light of the previous reports, the very low level of activation by KOS (20-40%) over background in the ICP22/47 and ICP27 experiments here is unexpected. The results are most likely due to over-

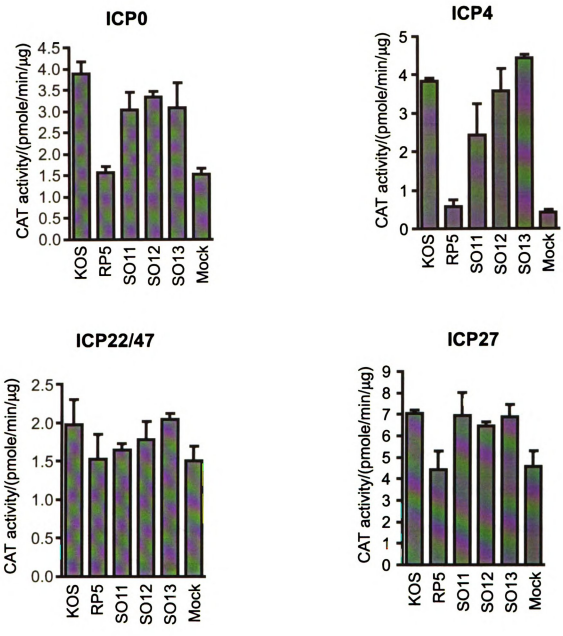

Figure 17: Loss of Ser411 does not affect the transcriptional activation of the IE genes during infection. HeLa cells were transfected with pAN5, pAN6, pAN7 or pICP27-CAT. These reporters express the CAT-gene from the ICP0, ICP4, ICP22/47 or ICP27 promoters, respectively. The cells were super infected in triplicate with KOS, RP5, SO11, SO12 or SO13 or mock infected. Extracts were made from the infected cells 2 hpi. The extract were tested for CAT activity, which is reported as pmole acetyl transferred per minute per µg of total protein. The error bars represents the standard deviation between the triplicates.

transfection of the reporter, giving rise to a very high background expression level. For the ICP0, ICP4 and ICP27 reporter plasmids, the expression levels induced by the mutant strains are most similar to that of KOS and distinctly different from that of RP5 and mock. This indicates that the activation of these genes are unaffected by mutations at Ser411. In ICP22/47, the low level of activation over background precludes any clear conclusion. The overall results of the experiment was that there was no evidence of any significant defects in IE gene expression in SO11, SO12 and SO13.

To rule out the possibility that an IE gene expression defect was masked by the expression of a complementing factor after infection had begun, the experiment was repeated in the presence of cycloheximide. As previously, HeLa cells were transfected with one of the CAT reporters driven by the four IE gene promoters, ICP0, ICP4, ICP22/47 and ICP27. The transfected cells were treated with cycloheximide a half hour before infection and for the first two hour of infection. The medium was switched to actinomycin-containing medium to allow the accumulated transcripts to be translated. The cells were lysed two hours after actinomycin addition and the extracts were assayed for CAT activity. The experiment was repeated for all four of the reporters. Representative results of these experiments are shown in figure 18. Again, both controls gave the expected result. KOS activated the IE genes 2-7 fold over the mock infected cells. The RP5 did not activate the IE reporter genes above the Mock level. The mutant strains activated the IE gene reporters well above the background, to levels similar to those of KOS. The ICP22/47 and ICP27 constructs that did not give conclusive results in the absence of cycloheximide showed in this experiment that there was no deficiency in IE gene expression on these reporters. The variation in activity between the SO strains

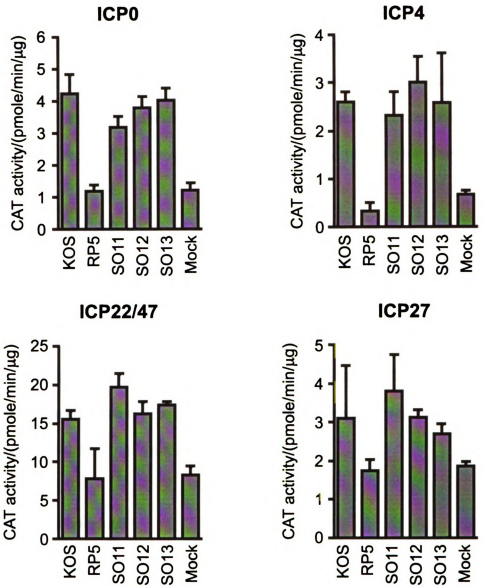

Figure 18: Loss of Ser411 did not affect IE gene activation in the absence of protein synthesis. HeLa cells were transfected with pAN5, pAN6, pAN7 or pICP27-CAT. These reporters express the CAT-gene from the ICP0, ICP4, ICP22/47 or ICP27 promoters, respectively. 48 hours after transfection, cycloheximide was added to the growth medium. After 30 minutes, the cells were superinfected in triplicate with KOS, RP5, SO11, SO12, or SO13 or mock infected at 10 moi. 2 hpi, the cycloheximide was removed and replaced with actinomycin D. Extracts were made from the infected cells 4 hpi. The extract were tested for CAT activity, which is reported as pmole acetyl transferred per minute per µg of total protein. The error bars represents the standard deviation between the triplicates.

were not conserved between the reporter constructs, nor were they conserved in replicates of the same reporters (not shown). The variations are not due to inherent differences in the potency of the IE gene activation between the strains. This experiment fails to demonstrate the existence of a factor, expressed subsequent to infection, which is capable of masking defects in IE gene expression caused by loss of the phosphorylation site at Ser411.

In summary, VP16 derived from infection with SO11 is phosphorylated at levels comparable to those of KOS. The site or sites of phosphorylation are all serine sites. There is no evidence of altered site specificity of the kinase to compensate. Furthermore, viral strains carrying alanine, glutamate or threonine substitutions at the Ser411 phosphorylation site proliferate with kinetics similar to that of KOS. These strains also do not demonstrate any deficiency in IE gene expression, regardless of whether subsequent translation is allowed.

Methods and Materials

Cell culture, virus preparation, *in vivo* labeling experiments, immunoprecipitation, SDS-PAGE, immunoblotting, peptide mapping and phosphoamino acid analysis was performed as described in chapter 2. Plasmids for the generation of the mutant virus strains were also generated as described in chapter 2, using the primers described in Table 2.

Generation of viral strains.

HSV-1 mutants were generated by co-transfection of HSV-1 genomic DNA from the VP16-null strain 8MA with a linear fragment of the mutant VP16 gene with flanking 5' and 3' UTRs. Homologous recombination occurred between the UTRs in the linear fragment and those of the 8MA DNA. The VP16 fragment was excised from pKOS-S375A, pSO365.4, pSO365.5 or pSO365.6 by digestion with *Eco*RI and *Pst*I. Vero cells were transfected with 5 μg of pKOS-S375A insert and 5 μg of viral DNA by calcium phosphate transfection (Graham *et al.* 1973). After transfection, the cells were overlaid with DMEM supplemented with 5% FCS and 0.9% SeaPlaque agarose and incubated for 3-4 days. Plaques were picked and re-purified by two additional rounds of plaque purification. A stock was generated from the last round of plaque purification (SJO2).

BHK-MMTV-VP16 cells were transfected with 1 μg of 8MA genomic DNA and 1 μg of either pSO365.4, pSO365.5 or pSO365.6 insert using Lipofectamine (Stratagene) in DMEM by standard protocols. The transfection was fed with DMEM supplemented with 2% FCS and 0.5 μg/mL Geneticin after 3 hours and the transfection was allowed to proceed for three days. The supernatant was harvested and used to infect Vero 16-8 cells as described for plaque assays in chapter 2. Plaques were screened for fluorescence using a Meridian Insight confocal laser scanning microscope, using a 20×objective. Plaques were screened and purified three times and stocks were generated in Vero 16-8 cells. (SO11, SO12 and SO13).

For all four strains, a small aliquot of DNA was extracted from 500 μL of a high titer stock. The stock was incubated for 2 hours with 1 mg of proteinase K and 1% SDS at 37°C. Protein was removed by phenol/chloroform extraction and subsequent 2-propanol precipitation. The DNA pellet was redissolved in 10 μL of 10 mM Tris·HCl,

pH=8.0. A fragment, spanning the mutation, was amplified by PCR and tested for loss of the *Acc*I site at the codon for aa 375 or 411, respectively. The PCR fragment was then submitted for automated sequencing to confirm the presence of the correct mutation.

Viral DNA preparation

5×10^6 Vero 16-8 cells were infected with 8MA at an moi of 0.01 as described in chapter 2. After three days, when 80% CPE was observed, the infected cells were harvested and sonicated as described in Chapter 2. The cellular debris was removed by centrifugation and the supernatant was overlaid on a 20% glycerol in PBSa. The sample was centrifuged for 90 minutes at 28 krpm in a sw28 swinging bucket rotor. The supernatant was gently removed and the virion pellet was lysed in virion lysis buffer (100 mM sodium chloride, 10 mM EDTA, 50 mM Tris·HCl, pH 8.0, and 0.5% lauryl sarcosinate.) Proteinase K was added to a final concentration of 0.2 mg/mL and the sample was incubated at 37°C for 2 hours. After incubation, equal volumes of cesium trifluoroacetate (CsTFA, Pharmacia) and sample were mixed and 25 μL of ethidium bromide was added (10mg/mL). The samples were centrifuged for 6 hours at 65 krpm in a vti90 rotor at 15°C. The purified DNA was removed, the ethidium bromide was removed by n-pentanol extraction and the CsTFA was removed by dialysis against TE (10 mM Tris·HCl pH 8.0, 1 mM EDTA). DNA concentration was determined by absorbance at 260 nm.

Chloramphenicol transferase assay

pAN5, pAN6, pAN7 or pICP27-CAT plasmids were introduced into HeLa cells using Lipofectamine and the Plus reagent (Stratagene). 24 hours after transfection, transfected cells were pooled to ensure consistent transfection efficiency and seeded at 2×10^5 cells/well in six well plates. 24 hours after seeding the cells were prepared for infection. When infecting in the absence of cycloheximide, the cells were washed in DMEM and infected at an moi of 5 or 0.1, for RP5. Infections were fed after 1 hour with DMEM supplemented with 2% FCS. The cells were washed and overlaid with PBSa 2hpi. The cells were dislodged from the culture dish with a disposable scraper and transferred to a microcentrifuge tube. The cells were pelleted at 200×g for 2 minutes in an eppendorf microcentrifuge. The supernatant was removed and the cells resuspended in 100 µL of 0.25 M Tris·HCl. Cells were disrupted by three repeated cycles of freezing and thawing. Each cycle consisted of 5 minutes in a dry ice ethanol bath followed by five minutes at 37°C. Extracts were tested for CAT activity by continuous organic extraction as described (Nielsen *et al.* 1989). 20 µL of sample was mixed with 20 µL of reaction buffer (5mM CAM, 500µg/mL BSA in 250 mM Tris·HCl (pH=7.8)) or 20 µL of (500µg/mL BSA in 250 mM Tris·HCl (pH=7.8)). Total volume was brought up to 80 µL with ddH_20. Reactions were started with 20 µL of [3H]-acetyl-CoA (20 µCi/mL; 150 µM) in 75 µM HCl. Samples were counted twice at either 30 minute or 45 minute intervals. Activity was normalized to total protein concentration, as determined by Bradford assay (BioRad)

To assay the activity of VP16 in the absence of protein translation, cycloheximide was added to the culture medium 30 minutes prior to infection. Infection was maintained in cycloheximide for another 2 hours, at which time the cycloheximide was washed out

and replaced with Actinomycin D. To allow translation of the CAT enzyme, the infection continued for another 2 hours in the presence of Actinomycin D. The cells were then harvested and assayed as above.

Growth curves

Stocks of KOS, RP5, SJO2, SO11, SO12 and SO13 were prepared and titered on Vero or Vero16-8 cells as described in Chapter 2. HeLa cells were plated on p60 tissue culture plates (p60s) at $2x10^5$ cells per p60. 24 hours after splitting the cells, they were infected with KOS, SJO2 or RP5 at an MOI of 10. Samples were harvested at 4 to 6 hour intervals for 24 hours. The samples were harvested by removing the cells with a disposable scraper and transferring the suspended cells and growth medium to a 15 mL conical tube. The cells were sonicated as described in chapter 2, to disrupt the cells and release the virions from the cellular debris. The insoluble cell debris was pelleted by centrifugation at 3000×g in a Sorvall Techospin centrifuge for 5 minutes at ambient temperatures. The titer of each sample was determined by plaque assay as described in chapter 2.

Discussion:

Function of Ser375 and Ser411 in VP16 during infection.

In this chapter, Ser375 and Ser411 were tested for their function in infection. Virus strains which carried mutations at one or the other of these sites were generated. These strains were tested for growth phenotypes and for transcriptional phenotypes. A Ser375Ala mutation was found to have no effect on the growth of the virus, yet the transcription of the IE genes was significantly reduced when the IE genes were activated solely by VP16. Under conditions where other factors could aid in the activation of the IE genes, their expression was near wild-type. Mutations at Ser411 were not found to have any effect on the growth of the virus, nor did these strains exhibit any defect in IE gene expression, whether examining only the VP16-derived activation or total IE gene activation.

Changing either serine to an alanine did not lead to the loss of phosphorylation of VP16. The loss of the serine did not affect the labeling of the carboxyterminal lysylendopeptidase C fragment either. The conclusion was that neither of these sites are major phosphorylation sites late in infection.

Because SJO2 grew with wild type kinetics, the hypothesis that VIC-derived transcription was required for the efficient proliferation of HSV-1 in infection had to be rejected. The hypothesis that Ser375 is the only *in vitro* CK2 phosphorylation site in the core domain of VP16 was also rejected. The hypothesis that phosphorylation of Ser411 served as a regulatory signal for transcriptional activation by VP16 or any other function required for viral proliferation in HeLa cells was also rejected.

Activation of the IE genes in infection by SJO2 overcomes the transcriptional deficiencies of the Ser375Ala mutation.

The investigation of the function of Ser375 of VP16 in the virus was prompted by the potential regulatory role that phosphorylation could have if it was a phosphorylation site. Furthermore, the site was known to be required for VIC formation *in vitro* and for IE gene activation *in vivo* . When testing the function of the Ser375Ala mutation under conditions that most stringently tested VP16 activation during infection, the protein was unable to activate the IE genes. This result was in accordance with the observation of previous studies of this particular mutation *in vivo* and *in vitro* (Greaves *et al.* 1990; Lai *et al.* 1997). Both Greaves *et al.* and Lai and Herr had discovered this mutation to disrupt the formation of the VIC complex and also prevents activation from an IE gene promoter. Furthermore, substitution with acidic residues or proline also disrupted the interaction. The conservative substitution with threonine did conserve the interaction and the activation potential (Greaves *et al.* 1990; O'Reilly *et al.* 1997). Lai *et al* also determined that the critical interaction affected by the mutation was that between Oct-1 and VP16 (Lai *et al.* 1997). None of the experiments addressed whether the function of VP16 relied on the phosphorylation of Ser375 or whether it was the serine *per se* that was required for the interaction with Oct-1.

In contrast to those experiments, when protein synthesis was allowed during infection, IE gene activation in the SJO2 was nearly indistinguishable from that of the wild type virus. Furthermore, this mutant virus strain was completely unaffected in its growth, replicating at the same speed as the wild type KOS. Because it was clear that the Ser375Ala mutation would not allow the VIC-dependent activation of the IE genes, the

IE genes had to be expressed by another mechanism. Here, two models to achieve this result will be presented.

The threshold model

Factors that have been shown to facilitate activation of the IE genes include the IE-genes themselves, especially ICP0 and ICP4. It is possible that the majority of IE gene expression seen in SJO2 infection is due to activation by these factors. In this model, it is proposed that VP16-S375A, even though it is a much weaker activator that the wild type, it is still capable of activating the IE genes at a low level. When translated, these IE gene transcripts then trigger the subsequent transcription cascade. This would mean that the threshold level for triggering this cascade would lie in the marginal difference that is observed between RP5 and SJO2 in figure 13.

The TAATGARAT is not the only significant response element through which VP16 acts

An alternative model might suggest that the S375A mutants inability to bind the IE promoters *in vitro* is not mirrored *in vivo*. One could imagine additional factors that facilitate interaction between VP16 and conserved elements in the IE promoters, apart from the TAATGARAT element. This is not an unlikely scenario, as deletion of the GA-element in the IE promoters decreases VP16-mediated activation (Triezenberg *et al.* 1988b). It is also know that HCF-1 interact with both Sp1 and GABP, as well as with VP16 (Gunther *et al.* 2000; Vogel *et al.* 2000). This suggests that VP16 could be recruited to the IE promoters through either the Sp1 sites or GA-elements. Both of these

interactions rely on surfaces of HCF-1 distinct from the β-propeller where VP16 interacts. This might suggest that HCF-1 can interact with both VP16 and either of these factors, recruiting VP16 to the promoter. In light of the effect of cycloheximide on IE gene expression by SJO2, the action of such a factor would be prevented by either the lack of protein synthesis or possibly by another effect of cycloheximide treatment. This could mean that the factor is a *de novo* synthesized protein whose expression is triggered by the infection. Both GABP and Sp1 are widely expressed genes.

. Ser411 was found in chapter 2 to be a likely phosphorylation site. While there was no direct correlation between the site and a function of VP16, the phosphorylation itself made it a likely candidate for regulation. There was no evidence that the mutations at Ser411 affected the activation of the IE genes in any way. Furthermore, there was no evidence that these mutations affected the growth of the virus. It was concluded that phosphorylation of Ser411 had no function in infection of HeLa cells.

The growth and transcription phenotypes are a little surprising if it is assumed that the bulk of IE gene expression comes from the VIC. Here, healthy growth and abundant activation of the IE genes was observed in a virus expressing a VP16 that is unable to bind the TAATGARAT *in vitro*.

Caveats

The identification of phosphorylation sites within VP16 were tempered by the following facts. The peptide mapping provided an incomplete picture of the phosphorylation of VP16. The method could only reliably identify phosphorylation between aa30-343 and 371-490. It did not address whether phosphorylation occurred

between aa1-29 or 344-370. Furthermore , while it did exclude phosphorylation of the serines between aa 30-343, it could not distinguish between the serines in the 371-490 fragment. There was positive evidence for phosphorylation of the 371-412 fragment. This site included serines at Ser375, Ser400 and Ser411. There was also positive evidence of both the 410-454 and the 410-490 fragments. These encompass the serines at 411, 419, 452 and , for the latter fragment, 462.

The phosphorylation sites that were identified by MS may not fully represent the sites in the protein. Six serines were not represented within the peptides identified by MS. Phosphorylated peptides representing three of the remaining twenty serines were found, but the lack of phosphorylated peptides representing the remaining seventeen sites can not be taken as rigorous evidence that no phosphorylation occurred there. It is possible that the ionization of a specific peptide in the MS can be negatively affected by the presence of a phosphogroup and therefore is underrepresented or absent in the MS.

The peptide mapping and the MS does not conflict about the phosphorylation of any residues. None of the sites within the 30-370 fragment that were identified by MS were found to be phosphorylated. Similarly, the labeled fragments encompassing 371-490, 371-454, 371-412, 410-490 and 410-454 were all found to have at least one serine residue that was either identified as a phosphorylation site by MS or was not found by the MS. However, both methods may fail to detect low abundance phosphorylated species. This means that if a small sub-population of VP16 in the cell is phosphorylated as a method of tagging it for a specific action, it is possible that neither method would be able to detect it.

The immunoprecipitation of VP16 from late in infection of HeLa cells is a "snap-shot" of the function of VP16. If the phosphorylation of VP16 is a regulatory function, the phosphorylatable residues must exist both in a phosphorylated and an unphosphorylated state. This means that as VP16 changes function from early to late in infection or in the virion or in reactivation from latency, the entire population of VP16 might change its phosphorylation state. The isolation of VP16 from a specific stage of infection will only address the phosphorylation state of VP16 when performing the activities required by VP16 at that stage. This means that phosphorylation could be quite different under different conditions and could include both serines that were found not to be phosphorylated in this study as well as both threonine and tyrosine residues.

The phosphorylation sites that were detected in the MS must be understood to be separated events. One can not draw any conclusion about whether these modifications occur in the same VP16 molecule or not. All of these modification might be present in a single VP16 molecule, but if the phosphorylation serves as a regulatory switch, a particular molecule is more likely to only have a subset of these modifications.

The immunoprecipitation of VP16 from infected cells is intended to isolate a representation of all of the VP16 populations in that particular stage of infection. It is however possible that the immunoprecipitation unintentionally fractionates the VP16 population. For instance, if a specific phosphorylation event causes VP16 to associate with an insoluble part of the cell lysate, the population of VP16 which carries this modification would be lost in the generation of the infected cell extract. Another cause might be that phosphorylation of the epitope recognized by the precipitating monoclonal antibody could interfere with the precipitation of a population of VP16, if it was modified

at this specific site. The fractionation of VP16 by events such as these are unlikely to completely remove VP16 from the immunoprecipitate, but they might cause an underrepresentation of a particular sub-population of VP16, causing it to go undetected in either the MS or the peptide mapping.

Specifically for SJO2, there is a concern in the selection of the mutant strain. Homologous recombination, selection and generation of SJO2 stock took place in the absence of complementation by a wt VP16. If the mutation of SJO2 at Ser375 does in fact generate an inviable or weakened virus, the lack of complementation introduces a selective pressure which favors strains that have acquired second-site compensatory mutations. Such mutations might hypothetically mask any deficiencies in the function of SJO2. This concern is tempered to some extent by the fact that in the absence of protein synthesis, SJO2 IE gene expression is similar to that of a Ser375 mutant of VP16 expressed from a plasmid. That means that none of the components that the virus carries into the host cell are capable of compensating for the deficiencies of VP16 375SA. It still leaves the possibility that some factor, expressed after infection, is capable of compensating for the mutant VP16. It is not possible to determine whether this compensation is due to a random mutation even or whether the function is present in the wild type virus. The most reliable method to address this issue is to generate additional strains, carrying the S375A mutation in VP16, but screening for the recombinants in the presence of a complementing wild type VP16. This would require a reliable method for screening the mutants. One such method has been developed and used in the generation of the SO series, but this has caveats associated with it, too.

In the generation of the SO strains, the major issue is the effects of adding a bulky domain to the activation domain of VP16. While there has been no report that EGFP is phosphorylated, it is a distinct possibility that the protein is a substrate for kinases. It might explain why there is no discernible loss of phosphorylation in the SO series. If the bulk of phosphorylation takes place within the EGFP, it would further mask small changes in phospholevels. Especially if these changes are already masked by the presence of additional phosphorylation sites within VP16 proper. It does not appear that there are gross changes in the level of phosphorylation, when comparing KOS and DG1. Yet, no real quantitative data is available to show this.

Furthermore, while fusing large peptides to the activation domain of VP16 does not seem to affect its function (McKnight *et al.* 1994) it may be that the EGFP itself complements the defects of the Ser411 mutants. At first glance, this suggestion seems unlikely, but one could imagine a number of ways that this could be the case. First, if the EGFP acts as a cryptic activation domain, it might complement loss of function of one or both of the VP16 ADs. Or the added strength might complement for lower affinity for the promoter response element. It is also possible that the EGFP packaged in the virion affects the kinetics of virion entry, intracellular transport of VP16 or virion packaging and egress. VP16 takes part in all of these events and changes in the activity due to the mutations that are introduced may be ameliorated by the presence of EGFP.

The viability of all of the strains in this dissertation were tested under conditions of single stage proliferation. Infection in this type of experiment is done at an MOI of 5-10. For RP5 and to some extent for in1814, a high MOI has been shown to reduce the apparent viability phenotype. The difference in the proliferation at high and low MOI of

these strains may be due to the expression of viral genes from multiple genomes. The mounting of a successful infectious event most likely relies on the presence of a threshold level of certain factors, whether this level is achieved through high levels of expression from a few templates or low levels of expression from a large number of templates. If testing SJO2, SO11, SO12 and SO13 under low MOI conditions, these strains might display a similar low MOI phenotype. The single stage growth curve also does not address whether the mutations affect re-infection of neighboring cells. If the mutations affect the ability of VP16 to quench the stress response in the primary host cell, neighboring cells may be able to mount a defense, possibly rendering them less susceptible to infection. In a single stage infection, all cells are infected simultaneously.

The growth of the mutant strains generated here were tested only in Vero cells. These cells are used in this assay because of their inherent susceptibility to infection with HSV-1. But the mutations at Ser375 or Ser411 could give rise to growth phenotypes that are either host-specific, either due to the presence or absence of a specific factor in the host cell or simply because a slow-growth phenotype inherent to these mutations are masked by the overall susceptibility of the host cell.

The transcription of the IE genes was tested in infection using a set of reporter genes. This method may not represent the effect of the mutations on the endogenous IE genes for several reasons. First, the environment of the IE promoters of the transfected reporter is clearly different from that of the viral genome. The reporter plasmids are introduced into the cell in the context of transfection reagent that by themselves may affect the expression of the genes. The plasmids may also be sequestered in a different nuclear compartment than the viral genome. The templates could also associate with

different factors, such as histones or polyamines, which would affect the expression of the genes encoded in these templates. Secondly, by looking at the expression from a plasmid reporter, artifactual misexpression of the viral genes could be masked. This could be due to mutations acquired in addition to those at Ser375 or Ser411 of VP16. For instance, a constitutive up-mutation in the ICP0 promoter could render transcription from that promoter independent of VP16 activation. The expression of the ICP0 gene would in turn cause the activation of the rest of the IE genes and subsequently trigger the viral gene expression cascade. In the presence of cycloheximide, because the up-mutation is not present in the reporter plasmid expression of the ICP0 gene would not be detected in the reporter assay. On the other hand, if the IE genes of the viral genome were tested directly, this defect would be much easier to identify. The misexpression would also be a result of cycloheximide treatment or the transfection reagent.

As for the proliferation phenotype, some transcriptional activation defects can be masked in infections at high MOI. Because the transcriptional activation assays in this dissertation were at high MOI, it is possible that similar defects in SJO2 and the SO series went undiscovered. The hypothesis is that the optimal expression of the IE genes can be achieved by high expression from a few templates or by lower expression from more templates. Therefore, the transcriptional assays that were done at high MOI may have missed transcription phenotypes of this particular type.

Finally, the observation that expression of the IE reporters in the transcription assay of SJO2 might be explained by a decrease in the protein stability of VP16-Ser375Ala, as compared to wild type VP16. To explain the wild type phenotype of SJO2 in the absence of cycloheximide, one would have to argue that either the protein is

directly destabilized by the drug or that the mutant protein is susceptible to degradation only in the absence of protein expression.

The findings of this dissertation are internally consistent. The mapping of the phosphorylation sites of VP16 by the peptide mapping and by MS did not show any conflict between assignment of phosphorylation sites. The exclusive identification of serines as phosphorylation sites in the MS was supported by the phosphoamino acid analysis. The mutagenesis of VP16 at either Ser375 or Ser411 did not lead to loss of phosphorylation of bulk VP16, consistent with the finding of multiple phosphorylation sites discovered in the MS. In particular, there was no evidence that the loss of Ser375 led to any appreciable loss of phosphorylation in the peptide mapping. This finding was in agreement with the MS, which found Ser375 to be unphosphorylated.

The loss of Ser375 did not cause an appreciable defect in proliferation with SJO2. Yet, a defect was discovered in IE gene activation by that strain, when translation was blocked during infection. The apparent disagreement between those two observations was resolved by the observation that when translation was not blocked, the expression of the IE genes were only modestly affected in SJO2 infection. These three observations were consistent with a model where IE gene expression in HSV-1 can be activated by VP16 alone, if VP16 is capable of interacting with Oct-1. If VP16 can not interact with Oct-1, the IE genes can be activated through a mechanism that requires de novo synthesis of protein upon infection. If protein expression is allowed, the expression of the IE genes may be required for the proliferation of the virus, but the expression of the IE genes are not dependent on the binding of VP16 to Oct-1. That means that the expression of the IE genes, when protein translation is allowed, is consistent with the growth phenotype. The

absence of expression of the IE genes in the absence of protein synthesis is also consistent with the model, if the translation-independent mechanism of IE gene activation relies on the binding of VP16 to Oct-1

Chapter Four

Discussion and Future Directions

Hypotheses

Hypothesis: The Ser375 dependent formation of the VIC does not require phosphorylation of VP16.

In retrospect, there is no good evidence to suggest that the interaction between VP16 and Oct-1 relies on phosphorylation. In my research, I have found no evidence to suggest that VP16 is phosphorylated at Ser375 late in infection and the evidence presented by O'Reilly *et al* is not convincing (O'Reilly *et al.* 1997). Furthermore, Babb *et al* demonstrate convincingly that the formation of the VIC occurs in the absence of phosphorylation of VP16 (Babb *et al.* 2001). While it can not be ruled out that phosphorylation of VP16 would further enhance this interaction, it is interesting that the interaction between Oct-1 and VP16 is disrupted by replacing Ser375 with acidic residues (O'Reilly *et al.* 1997). Although it is not universally true that substitution of a phosphoresidue can be functionally replaced by an acidic residue, it does add support to the hypothesis that the mechanism of interaction does not simply rely on the presence of a negative charge.

Therefore, I hypothesize that if a) there is no evidence of phosphorylation of VP16 at Ser375 in infection or by the kinases in a nuclear extract, b) if the formation of the VIC does not require phosphorylation of any kind and c) if replacement of the serine with neither aspartate nor glutamate conserves the transcriptional activity of VP16, then phosphorylation of VP16 at Ser375 is not required for activation. because VP16 isolated

late in infection still is not phosphorylated at Ser375, it is unlikely that phosphorylation of Ser375 serves to prevent the interaction between VP16 and Oct-1.

Hypothesis: Ser400 is a major site of phosphorylation.

In this dissertation, I have demonstrated that Ser375 is very unlikely to be a phosphorylation site. I have also demonstrated that while mapping phosphorylation sites of VP16 can not rule out Ser411, there is no functional evidence to show that Ser411 need be a phosphorylation site. Furthermore, the site is not conserved between the homologs of VP16 (Figure 9). However, it is clear that the 371-413 fragment of RP5 is phosphorylated at a serine. The only remaining serine in the fragment is Ser400. While the MS data did not find evidence that this site is phosphorylated, it is only represented by a few spectra and may have been missed by the MS. And in contrast to Ser411, the putative phosphorylation at Ser400 is conserved between HSV-1, HSV-1, VZV, EHV-1 and BHV-1. Based on these facts and observations, I hypothesize that the primary phosphorylation site of the 371-413 fragment is Ser400.

Hypothesis: VP16 requires HCF-1 to activate IE genes during infection by a mechanism other than the VIC complex.

In my research, I discovered that mutating VP16 at Ser375 severely diminishes the activation of the IE genes by VP16. However, the same mutation does not affect viability of SJO2 in infection, nor does it abolish the activation of the IE genes through another mechanism. It is clear that the loss of the activation domain of RP5 and V422, a similar truncation mutant, has a significant effect on the activation of the IE genes

(Smiley *et al.* 1997; Tal-Singer *et al.* 1999) as well as proliferation. It is also clear that the in1814 insertion, which most strongly affects the interaction with HCF-1, abolishes activation of the IE genes (Ace *et al.* 1989).

I hypothesize that the interaction with HCF-1 is required for the activation of the IE genes through VP16. This requirement can be divided into two aspects: Nuclear localization and direct activation. The formation of the VIC relies on the presence of all three members of the complex in the nucleus. It has been suggested that the interaction of VP16 and HCF-1 is required for the nuclear localization of VP16. (Mahajan *et al.* 2002). Therefore, any disruption of this interaction will initially prevent activation of the IE genes due to the retention of VP16 in the cytoplasm. However, if the interaction is conserved, and facilitated the nuclear localization of VP16, the disruption of the VIC through abolishing the interaction with Oct-1 by the Ser375Ala mutation is not sufficient to prevent the expression of the IE genes. RP5 and V422 can not activate the IE genes, even though they presumably make it to the nucleus, therefore the VP16 AD must be required for activation by both the VIC dependent and the VIC independent pathway.

In this hypothesis, I do not distinguish between a model in which the interaction with HCF-1 is only required for VIC-independent activation due to its role in nuclear localization of VP16 or whether it has a direct role in activation of the IE genes.

Hypothesis: The luman family of proteins may be phosphorylated at a position homologous to Ser353 of VP16.

VP16 uses host factors for DNA binding. The only cellular proteins with a similar function to that of the core domain of VP16 are those of the Luman family of

transcription factors. These factors has a very limited sequence similarity, except for the 4 aa HBM. These factors interact with HCF-1 in a similar manner to VP16 through HBM (E/D-H-x-Y) . The HBM is also conserved in the α-herpesviruses. However, the phosphorylation site proximal to this site, Ser353 does not have a good homology in any of the herpes viruses. Even HSV-2, which is otherwise very conserved and does have a conserved serine at this site, is quite divergent in this motif.

Curiously, if the HBM motif of the cellular HBM-proteins is used as an anchor, Ser353, which is 7 amino acids aminoterminal to the HBM motif, aligns with an invariant serine in Luman and Zf, as well as their homologues in B. taurus, M. musculus and R. norwegicus. Furthermore, the three preceding amino acids (FTT) are either identical or conservative substitutions. I propose that the interaction surface that VP16 mimics to interact with HCF-1 includes this motif. Because this site was found to be phosphorylated in VP16, it is also possible that it is phosphorylated in the cellular homologs. That means that it could be a regulatory site for interaction with HCF-1, not only for VP16, but also for the cellular factors. In this model, I favor the proposal that the interaction between VP16 and HCF-1 is disrupted, rather than facilitated by phosphorylation. This suggestion is based on the fact that VIC can form from bacterially expressed factors, which are likely to be unmodified. This idea is also in agreement with the observation that the site is phosphorylated late in infection. At this time, prevention of interaction with HCF-1 could be required to ensure the availability of VP16 for other functions. This model is supported, in the observation that late in infection, VP16 is separated into two populations, one that co-localizes with HCF-1 in the nucleus of the infected cell and one that does not (LaBoissiere *et al.* 2000).

The phosphorylation sites at Ser18, Ser353 and Ser452 may be involved in regulating other VP16 functions.

In addition to the specific hypotheses that I have presented above, there are still more possible functions for the phosphorylation of VP16. These other functions of VP16 that may change during infection with HSV-1 include the putative repression of the vhs, the interaction with VP22 and the role in DNA encapsidation and nuclear egress. Regulation of these processes may also be achieved through phosphorylation of VP16. I did not address the involvement of the mutant versions of VP16 in any of these activities in my project, but it may be possible to develop specific hypotheses about each of these functions and the putative role of phosphorylation in their regulation. These models can be can be generalized to the role of phosphorylation in regulation of interaction between partners. Phosphorylation of one or more of the discovered phosphorylation sites could serve to either facilitate or prevent interaction between VP16 and these factors.

Future research: Addressing the hypotheses experimentally

The reagents that have been generated in the course of this dissertation are obvious starting points for the continuation of this project. Also, the determination of additional phosphorylation sites would prompt the generation of additional mutant strains, carrying knock-outs at this site. Finally, the continuing investigation of phosphorylation of VP16 during other times in infection would also assist in understanding the complete role of VP16 in infection.

Investigate the SJO2 phenotype

The first set of these experiments involves the continuing examination of the phosphorylation sites at Ser375 and 411 during infection. Especially the Ser375 strain promises to add to our knowledge of VP16-mediated activation. If one hypothesizes, based on the GABP and Sp1 interactions, (Gunther *et al.* 2000; Vogel *et al.* 2000) that VP16 gets recruited to the promoter during infection, it should be possible to detect it there by chromatin immunoprecipitation. Depending on whether VP16 was found at the promoter or not, it would be possible to attribute the effect to a deficiency in promoter binding or transactivation. Secondly, if VP16 is found at the promoter, it would be interesting to see whether it remains there in the presence of cycloheximide. If not, it would suggest that in the absence of the VIC, VP16 relies either on a newly synthesized protein factor, or more generally, on a factor which changes character in the presence of cycloheximide. The more general interpretation must be kept in mind, because cycloheximide may affect the cell in ways other than blocking protein synthesis.

Another aspect that should be addressed is the potential low multiplicity effect of the mutation. In1814 and RP5 both have demonstrated greater problems in proliferation at lower multiplicities of infection. It is possible that SJO2 has similar problems at lower moi, but to a lesser extent. SJO2 may be fully rescued at high moi, in contrast to RP5 and in1814, where the phenotype is only partially complemented. But if SJO2 were to exhibit a mutant phenotype, similar to those of in1814 and RP5, even if it is less severe, it would suggest that the mutation in all three work through the same mechanism.

Ser18, Ser343 and Ser452 phosphorylation

The obvious next step in regards to the phosphorylation of these sites is mutagenesis. Because there is a wide varieties of effects these sites might have on the virus, it would be prudent, to remain in the context of the virus. It would be possible to use the EGFP-tag screen to identify these mutants, leaving the possibility of generating them in complementing cell lines. The continued research into these sites will be driven by the phenotypes that are observed as a result of the mutations.

Other approaches

An intriguing possibility is that VP16 might be recruited to the IE promoters through Sp1 or GABP sites. This mechanism could be investigated outside of the virus. The interactions could be observed *in vitro* or by transfection. This method would facilitate a greater range of mutations that can be introduced and tested for their relevance. The interactions could be examined in transfection, both by examining the recruitment of VP16 to the promoter, for instance by chromatin immunoprecipitation but also by standard promoter expression assays.

Luman homology

Finally, the homology with the Luman family could be tested. If the hypothesis holds true, I would expect both Luman and Zf to be phosphorylated. I would also expect alanine substitutions at that site to affect the function of Luman and Zf, as well as of VP16. This line of investigation would be started in the same manner as this project was, simply by verifying the phosphorylation of the protein and then proceeding to map it by MS.

LIST OF REFERENCES

Ace, C. I., T. A. McKee, J. M. Ryan, J. M. Cameron and C. M. Preston (1989). "Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression." J Virol **63**: 2260-9.

Adams, J., R. Kelso and L. Cooley (2000). "The kelch repeat superfamily of proteins: propellers of cell function." Trends Cell Biol **10**: 17-24.

Andrew, C. D., J. Warwicker, G. R. Jones and A. J. Doig (2002). "Effect of phosphorylation on alpha-helix stability as a function of position." Biochemistry **41**: 1897-905.

Arnosti, D. N., C. M. Preston, M. Hagmann, W. Schaffner, R. G. Hope, G. Laughlan and B. F. Luisi (1993). "Specific transcriptional activation in vitro by the herpes simplex virus protein VP16." Nucleic Acids Res **21**: 5570-6.

Ashcroft, M., M. H. Kubbutat and K. H. Vousden (1999). "Regulation of p53 function and stability by phosphorylation." Mol Cell Biol **19**: 1751-8.

Babb, R., C. C. Huang, D. J. Aufiero and W. Herr (2001). "DNA recognition by the herpes simplex virus transactivator VP16: a novel DNA-binding structure." Mol Cell Biol **21**: 4700-12.

Bagby, S., T. K. Mal, D. Liu, E. Raddatz, Y. Nakatani and M. Ikura (2000). "TFIIA-TAF regulatory interplay: NMR evidence for overlapping binding sites on TBP." FEBS Lett **468**: 149-54.

Barlev, N. A., R. Candau, L. Wang, P. Darpino, N. Silverman and S. L. Berger (1995). "Characterization of physical interactions of the putative transcriptional adaptor, ADA2, with acidic activation domains and TATA-binding protein." J Biol Chem **270**: 19337-44.

Baru, M., M. Shlissel and H. Manor (1991). "The yeast GAL4 protein transactivates the polyomavirus origin of DNA replication in mouse cells." J Virol **65**: 3496-503.

Bauer, D. and R. Tampe (2002). "Herpes viral proteins blocking the transporter associated with antigen processing TAP--from genes to function and structure." Curr Top Microbiol Immunol **269**: 87-99.

Bennett-Cook, E. R. and J. A. Hassell (1991). "Activation of polyomavirus DNA replication by yeast GAL4 is dependent on its transcriptional activation domains." Embo J **10**: 959-69.

Berger, S. L. (2002). "Histone modifications in transcriptional regulation." Current Opinion in Genetics & Development **12**: 142–148.

Berger, S. L., B. Pina, N. Silverman, G. A. Marcus, J. Agapite, J. L. Regier, S. J. Triezenberg and L. Guarente (1992). "Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains." Cell **70**: 251-65.

Bhella, D., F. J. Rixon and D. J. Dargan (2000). "Cryomicroscopy of human cytomegalovirus virions reveals more densely packed genomic DNA than in herpes simplex virus type 1." J Mol Biol **295**: 155-61.

Blom, N., S. Gammeltoft and S. Brunak (1999). "Sequence and structure-based prediction of eukaryotic protein phosphorylation sites." J Mol Biol **294**: 1351-62.

Boutell, C. and R. Everett (2003). "The HSV-1 regulatory protein ICP0 interacts with and ubiquitinates p53." J Biol Chem **Epub ahead of print**.

Bowman, B. R., M. L. Baker, F. J. Rixon, W. Chiu and F. A. Quiocho (2003). "Structure of the herpesvirus major capsid protein." Embo J **22**: 757-65.

Bresnahan, W. A. and T. Shenk (2000). "A subset of viral transcripts packaged within human cytomegalovirus particles." Science **288**: 2373-6.

Brownell, J. E., J. Zhou, T. Ranalli, R. Kobayashi, D. G. Edmondson, S. Y. Roth and C. D. Allis (1996). "Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation." Cell **84**: 843-51.

Burton, E. A., C. S. Hong and J. C. Glorioso (2003). "The Stable 2.0-Kilobase Intron of the Herpes Simplex Virus Type 1 Latency-Associated Transcript Does Not Function as an Antisense Repressor of ICP0 in Nonneuronal Cells." J Virol **77**: 3516-30.

Campbell, M. E., J. W. Palfreyman and C. M. Preston (1984). "Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription." J Mol Biol **180**: 1-19.

Candau, R., J. X. Zhou, C. D. Allis and S. L. Berger (1997). "Histone acetyltransferase activity and interaction with ADA2 are critical for GCN5 function in vivo." Embo J **16**: 555-65.

Chen, I. H., K. S. Sciabica and R. M. Sandri-Goldin (2002). "ICP27 interacts with the RNA export factor Aly/REF to direct herpes simplex virus type 1 intronless mRNAs to the TAP export pathway." J Virol **76**: 12877-89.

Chi, J. H. and D. W. Wilson (2000). "ATP-Dependent localization of the herpes simplex virus capsid protein VP26 to sites of procapsid maturation." J Virol **74**: 1468-76.

Chiu, W. and F. J. Rixon (2002). "High resolution structural studies of complex icosahedral viruses: a brief overview." Virus Res **82**: 9-17.

Chrivia, J. C., R. P. Kwok, N. Lamb, M. Hagiwara, M. R. Montminy and R. H. Goodman (1993). "Phosphorylated CREB binds specifically to the nuclear protein CBP." Nature **365**: 855-9.

Cress, W. D. and S. J. Triezenberg (1991). "Critical structural elements of the VP16 transcriptional activation domain." Science **251**: 87-90.

Desai, P., J. C. Akpa and S. Person (2003). "Residues of VP26 of herpes simplex virus type 1 that are required for its interaction with capsids." J Virol **77**: 391-404.

Desai, P., N. A. DeLuca and S. Person (1998). "Herpes simplex virus type 1 VP26 is not essential for replication in cell culture but influences production of infectious virus in the nervous system of infected mice." Virology **247**: 115-24.

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

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

Doig, A. J. (2002). "Recent advances in helix-coil theory." Biophys Chem **101-102**: 281-93.

Doig, A. J. and R. L. Baldwin (1995). "N- and C-capping preferences for all 20 amino acids in alpha-helical peptides." Protein Sci **4**: 1325-36.

Dolter, K. E., R. Ramaswamy and T. C. Holland (1994). "Syncytial mutations in the herpes simplex virus type 1 gK (UL53) gene occur in two distinct domains." J Virol **68**: 8277-81.

Elgadi, M. M., C. E. Hayes and J. R. Smiley (1999). "The herpes simplex virus vhs protein induces endoribonucleolytic cleavage of target RNAs in cell extracts." J Virol **73**: 7153-64.

Elliott, G., G. Mouzakitis and P. O'Hare (1995). "VP16 interacts via its activation domain with VP22, a tegument protein of herpes simplex virus, and is relocated to a novel macromolecular assembly in coexpressing cells." J Virol **69**: 7932-41.

Elliott, G. and P. O'Hare (1997). "Intercellular trafficking and protein delivery by a herpesvirus structural protein." Cell **88**: 223-33.

Everett, R. D. (2000). "ICP0, a regulator of herpes simplex virus during lytic and latent infection." Bioessays **22**: 761-70.

Everett, R. D. and G. G. Maul (1994). "HSV-1 IE protein Vmw110 causes redistribution of PML." Embo J **13**: 5062-9.

Everett, R. D., G. Sourvinos and A. Orr (2003). "Recruitment of Herpes Simplex Virus Type 1 Transcriptional Regulatory Protein ICP4 into Foci Juxtaposed to ND10 in Live, Infected Cells." J Virol **77**: 3680-9.

Everly, D. N., Jr., P. Feng, I. S. Mian and G. S. Read (2002). "mRNA degradation by the virion host shutoff (Vhs) protein of herpes simplex virus: genetic and biochemical evidence that Vhs is a nuclease." J Virol **76**: 8560-71.

Fenwick, M. L. and R. D. Everett (1990). "Inactivation of the shutoff gene (UL41) of herpes simplex virus types 1 and 2." J Gen Virol **71**: 2961-7.

Ferdous, A., F. Gonzalez, L. Sun, T. Kodadek and S. A. Johnston (2001). "The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II." Mol Cell **7**: 981-91.

Gaffney, D. F., J. McLauchlan, J. L. Whitton and J. B. Clements (1985). "A modular system for the assay of transcription regulatory signals: the sequence TAATGARAT is required for herpes simplex virus immediate early gene activation." Nucleic Acids Res **13**: 7847-63.

Gallagher, S. R. (1999). One-dimensional SDS gel electrophoresis of proteins. Current Protocols in Current Biology. F. M. Ausubel. **2**.

Gandhi, M. and R. M. Greenblatt (2002). "Human herpesvirus 8, Kaposi's sarcoma, and associated conditions." Clin Lab Med **22**: 883-910.

Gibson, W. and B. Roizman (1974). "Proteins specified by herpes simplex virus. Staining and radiolabeling properties of B capsid and virion proteins in polyacrylamide gels." J Virol **13**: 155-65.

Gonzalez, F., A. Delahodde, T. Kodadek and S. A. Johnston (2002). "Recruitment of a 19S proteasome subcomplex to an activated promoter." Science **296**: 548-50.

Goto, H., S. Motomura, A. C. Wilson, R. N. Freiman, Y. Nakabeppu, K. Fukushima, M. Fujishima, W. Herr and T. Nishimoto (1997). "A single-point mutation in

HCF causes temperature-sensitive cell-cycle arrest and disrupts VP16 function." Genes Dev **11**: 726-37.

Graham, F. L. and A. J. van der Eb (1973). "A new technique for the assay of infectivity of human adenovirus 5 DNA." Virology **52**: 456-67.

Grant, P. A., D. Schieltz, M. G. Pray-Grant, D. J. Steger, J. C. Reese, J. R. Yates, 3rd and J. L. Workman (1998). "A subset of TAF(II)s are integral components of the SAGA complex required for nucleosome acetylation and transcriptional stimulation." Cell **94**: 45-53.

Greaves, R. F. and P. O'Hare (1990). "Structural requirements in the herpes simplex virus type 1 transactivator Vmw65 for interaction with the cellular octamer-binding protein and target TAATGARAT sequences." J Virol **64**: 2716-24.

Greensides, D. R. (2002). VP16 localization during herpes simplex-1 infection. Cell and Molecular Biology. East Lansing, Michigan State University: 209.

Greijer, A. E., C. A. Dekkers and J. M. Middeldorp (2000). "Human cytomegalovirus virions differentially incorporate viral and host cell RNA during the assembly process." J Virol **74**: 9078-82.

Grossmann, J. G., A. J. Sharff, P. O'Hare and B. Luisi (2001). "Molecular shapes of transcription factors TFIIB and VP16 in solution: implications for recognition." Biochemistry **40**: 6267-74.

Gu, B., R. Rivera-Gonzalez, C. A. Smith and N. A. DeLuca (1993). "Herpes simplex virus infected cell polypeptide 4 preferentially represses Sp1-activated over basal transcription from its own promoter." Proc Natl Acad Sci U S A **90**: 9528-32.

Gunther, M., M. Laithier and O. Brison (2000). "A set of proteins interacting with transcription factor Sp1 identified in a two-hybrid screening." Mol Cell Biochem **210**: 131-42.

Halford, W. P., C. D. Kemp, J. A. Isler, D. J. Davido and P. A. Schaffer (2001). "ICP0, ICP4, or VP16 expressed from adenovirus vectors induces reactivation of latent herpes simplex virus type 1 in primary cultures of latently infected trigeminal ganglion cells." J Virol **75**: 6143-53.

Harper, E. T. and G. D. Rose (1993). "Helix stop signals in proteins and peptides: the capping box." Biochemistry **32**: 7605-9.

Heine, J. W., R. W. Honess, E. Cassai and B. Roizman (1974). "Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains." J Virol **14**: 640-51.

Herr, W. (1998). "The herpes simplex virus VP16-induced complex: mechanisms of combinatorial transcriptional regulation." Cold Spring Harb Symp Quant Biol **63**: 599-607.

Hippenmeyer, P. and M. Highkin (1993). "High level, stable production of recombinant proteins in mammalian cell culture using the herpesvirus VP16 transactivator." Biotechnology (N Y) **11**: 1037-41.

Homa, F. L. and J. C. Brown (1997). "Capsid assembly and DNA packaging in herpes simplex virus." Rev Med Virol **7**: 107-122.

Hope, I. A., S. Mahadevan and K. Struhl (1988). "Structural and functional characterization of the short acidic transcriptional activation region of yeast GCN4 protein." Nature **333**: 635-40.

Hukkanen, V., E. Broberg, A. Salmi and J. P. Eralinna (2002). "Cytokines in experimental herpes simplex virus infection." Int Rev Immunol **21**: 355-71.

Hutchinson, L., F. L. Graham, W. Cai, C. Debroy, S. Person and D. C. Johnson (1993). "Herpes simplex virus (HSV) glycoproteins B and K inhibit cell fusion induced by HSV syncytial mutants." Virology **196**: 514-31.

Jamieson, D. R., L. H. Robinson, J. I. Daksis, M. J. Nicholl and C. M. Preston (1995). "Quiescent viral genomes in human fibroblasts after infection with herpes simplex virus type 1 Vmw65 mutants." J Gen Virol **76**: 1417-31.

Jans, D. A. and S. Hubner (1996). "Regulation of protein transport to the nucleus: central role of phosphorylation." Physiol Rev **76**: 651-85.

Jones, K. A. and R. Tjian (1985). "Sp1 binds to promoter sequences and activates herpes simplex virus 'immediate-early' gene transcription in vitro." Nature **317**: 179-82.

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 Res **18**: 6871-80.

Kennedy, P. G. and A. Chaudhuri (2002). "Herpes simplex encephalitis." J Neurol Neurosurg Psychiatry **73**: 237-8.

Khodarev, N. N., S. J. Advani, N. Gupta, B. Roizman and R. R. Weichselbaum (1999). "Accumulation of specific RNAs encoding transcriptional factors and stress response proteins against a background of severe depletion of cellular RNAs in cells infected with herpes simplex virus 1." Proc Natl Acad Sci U S A **96**: 12062-7.

Knez, J., P. T. Bilan and J. P. Capone (2003). "A Single Amino Acid Substitution in Herpes Simplex Virus Type 1 VP16 Inhibits Binding to the Virion Host Shutoff Protein and Is Incompatible with Virus Growth." J Virol **77**: 2892-902.

Kobayashi, A., T. Miyake, M. Kawaichi and T. Kokubo (2003). "Mutations in the histone fold domain of the TAF12 gene show synthetic lethality with the TAF1 gene lacking the TAF N-terminal domain (TAND) by different mechanisms from those in the SPT15 gene encoding the TATA box-binding protein (TBP)." Nucleic Acids Res **31**: 1261-74.

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." Embo J **8**: 4229-38.

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." Proc Natl Acad Sci U S A **81**: 4065-9.

Kristie, T. M. and B. Roizman (1986). "DNA-binding site of major regulatory protein alpha 4 specifically associated with promoter-regulatory domains of alpha genes of herpes simplex virus type 1." Proc Natl Acad Sci U S A **83**: 4700-4.

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 Dev **4**: 2383-96.

Kristie, T. M., J. L. Vogel and A. E. Sears (1999). "Nuclear localization of the C1 factor (host cell factor) in sensory neurons correlates with reactivation of herpes simplex virus from latency." Proc Natl Acad Sci U S A **96**: 1229-33.

Kuo, M. H., J. E. Brownell, R. E. Sobel, T. A. Ranalli, R. G. Cook, D. G. Edmondson, S. Y. Roth and C. D. Allis (1996). "Transcription-linked acetylation by Gcn5p of histones H3 and H4 at specific lysines." Nature **383**: 269-72.

Kuo, M. H., J. Zhou, P. Jambeck, M. E. Churchill and C. D. Allis (1998). "Histone acetyltransferase activity of yeast Gcn5p is required for the activation of target genes in vivo." Genes Dev **12**: 627-39.

La Boissiere, S., T. Hughes and P. O'Hare (1999). "HCF-dependent nuclear import of VP16." Embo J **18**: 480-9.

LaBoissiere, S. and P. O'Hare (2000). "Analysis of HCF, the cellular cofactor of VP16, in herpes simplex virus- infected cells." J Virol **74**: 99-109.

Lai, J. S., M. A. Cleary and W. Herr (1992). "A single amino acid exchange transfers VP16-induced positive control from the Oct-1 to the Oct-2 homeo domain." Genes Dev **6**: 2058-65.

Lai, J. S. and W. Herr (1997). "Interdigitated residues within a small region of VP16 interact with Oct- 1, HCF, and DNA." Mol Cell Biol **17**: 3937-46.

Lam, Q., C. A. Smibert, K. E. Koop, C. Lavery, J. P. Capone, S. P. Weinheimer and J. R. Smiley (1996). "Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion host shutoff function." Embo J **15**: 2575-81.

Leib, D. A. (2002). "Counteraction of interferon-induced antiviral responses by herpes simplex viruses." Curr Top Microbiol Immunol **269**: 171-85.

Leinbach, S. S. and W. C. Summers (1980). "The structure of herpes simplex virus type 1 DNA as probed by micrococcal nuclease digestion." J Gen Virol **51**: 45-59.

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

Lentine, A. F. and S. L. Bachenheimer (1990). "Intracellular organization of herpes simplex virus type 1 DNA assayed by staphylococcal nuclease sensitivity." Virus Res **16**: 275-92.

Li, R. and M. R. Botchan (1993). "The acidic transcriptional activation domains of VP16 and p53 bind the cellular replication protein A and stimulate in vitro BPV-1 DNA replication." Cell **73**: 1207-21.

Li, T., Y. Jin, A. K. Vershon and C. Wolberger (1998). "Crystal structure of the MATa1/MATalpha2 homeodomain heterodimer in complex with DNA containing an A-tract." Nucleic Acids Res **26**: 5707-18.

Li, T., M. R. Stark, A. D. Johnson and C. Wolberger (1995). "Crystal structure of the MATa1/MAT alpha 2 homeodomain heterodimer bound to DNA." Science **270**: 262-9.

Liesegang, T. J. (2001). "Herpes simplex virus epidemiology and ocular importance." Cornea **20**: 1-13.

Liu, D., R. Ishima, K. I. Tong, S. Bagby, T. Kokubo, D. R. Muhandiram, L. E. Kay, Y. Nakatani and M. Ikura (1998). "Solution structure of a TBP-TAF(II)230 complex: protein mimicry of the minor groove surface of the TATA box unwound by TBP." Cell **94**: 573-83.

Liu, Y., W. Gong, C. C. Huang, W. Herr and X. Cheng (1999). "Crystal structure of the conserved core of the herpes simplex virus transcriptional regulatory protein VP16." Genes Dev **13**: 1692-703.

Lium, E. K., C. A. Panagiotidis, X. Wen and S. Silverstein (1996). "Repression of the alpha0 gene by ICP4 during a productive herpes simplex virus infection." J Virol **70**: 3488-96.

Long, M. C., V. Leong, P. A. Schaffer, C. A. Spencer and S. A. Rice (1999). "ICP22 and the UL13 protein kinase are both required for herpes simplex virus-induced modification of the large subunit of RNA polymerase II." J Virol **73**: 5593-604.

Lu, R. and V. Misra (2000a). "Potential role for luman, the cellular homologue of herpes simplex virus VP16 (alpha gene trans-inducing factor), in herpesvirus latency." J Virol **74**: 934-43.

Lu, R. and V. Misra (2000b). "Zhangfei: a second cellular protein interacts with herpes simplex virus accessory factor HCF in a manner similar to Luman and VP16." Nucleic Acids Res **28**: 2446-54.

Lu, R., P. Yang, P. O'Hare and V. Misra (1997). "Luman, a new member of the CREB/ATF family, binds to herpes simplex virus VP16-associated host cellular factor." Mol Cell Biol **17**: 5117-26.

Lu, R., P. Yang, S. Padmakumar and V. Misra (1998). "The herpesvirus transactivator VP16 mimics a human basic domain leucine zipper protein, luman, in its interaction with HCF." J Virol **72**: 6291-7.

Mahajan, S. S., M. M. Little, R. Vazquez and A. C. Wilson (2002). "Interaction of HCF-1 with a cellular nuclear export factor." J Biol Chem **277**: 44292-9.

Mahajan, S. S. and A. C. Wilson (2000). "Mutations in host cell factor 1 separate its role in cell proliferation from recruitment of VP16 and LZIP." Mol Cell Biol **20**: 919-28.

Marcus, G. A., N. Silverman, S. L. Berger, J. Horiuchi and L. Guarente (1994). "Functional similarity and physical association between GCN5 and ADA2: putative transcriptional adaptors." Embo J **13**: 4807-15.

Matis, J. and M. Kudelova (2001). "Early shutoff of host protein synthesis in cells infected with herpes simplex viruses." Acta Virol **45**: 269-77.

Maul, G. G., H. H. Guldner and J. G. Spivack (1993). "Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early gene 1 product (ICP0)." J Gen Virol **74**: 2679-90.

McFarlane, M., J. I. Daksis and C. M. Preston (1992). "Hexamethylene bisacetamide stimulates herpes simplex virus immediate early gene expression in the absence of trans-induction by Vmw65." J Gen Virol **73**: 285-92.

McKnight, J. L., M. Doerr and Y. Zhang (1994). "An 85-kilodalton herpes simplex virus type 1 alpha trans-induction factor (VP16)-VP13/14 fusion protein retains the transactivation and structural properties of the wild-type molecule during virus infection." J Virol **68**: 1750-7.

McKnight, J. L., T. M. Kristie and B. Roizman (1987a). "Binding of the virion protein mediating alpha gene induction in herpes simplex virus 1-infected cells to its cis site requires cellular proteins." Proc Natl Acad Sci U S A **84**: 7061-5.

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

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." J Gen Virol **63**: 297-305.

McMahan, L. and P. A. Schaffer (1990). "The repressing and enhancing functions of the herpes simplex virus regulatory protein ICP27 map to C-terminal regions and are required to modulate viral gene expression very early in infection." J Virol **64**: 3471-85.

McNabb, D. S. and R. J. Courtney (1992). "Characterization of the large tegument protein (ICP1/2) of herpes simplex virus type 1." Virology **190**: 221-32.

Meredith, D. M., J. A. Lindsay, I. W. Halliburton and G. R. Whittaker (1991). "Post-translational modification of the tegument proteins (VP13 and VP14) of herpes simplex virus type 1 by glycosylation and phosphorylation." J Gen Virol **72**: 2771-5.

Mettenleiter, T. C. (2000). "Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis--state of the art, June 1999." Vet Res **31**: 99-115.

Miranda-Saksena, M., R. A. Boadle, P. Armati and A. L. Cunningham (2002). "In rat dorsal root ganglion neurons, herpes simplex virus type 1 tegument forms in the cytoplasm of the cell body." J Virol **76**: 9934-51.

Moriuchi, H., M. Moriuchi, R. Pichyangkura, S. J. Triezenberg, S. E. Straus and J. I. Cohen (1995). "Hydrophobic cluster analysis predicts an amino-terminal domain

of varicella-zoster virus open reading frame 10 required for transcriptional activation." Proc Natl Acad Sci U S A **92**: 9333-7.

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

Mossman, K. L., P. F. Macgregor, J. J. Rozmus, A. B. Goryachev, A. M. Edwards and J. R. Smiley (2001). "Herpes simplex virus triggers and then disarms a host antiviral response." J Virol **75**: 750-8.

Mossman, K. L., R. Sherburne, C. Lavery, J. Duncan and J. R. Smiley (2000). "Evidence that herpes simplex virus VP16 is required for viral egress downstream of the initial envelopment event." J Virol **74**: 6287-99.

Mossman, K. L. and J. R. Smiley (1999). "Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 renders expression of the immediate-early genes almost entirely dependent on ICP0." J Virol **73**: 9726-33.

Narlikar, G. J., H. Y. Fan and R. E. Kingston (2002). "Cooperation between complexes that regulate chromatin structure and transcription." Cell **108**: 475-87.

Nedyalkov, Y. A. and S. J. Triezenberg (2003). in press.

Nguyen-Huynh, A. T. and P. A. Schaffer (1998). "Cellular transcription factors enhance herpes simplex virus type 1 oriS- dependent DNA replication." J Virol **72**: 3635-45.

Nielsen, D. A., T. C. Chang and D. J. Shapiro (1989). "A highly sensitive, mixed-phase assay for chloramphenicol acetyltransferase activity in transfected cells." Anal Biochem **179**: 19-23.

Nishikawa, J., T. Kokubo, M. Horikoshi, R. G. Roeder and Y. Nakatani (1997). "Drosophila TAF(II)230 and the transcriptional activator VP16 bind competitively to the TATA box-binding domain of the TATA box-binding protein." Proc Natl Acad Sci U S A **94**: 85-90.

Ogle, W. O. and B. Roizman (1999). "Functional anatomy of herpes simplex virus 1 overlapping genes encoding infected-cell protein 22 and US1.5 protein." J Virol **73**: 4305-15.

O'Hare, P. and G. Williams (1992). "Structural studies of the acidic transactivation domain of the Vmw65 protein of herpes simplex virus using 1H NMR." Biochemistry **31**: 4150-6.

Ojala, P. M., B. Sodeik, M. W. Ebersold, U. Kutay and A. Helenius (2000). "Herpes simplex virus type 1 entry into host cells: reconstitution of capsid binding and uncoating at the nuclear pore complex in vitro." Mol Cell Biol **20**: 4922-31.

O'Reilly, D., O. Hanscombe and P. O'Hare (1997). "A single serine residue at position 375 of VP16 is critical for complex assembly with Oct-1 and HCF and is a target of phosphorylation by casein kinase II." Embo J **16**: 2420-30.

Orphanides, G., T. Lagrange and D. Reinberg (1996). "The general transcription factors of RNA polymerase II." Genes Dev **10**: 2657-83.

Ottosen, S. Personal communication.

Parker, D., K. Ferreri, T. Nakajima, V. J. LaMorte, R. Evans, S. C. Koerber, C. Hoeger and M. R. Montminy (1996). "Phosphorylation of CREB at Ser-133 induces complex formation with CREB- binding protein via a direct mechanism." Mol Cell Biol **16**: 694-703.

Pathakamuri, J. A. and D. A. Theilmann (2002). "The acidic activation domain of the baculovirus transactivator IE1 contains a virus-specific domain essential for DNA replication." J Virol **76**: 5598-604.

Payne, L. N. and K. Venugopal (2000). "Neoplastic diseases: Marek's disease, avian leukosis and reticuloendotheliosis." Rev Sci Tech **19**: 544-64.

Pereira, L. A., J. A. van der Knaap, V. van den Boom, F. A. van den Heuvel and H. T. Timmers (2001). "TAF(II)170 interacts with the concave surface of TATA-binding protein to inhibit its DNA binding activity." Mol Cell Biol **21**: 7523-34.

Pomerantz, J. L., T. M. Kristie and P. A. Sharp (1992). "Recognition of the surface of a homeo domain protein." Genes Dev **6**: 2047-57.

Poppers, J., M. Mulvey, D. Khoo and I. Mohr (2000). "Inhibition of PKR activation by the proline-rich RNA binding domain of the herpes simplex virus type 1 Us11 protein." J Virol **74**: 11215-21.

Preston, C. M. and M. McFarlane (1998). "Cytodifferentiating agents affect the replication of herpes simplex virus type 1 in the absence of functional VP16." Virology **249**: 418-26.

Preston, V. G., J. W. Palfreyman and B. M. Dutia (1984). "Identification of a herpes simplex virus type 1 polypeptide which is a component of the virus-induced ribonucleotide reductase." J Gen Virol **65**: 1457-66.

Prod'hon, C., I. Machuca, H. Berthomme, A. Epstein and B. Jacquemont (1996). "Characterization of regulatory functions of the HSV-1 immediate-early protein ICP22." Virology **226**: 393-402.

Ptashne, M. and A. Gann (1997). "Transcriptional activation by recruitment." Nature **386**: 569-77.

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

Regier, J. L., F. Shen and S. J. Triezenberg (1993). "Pattern of aromatic and hydrophobic amino acids critical for one of two subdomains of the VP16 transcriptional activator." Proc Natl Acad Sci U S A **90**: 883-7.

Rice, S. A., M. C. Long, V. Lam, P. A. Schaffer and C. A. Spencer (1995). "Herpes simplex virus immediate-early protein ICP22 is required for viral modification of host RNA polymerase II and establishment of the normal viral transcription program." J Virol **69**: 5550-9.

Roizman, B., R. Whitley and C. Lopez (1993). Human Herpesviruses. New York, Raven Press.

Roller, R. J., L. L. Monk, D. Stuart and B. Roizman (1996). "Structure and function in the herpes simplex virus 1 RNA-binding protein U(s)11: mapping of the domain required for ribosomal and nucleolar association and RNA binding in vitro." J Virol **70**: 2842-51.

Sadowski, I., J. Ma, S. Triezenberg and M. Ptashne (1988). "GAL4-VP16 is an unusually potent transcriptional activator." Nature **335**: 563-4.

Salghetti, S. E., A. A. Caudy, J. G. Chenoweth and W. P. Tansey (2001). "Regulation of transcriptional activation domain function by ubiquitin." Science **293**: 1651-3.

Salghetti, S. E., M. Muratani, H. Wijnen, B. Futcher and W. P. Tansey (2000). "Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis." Proc Natl Acad Sci U S A **97**: 3118-23.

Sandri-Goldin, R. M. (1994). "Properties of an HSV-1 regulatory protein that appears to impair host cell splicing." Infect Agents Dis **3**: 59-67.

Schaerer-Uthurralt, N., M. Erard, K. Kindbeiter, J. J. Madjar and J. J. Diaz (1998). "Distinct domains in herpes simplex virus type 1 US11 protein mediate post-transcriptional transactivation of human T-lymphotropic virus type I envelope

glycoprotein gene expression and specific binding to the Rex responsive element." J Gen Virol **79**: 1593-602.

Schagger, H. and G. v. Jagow (1987). "Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 KDa." Anal. Biochem. **166**: 368-379.

Schmelter, J., J. Knez, J. R. Smiley and J. P. Capone (1996). "Identification and characterization of a small modular domain in the herpes simplex virus host shutoff protein sufficient for interaction with VP16." J Virol **70**: 2124-31.

Sciabica, K. S., Q. J. Dai and R. M. Sandri-Goldin (2003). "ICP27 interacts with SRPK1 to mediate HSV splicing inhibition by altering SR protein phosphorylation." Embo J **22**: 1608-19.

Sciortino, M. T., M. Suzuki, B. Taddeo and B. Roizman (2001). "RNAs extracted from herpes simplex virus 1 virions: apparent selectivity of viral but not cellular RNAs packaged in virions." J Virol **75**: 8105-16.

Sciortino, M. T., B. Taddeo, A. P. Poon, A. Mastino and B. Roizman (2002). "Of the three tegument proteins that package mRNA in herpes simplex virions, one (VP22) transports the mRNA to uninfected cells for expression prior to viral infection." Proc Natl Acad Sci U S A **99**: 8318-23.

Scott, E. S. and P. O'Hare (2001). "Fate of the inner nuclear membrane protein lamin B receptor and nuclear lamins in herpes simplex virus type 1 infection." J Virol **75**: 8818-30.

Sears, A. E., I. W. Halliburton, B. Meignier, S. Silver and B. Roizman (1985). "Herpes simplex virus 1 mutant deleted in the alpha 22 gene: growth and gene expression in permissive and restrictive cells and establishment of latency in mice." J Virol **55**: 338-46.

Shaw, P., J. Knez and J. P. Capone (1995). "Amino acid substitutions in the herpes simplex virus transactivator VP16 uncouple direct protein-protein interaction and DNA binding from complex assembly and transactivation." J Biol Chem **270**: 29030-7.

Shen, F., S. J. Triezenberg, P. Hensley, D. Porter and J. R. Knutson (1996a). "Critical amino acids in the transcriptional activation domain of the herpesvirus protein VP16 are solvent-exposed in highly mobile protein segments. An intrinsic fluorescence study." J Biol Chem **271**: 4819-26.

Shen, F., S. J. Triezenberg, P. Hensley, D. Porter and J. R. Knutson (1996b). "Transcriptional activation domain of the herpesvirus protein VP16 becomes

conformationally constrained upon interaction with basal transcription factors." J Biol Chem **271**: 4827-37.

Shieh, S. Y., M. Ikeda, Y. Taya and C. Prives (1997). "DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2." Cell **91**: 325-34.

Simmen, K. A., A. Newell, M. Robinson, J. S. Mills, G. Canning, R. Handa, K. Parkes, N. Borkakoti and R. Jupp (1997). "Protein interactions in the herpes simplex virus type 1 VP16-induced complex: VP16 peptide inhibition and mutational analysis of host cell factor requirements." J Virol **71**: 3886-94.

Smibert, C. A., B. Popova, P. Xiao, J. P. Capone and J. R. Smiley (1994). "Herpes simplex virus VP16 forms a complex with the virion host shutoff protein vhs." J Virol **68**: 2339-46.

Smiley, J. R. and J. Duncan (1997). "Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 produces a phenotype similar to that of the in1814 linker insertion mutation." J Virol **71**: 6191-3.

Smiley, J. R., M. M. Elgadi and H. A. Saffran (2001). "Herpes simplex virus vhs protein." Methods Enzymol **342**: 440-51.

Smith, J. S. and N. J. Robinson (2002). "Age-specific prevalence of infection with herpes simplex virus types 2 and 1: a global review." J Infect Dis **186 Suppl 1**: S3-28.

Sodeik, B., M. W. Ebersold and A. Helenius (1997). "Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus." J Cell Biol **136**: 1007-21.

Stark, M. R., D. Escher and A. D. Johnson (1999). "A trans-acting peptide activates the yeast a1 repressor by raising its DNA-binding affinity." Embo J **18**: 1621-9.

Steiner, I., J. G. Spivack, S. L. Deshmane, C. I. Ace, C. M. Preston and N. W. Fraser (1990). "A herpes simplex virus type 1 mutant containing a nontransinducing Vmw65 protein establishes latent infection in vivo in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia." J Virol **64**: 1630-8.

Stern, S. and W. Herr (1991). "The herpes simplex virus trans-activator VP16 recognizes the Oct-1 homeo domain: evidence for a homeo domain recognition subdomain." Genes Dev **5**: 2555-66.

Stern, S., M. Tanaka and W. Herr (1989). "The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16." Nature **341**: 624-30.

Su, Y. H., M. J. Moxley, A. K. Ng, J. Lin, R. Jordan, N. W. Fraser and T. M. Block (2002). "Stability and circularization of herpes simplex virus type 1 genomes in quiescently infected PC12 cultures." J Gen Virol **83**: 2943-50.

Sullivan, S. M., P. J. Horn, V. A. Olson, A. H. Koop, W. Niu, R. H. Ebright and S. J. Triezenberg (1998). "Mutational analysis of a transcriptional activation region of the VP16 protein of herpes simplex virus." Nucleic Acids Res **26**: 4487-96.

Suszkiw, J. (2001). "Cracking the Code of Marek's Disease." Agricultural Research Magazine **49**: 16-17.

Suzuki, N., W. Peter, T. Ciesiolka, P. Gruss and H. R. Scholer (1993). "Mouse Oct-1 contains a composite homeodomain of human Oct-1 and Oct-2." Nucleic Acids Res **21**: 245-52.

Taddeo, B., A. Esclatine and B. Roizman (2002). "The patterns of accumulation of cellular RNAs in cells infected with a wild-type and a mutant herpes simplex virus 1 lacking the virion host shutoff gene." Proc Natl Acad Sci U S A **99**: 17031-6.

Tal-Singer, R., R. Pichyangkura, E. Chung, T. M. Lasner, B. P. Randazzo, J. Q. Trojanowski, N. W. Fraser and S. J. Triezenberg (1999). "The transcriptional activation domain of VP16 is required for efficient infection and establishment of latency by HSV-1 in the murine peripheral and central nervous systems." Virology **259**: 20-33.

Thomas, S. K., G. Gough, D. S. Latchman and R. S. Coffin (1999). "Herpes simplex virus latency-associated transcript encodes a protein which greatly enhances virus growth, can compensate for deficiencies in immediate-early gene expression, and is likely to function during reactivation from virus latency." J Virol **73**: 6618-25.

Thomas, S. K., C. E. Lilley, D. S. Latchman and R. S. Coffin (2002). "A protein encoded by the herpes simplex virus (HSV) type 1 2-kilobase latency-associated transcript is phosphorylated, localized to the nucleus, and overcomes the repression of expression from exogenous promoters when inserted into the quiescent HSV genome." J Virol **76**: 4056-67.

Triezenberg, S. J., R. C. Kingsbury and S. L. McKnight (1988a). "Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression." Genes Dev **2**: 718-29.

Triezenberg, S. J., K. L. LaMarco and S. L. McKnight (1988b). "Evidence of DNA: protein interactions that mediate HSV-1 immediate early gene activation by VP16." Genes Dev **2**: 730-42.

Uesugi, M., O. Nyanguile, H. Lu, A. J. Levine and G. L. Verdine (1997). "Induced alpha helix in the VP16 activation domain upon binding to a human TAF." Science **277**: 1310-3.

Unger, T., T. Juven-Gershon, E. Moallem, M. Berger, R. Vogt Sionov, G. Lozano, M. Oren and Y. Haupt (1999a). "Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2." Embo J **18**: 1805-14.

Unger, T., R. V. Sionov, E. Moallem, C. L. Yee, P. M. Howley, M. Oren and Y. Haupt (1999b). "Mutations in serines 15 and 20 of human p53 impair its apoptotic activity." Oncogene **18**: 3205-12.

Vacratsis, P. O., B. R. Phinney, D. A. Gage and K. A. Gallo (2002). "Identification of in Vivo Phosphorylation Sites of MLK3 by Mass Spectrometry and Phosphopeptide Mapping." Biochemistry **41**: 5613 -5624.

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

Van Sant, C., R. Hagglund, P. Lopez and B. Roizman (2001). "The infected cell protein 0 of herpes simplex virus 1 dynamically interacts with proteasomes, binds and activates the cdc34 E2 ubiquitin- conjugating enzyme, and possesses in vitro E3 ubiquitin ligase activity." Proc Natl Acad Sci U S A **98**: 8815-20.

Vogel, J. L. and T. M. Kristie (2000). "The novel coactivator C1 (HCF) coordinates multiprotein enhancer formation and mediates transcription activation by GABP." Embo J **19**: 683-90.

Warner, S. C., P. Desai and S. Person (2000). "Second-site mutations encoding residues 34 and 78 of the major capsid protein (VP5) of herpes simplex virus type 1 are important for overcoming a blocked maturation cleavage site of the capsid scaffold proteins." Virology **278**: 217-26.

Weinheimer, S. P., B. A. Boyd, S. K. Durham, J. L. Resnick and D. R. O'Boyle, 2nd (1992). "Deletion of the VP16 open reading frame of herpes simplex virus type 1." J Virol **66**: 258-69.

Werstuck, G. and J. P. Capone (1989). "Mutational analysis of the herpes simplex virus trans-inducing factor Vmw65." Gene **75**: 213-24.

White, C. A., N. D. Stow, A. H. Patel, M. Hughes and V. G. Preston (2003). "Herpes simplex virus type 1 portal protein UL6 interacts with the putative terminase subunits UL15 and UL28." J Virol **77**: 6351-8.

Wilcox, K. W., A. Kohn, E. Sklyanskaya and B. Roizman (1980). "Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA." J Virol **33**: 167-82.

Wilson, A. C., R. N. Freiman, H. Goto, T. Nishimoto and W. Herr (1997). "VP16 targets an amino-terminal domain of HCF involved in cell cycle progression." Mol Cell Biol **17**: 6139-46.

Wilson, A. C., K. LaMarco, M. G. Peterson and W. Herr (1993). "The VP16 accessory protein HCF is a family of polypeptides processed from a large precursor protein." Cell **74**: 115-25.

Wilson, A. C., J. E. Parrish, H. F. Massa, D. L. Nelson, B. J. Trask and W. Herr (1995a). "The gene encoding the VP16-accessory protein HCF (HCFC1) resides in human Xq28 and is highly expressed in fetal tissues and the adult kidney." Genomics **25**: 462-8.

Wilson, A. C., M. G. Peterson and W. Herr (1995b). "The HCF repeat is an unusual proteolytic cleavage signal." Genes Dev **9**: 2445-58.

Woychik, N. A. and M. Hampsey (2002). "The RNA polymerase II machinery: structure illuminates function." Cell **108**: 453-63.

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

Wysocka, J., M. P. Myers, C. D. Laherty, R. N. Eisenman and W. Herr (2003b). "Human Sin3 deacetylase and trithorax-related Set1/Ash2 histone H3-K4 methyltransferase are tethered together selectively by the cell- proliferation factor HCF-1." Genes Dev **17**: 896-911.

Xiao, P. and J. P. Capone (1990). "A cellular factor binds to the herpes simplex virus type 1 transactivator Vmw65 and is required for Vmw65-dependent protein-DNA complex assembly with Oct-1." Mol Cell Biol **10**: 4974-7.

Zelus, B. D., R. S. Stewart and J. Ross (1996). "The virion host shutoff protein of herpes simplex virus type 1: messenger ribonucleolytic activity in vitro." J Virol **70**: 2411-9.

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

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." J Virol **73**: 3210-8.