THE MODULATION OF SPHINGOLIPIDS BY HUMAN CYTOMEGALOVIRUS AND ITS INFLUENCE ON VIRAL PROTEIN ACCUMULATION AND GROWTH

DISSERTATION

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By

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ABSTRACT

Human cytomegalovirus (HCMV) is a β-herpes virus which can cause serious disease and even death in congenitally-infected infants and in immunocompromised individuals or immunosuppressed transplant recipients. This virus promotes its own replication by exploiting many cellular signaling pathways. Sphingolipids are structural components of cell membranes which can act as critical mediators of cell signaling. Sphingosine kinase (SphK) can phosphorylate dihydrosphingosine (dhSph) and sphingosine (Sph) to produce dihydrosphingosine-1-phosphate (dhS1P) and sphingosine-1-phosphate (S1P), respectively, which can activate many signaling pathways through binding to G-protein coupled S1P receptors, named $S1P_{1-5}$. An area of research which has yet to be elucidated is whether HCMV modulates sphingolipids and their signaling pathways. Our data show that HCMV infection results in increased accumulation and activity of sphingosine kinase (SphK) within different cell types. This occurs during early times of infection in that it occurs after virus entry but before replication of viral DNA. Measuring the levels of transcripts encoding key enzymes of the sphingolipid metabolic pathway during HCMV infection revealed a temporal regulation of both synthetic and degradative enzymes of this pathway. Using mass spectrometry we were able to generate a sphingolipidomic profile of HCMV-infected cells which suggests an

enhancement of *de novo* sphingolipid synthesis at early times of infection. This was followed by a decrease in the levels of several sphingolipids at 48 hrs correlating with the upregulation of degradative enzymes. Then by knocking down SphK1 expression with siRNA we showed that this enzyme may function within HCMV infected cells to sustain levels of the immediate early (IE) transactivator, IE1. Later, we show evidence which suggest that *de novo* sphingolipid biosynthesis is necessary for the production of optimal levels of infectious virus progeny. However, our results also indicate that this pathway may have a product which suppresses the levels of IE1 protein accumulation. Through exogenous addition of dhSph, an intermediate of *de novo* sphingolipid synthesis, we then show that this lipid can inhibit HCMV protein accumulation. Exogenous addition of dhS1P, however, results in increased accumulation of HCMV gene products, although only at early times during infection. Moreover, our results indicate that pretreatment of cells with dhS1P or S1P prior to infection results in reduced accumulation of HCMV early and late gene products, thus providing evidence for a time-dependent need for increased dhS1P levels by HCMV. Finally, our results, using a drug inhibitor to suppress SphK activity during infection with HCMV, suggest that the stimulation of SphK activity may act to promote virus replication. The results of these studies indicate that HCMV modulates sphingolipid metabolism to temporally regulate the levels of dhSph and dhS1P in order to optimize viral protein accumulation and growth.

DEDICATION

This dissertation is dedicated to my wife, Kimberly Machesky, whose unconditional love and support made this possible.

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FIELDS OF STUDY

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
α	alpha
β	beta
BSA	bovine serum albumin
°C	degrees Celsius
dhSph	dihydrosphingosine
dhS1P	dihydrosphingosine-1-phosphate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified eagle's medium
E	early
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
γ	gamma
GAPDH	glyceraldehyde dehydrogenase
gB	glycoprotein B

gH	glycoprotein H
g	gram(s)
h or hrs	hour(s)
HCMV	human cytomegalovirus
HFF	human foreskin fibroblasts
HIV	human immunodeficiency virus
IE	immediate early
IFN	interferon
k	kilo
kbp	kilobase pairs
L	late or liter(s)
LC	liquid chromatography
MS	mass spectrometry
mRNA	messenger ribonucleic acid
μ	micro
min	minute(s)
MAPK	mitogen-activated protein kinase
mol	mole(s)
M	moles per liter
n	nano
NFκB	nuclear factor-kappaB
PBS	phosphate buffered saline
PI3-K	phosphoinositide 3-kinase

PLC	phospholipase C
pp	phosphoprotein
PFU	plaque forming units
PCR	polymerase chain reaction
PKC	protein kinase C
RNA	ribonucleic acid
rt	room temperature
SPT	serine palmitoyltransferase
SPTLC	serine palmitoyltransferase long chain base subunit
Sph	sphingosine
SphK	sphingosine kinase
S1P	sphingosine-1-phosphate
SPL	S1P lyase
SPP	S1P phosphatase
TLC	thin layer chromatography
UV	ultraviolet
U_L or UL	unique long
U_S or US	unique short

CHAPTER 1

INTRODUCTION

Human Cytomegalovirus

Human Cytomegalovirus (HCMV) belongs to the family *herpesviridae*. The genome is a linear double stranded DNA within an icosahedral capsid surrounded by an amorphous tegument contained within a lipid bilayer envelope (Wright, Goodheart et al. 1964; McGavran and Smith 1965; Spaete, Gehrz et al. 1994; Britt and Mach 1996). The HCMV genome is the largest among herpes viruses at 235 kbp and consists of two unique components termed U_L (unique long) and U_S (unique short), which are bracketed by inverted repeats (Somogyi, Colimon et al. 1986; Mocarski, Liu et al. 1987). This virus is a member of the β-herpes virus subgroup characterized by restricted host range, salivary gland tropism and long replication cycle which results in a slow progression in culture (Furukawa, Fioretti et al. 1973; Atula, Grenman et al. 1998; Ellsmore, Reid et al. 2003). The name Cytomegalovirus was proposed by Weller et al. (Weller, Hanshaw et al. 1960) to illustrate the characteristic cytopathic effects, which include cytoplasmic and nuclear inclusions as well as cell enlargement (Albrecht, Cavallo et al. 1980), and also to designate it as a causative agent in congenitally acquired cytomegalic inclusion disease.

Clinical Aspects of HCMV Infection

HCMV is endemic in the human population where approximately 60-90% of adults are estimated to harbor a latent infection (Pass 1985). Although HCMV is not highly contagious, transmission can occur in a variety of ways, all involving direct contact with infectious material. Infected individuals can secrete infectious virus after primary infection or reactivation of latent infections from urine, saliva, tears, semen, cervical secretions, breast milk and blood (Pass 1985; Hibberd 1995). Typical HCMV infection occurs through contact between infectious virus and mucosal surfaces, specifically the epithelium of the respiratory, upper alimentary and genitourinary tracts (Sinzger and Jahn 1996). Infection can also be established by blood transfusion, transplanted organs (Weber and Doerr 1994; Hibberd 1995; Reinke, Prosch et al. 1999) and from mother to fetus or infant (Stagno, Reynolds et al. 1975; Stagno, Pass et al. 1982; Vochem, Hamprecht et al. 1998; Meier, Lienicke et al. 2005). Transmission from mother to fetus or newborn is common and one of the main reasons HCMV is so prevalent in the population.

When this virus infects healthy adults or full term infants, it does not normally result in the manifestation of serious clinically apparent disease (Stagno, Reynolds et al. 1975; Granstrom, Leinikki et al. 1977; Cohen and Corey 1985). Infection normally progresses through initial virus growth within mucosal epithelial as well as endothelial and fibroblast cells (Sinzger and Jahn 1996). This is followed by spread within the host through the infection of leukocytes when the virus enters a systemic phase of infection, which allows it to establish subsequent infections at other sites within the body (Sinzger and Jahn 1996; Revello, Zavattoni et al. 1998). HCMV eventually establishes latency

within myeloid progenitor cells, where it persists for the life of the host (Kondo, Xu et al. 1996; Khaiboullina, Maciejewski et al. 2004; Sinclair and Sissons 2006). The virus can then periodically reactivate when the progenitor cells are induced to differentiate during immune activation (Soderberg-Naucler, Fish et al. 1997; Soderberg-Naucler and Nelson 1999; Sinclair and Sissons 2006).

However, a notable exception to the typical asymptomatic infection is that HCMV accounts for approximately 8% of all cases of mononucleosis and can cause this disease within healthy individuals, resulting in clinical features similar to Epstein-Barr virus-induced disease (Klemola, Von Essen et al. 1970). There are also several other contexts in which HCMV infection can result in serious diseases. Congenital HCMV infection can result in a variety of neurological abnormalities or even death (Bale, Blackman et al. 1990; Ramsay, Miller et al. 1991; Boppana, Pass et al. 1992; Ross, Dollard et al. 2006). Exposure to the virus in this manner is estimated to be the leading cause of sensorineural deafness as well as the primary infectious cause of brain damage in children (Boppana, Fowler et al. 1997; Fowler, McCollister et al. 1997; Pass, Fowler et al. 2006). Unfortunately, there are currently no effective treatments for congenital HCMV infection, since ganciclovir treatment only has a limited benefit (Whitley 2004; Ross, Dollard et al. 2006).

Immunocompromised individuals, such as immunosuppressed transplant recipients or AIDS patients, represent another scenario in which HCMV infection can be particularly destructive. This problem is unfortunately common to these types of patients because HCMV infection can occur by primary infection, re-infection or reactivation of latent virus (Reinke, Prosch et al. 1999). HCMV infection in solid organ and bone

marrow transplant recipients in particular can cause significant morbidity and even mortality (Emanuel, Cunningham et al. 1988; Reed, Bowden et al. 1988; Hillyer, Snydman et al. 1990; Patel, Snydman et al. 1996; Murray, Amsterdam et al. 1997). Some of the life-threatening symptoms of CMV disease include pneumonia, hepatitis, gastrointestinal ulceration and impaired function of the transplanted organ (Shabtai, Luft et al. 1988; Hillyer, Snydman et al. 1990; Murray, Amsterdam et al. 1997; Knipe and Howley 2001).

Treatment regimens involving intravenous administration of ganciclovir, foscarnet, cidofovir or anti-cytomegalovirus antibody can be utilized in these contexts and have demonstrated reasonable success (Emanuel, Cunningham et al. 1988; Reed, Bowden et al. 1988; Prentice 1989; Skarp-Orberg, Hokeberg et al. 1990; Knipe and Howley 2001). However, the emergence of drug resistant strains of HCMV makes these infections increasingly difficult to manage (Erice 1999; Baldanti, Lurain et al. 2004; Gilbert and Boivin 2005). The acute effects of HCMV infection are a substantial public health problem, but the virus has also been associated with several chronic diseases. Recent studies have suggested that HCMV may be linked to atherosclerosis, coronary restenosis, immunological senescence in the elderly and possibly cancer (Speir, Modali et al. 1994; Horvath, Cerny et al. 2000; Cobbs, Harkins et al. 2002; Harkins, Volk et al. 2002; Samanta, Harkins et al. 2003; Koch, Solana et al. 2006; Pawelec, Koch et al. 2006). Although still controversial, these studies raise the possibility that HCMV infection of healthy persons may have detrimental consequences later in life.

Life Cycle of HCMV

The HCMV life cycle begins with the attachment of the virus to the cell surface. At this time, the initial binding event is thought to begin with a transient interaction between heparin sulfate proteoglycan surrounding the cells plasma membrane and the viral glycoprotein B (gB), which is expressed on the outer membrane of the virus (Boyle and Compton 1998). This is followed by a stable interaction between gB and epidermal growth factor receptor (EGFR) (Wang, Huong et al. 2003) in conjunction with a second viral glycoprotein, glycoprotein H (gH), binding to αvβ3 integrin (Wang, Huang et al. 2005). However, one recent study suggests that the HCMV binding event involves viral glycoproteins interacting with β1 and β3 heterodimers without the involvement of EGFR (Isaacson, Feire et al. 2007). After binding to the cell surface, the virus penetrates the host cell membrane by fusing it with the virion envelope by a process not fully understood, yet known to involve the gH/gL complex of glycoproteins expressed on the surface of the virus (Keay and Baldwin 1991; Milne, Paterson et al. 1998). This fusion event results in the release of the nucleocapsid into the cytoplasm, where it is quickly transported to the nucleus through intact microtubule networks utilizing a process that also requires actin depolymerization (Jones, Lewis et al. 1986; Ogawa-Goto, Tanaka et al. 2003; Wang, Huang et al. 2005). The nucleocapsid then transfers the viral DNA genome into the nucleus by a means which is not well understood.

Once the viral DNA is inside the nucleus, gene expression begins with immediate early (IE), or α genes, which are expressed with the assistance of tegument proteins, but do not require the expression of any other viral genes. IE genes are generally transactivators that regulate their own transcription and activate transcription of early (E)

or β genes. Most E gene products function to replicate viral DNA, and their expression depends on the expression of IE gene products. Viral DNA replication results in the synthesis of long concatemeric DNA molecules, which provide the templates for late (L) or γ gene expression. L gene products are primarily needed for virus assembly and egress as well as the virion structural components (Knipe and Howley 2001).

At late times of infection, within the cell's nucleus, these structural components begin to assemble preformed capsids, into which mature genomes are packaged (Reddehase 2006). These newly formed nucloecapsids mature and accumulate within the nucleus prior to undergoing a process of envelopment and de-envelopment at the inner and outer nuclear membrane, respectively (Smith and De Harven 1973; Fons, Graves et al. 1986; Reddehase 2006). These cytoplasmic nucleocapsids obtain tegument proteins which coat these structures, as well as both cellular and viral RNAs (Smith and De Harven 1973; Terhune, Schroer et al. 2004). Then the encased nucleocapsids undergo a second stage of envelopment at the cytoplasmic cisternae, which produces particles with a double membrane (Reddehase 2006). This double membrane allows the outer leaflet to fuse with the plasma membrane releasing virus particles containing a single membrane, which can subsequently infect nearby cells (Reddehase 2006). The aforementioned process is known as a lytic infection because virus growth in this manner eventually results in lysis of the infected cell, providing a second means by which the virus can be released (Knipe and Howley 2001; Reddehase 2006).

HCMV infection of some cell types, namely myeloid progenitor cells, can result in a latent infection (Kondo, Xu et al. 1996; Khaiboullina, Maciejewski et al. 2004). From a molecular standpoint, a latent infection is one in which the infected cell maintains

copies of the viral genome but gene expression is limited to a small number of latancy-associated transcripts, and no virus progeny are produced unless reactivation occurs (Sinclair and Sissons 2006). The precise triggers that cause latent infections and their reactivation are poorly understood.

HCMV and Cell Signaling

HCMV affects many cell signaling pathways throughout the course of infection. Many of these cell signaling events occur during attachment and entry of HCMV. The binding of glycoprotein B (gB) to the epidermal growth factor receptor (EGFR) results in the activation of phosphoinositide 3-kinase (PI3-K) and phospholipase C γ (PLC γ) (Wang, Huong et al. 2003). PI3-K phosphorylates Akt (protein kinase B), which prevents apoptosis and regulates cellular metabolism, while the hydrolyzation action of PLC γ releases second messengers that induce protein kinase C (PKC) activity and calcium influx (Zwick, Hackel et al. 1999). These activities appear to prepare the host cell for subsequent viral replication and are necessary for productive HCMV replication (Slobbe-van Drunen, Vossen et al. 1997; Johnson, Wang et al. 2001; Wang, Huong et al. 2003). The attachment of HCMV also involves the binding of glycoprotein H (gH) to αvβ3 integrin resulting in phosphorylation of the β3 integrin subunit, which then activates Src (Wang, Huang et al. 2005). The downstream activity of Src, namely the inhibition of RhoA, is enhanced by the coordinated signaling between EGFR and αvβ3 that occurs upon the binding and entry of HCMV (Wang, Huang et al. 2005). This inhibition of RhoA appears to be critical for successful infection by promoting disruption of cell

microfilaments necessary for capsid transport to the nucleus (Jones, Lewis et al. 1986; Wang, Huang et al. 2005).

The HCMV glycoprotein B-initiated signaling events also result in the stimulation of the interferon-responsive pathway (Boyle, Pietropaolo et al. 1999). This pathway activates antiviral cellular defenses that can result in the inhibition of transcription, translation and cellular apoptosis (Stark, Kerr et al. 1998). The virus is able to avoid these consequences by its ability to down regulate the expression of interferon (IFN) responsive genes through the actions of pp65 (Browne and Shenk 2003). This is most likely accomplished through the prevention of IFN response factor 3 activation, an activity that is directly attributed to pp65 expression (Abate, Watanabe et al. 2004). HCMV further ensures its avoidance of the antiviral actions associated with the IFN-responsive pathway through the ability of IE viral gene products to suppress IFN production and downstream signaling of this pathway (Taylor and Bresnahan 2005; Paulus, Krauss et al. 2006). Infection with this virus has also been shown to result in decreased expression of Janus kinase 1 and p48, which are two essential components involved in IFN-alpha signaling (Miller, Zhang et al. 1999).

The interactions of the virus binding to its cellular receptors also result in activation of mitogen-activated protein kinases (MAPK). At 10 to 30 minutes post infection, HCMV results in the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) through the activity of MEK1/2 (Johnson, Ma et al. 2001). This activity appears to be necessary for HCMV replication because the use of inhibitors to MEK1/2 reduced viral gene expression and decreased virus growth (Rodems and Spector 1998; Johnson, Ma et al. 2001). Another MAPK, p38, is also activated within monocytes upon

the receptor-ligand interactions during the immediate early phase of HCMV infection (Yurochko and Huang 1999). These interactions also result in the enhanced expression and activity of cyclooxygenase-2 (COX2), resulting in the increase of its product prostaglandin E(2) (Zhu, Cong et al. 2002). Inhibition of COX2, resulting in decreased levels of prostaglandin E(2), reduced virus growth and suppressed the accumulation of the *UL122* gene product, IE2, thus implicating COX2 activity in the promotion of IE gene expression (Zhu, Cong et al. 2002).

Immediate early gene expression coincides with a second stage of cell signaling that involves a reactivation of both ERK1/2 and p38, further illustrating their importance in HCMV replication (Rodems and Spector 1998; Johnson, Huong et al. 2000). This phase also involves the disruption of the cell cycle through HCMV induced activation of E2Fs, cyclins, p53 and the retinoblastoma gene product (Rb) (Jault, Jault et al. 1995; Poma, Kowalik et al. 1996). The *UL123* and *UL122* gene products, IE1 and IE2, respectively, have been shown to interact with proteins directly involved in the regulation of the cell cycle, and are therefore thought to be directly responsible for the HCMV induced alteration of cell cycle progression (Hagemeier, Caswell et al. 1994; Poma, Kowalik et al. 1996; Zhang, Huong et al. 2003).

HCMV accomplishes some of its cell signaling actions through host mimicry (Michelson 2004). For example, the HCMV UL146 gene product codes for an interleukin 8 homolog, which has been shown to be fully functional and has been proposed to attract leukocytes to sites of infection (Penfold, Dairaghi et al. 1999). Additional examples of this mimicry are the four G-protein coupled receptor (GPCR) homologs that were identified by sequence homology within the HCMV genome (Chee,

Satchwell et al. 1990). These GPCR homologs are encoded by the US27, US28, UL33 and UL78 genes (Chee, Satchwell et al. 1990). The best characterized of these is the US28 gene product, as it has been demonstrated to bind to several CC chemokine ligands (Gao and Murphy 1994). Sequestration of CC chemokines by US28 has been proposed to assist HCMV in evading the immune system (Bodaghi, Jones et al. 1998). US28 can also signal in an agonist-independent manner, resulting in sustained activation of phospholipase C and Nuclear Factor-kappaB (NF-κB) signaling (Casarosa, Bakker et al. 2001; Waldhoer, Kledal et al. 2002). This gene product can activate focal adhesion kinase and Src, resulting in smooth muscle cell migration (Streblow, Soderberg-Naucler et al. 1999; Streblow, Vomaske et al. 2003). Furthermore, the constitutive activity of US28 has been shown to promote tumorigenesis *in vivo* (Maussang, Verzijl et al. 2006).

Sphingolipids

Since the late 1800s, sphingolipids have been known to be constituents of the cell membrane. In 1884 J.L.W. Thudichum first used the name "sphingosin" to describe an enigmatic compound he had found within the brain (Thudichum 1884). Since then, many studies have illustrated roles for sphingolipids as both structural components of cell membranes as well as critical mediators of cell signalling (Futerman and Hannun 2004).

Biosynthesis of Sphingolipids

De novo sphingolipid biosynthesis starts with the activity of serine palmitoyltransferase (SPT), which resides at the cytosolic face of the endoplasmic reticulum (ER) (Mandon, Ehses et al. 1992). SPT condenses serine and palmitoylCoA to

form 3-ketosphinganine (Stoffel and Bister 1974; Mandon, van Echten et al. 1991), which is then reduced to dihydrosphingosine (dhSph) or sphinganine by the activity of 3-ketosphinganine reductase (Stoffel and Bister 1974; Williams, Wang et al. 1984). Ceramide synthase then adds an amide-linked fatty acid to form dihydroceramide, and afterward the 4-trans double bond is introduced by dihydroceramide desaturase, which produces ceramide (Rother, van Echten et al. 1992; Venkataraman, Riebeling et al. 2002). Ceramide is then transported to the golgi where it can then be converted into many complex sphingolipids like glycosphingolipids and sphingomyelin (Stoffel and Bister 1974; Kolter, Doering et al. 1999).

Sphingomyelin is produced by the addition of phosphorylcholine to the 1-hydroxyl end of ceramide by sphingomyelin synthase (Ullman and Radin 1974; Voelker and Kennedy 1982) and can occur at either the golgi or the plasma membrane (van den Hill, van Heusden et al. 1985; Malgat, Maurice et al. 1986; Futerman, Stieger et al. 1990; Jeckel, Karrenbauer et al. 1990). Sphingomyelin can then be converted back to ceramide through the hydrolytic activity of sphingomyelinase (SMase) (Spence, Clarke et al. 1983; Merrill and Jones 1990; Spence 1993). Ceramide degradation through the activity of ceramidase can occur by removal of the acylated fatty acid to form sphingosine (Hassler and Bell 1993). Sphingosine kinase (SphK) can then phosphorylate sphingosine to form sphingosine-1-phosphate (S1P) (Keenan and Haegelin 1969). Sphingosine-1-phosphate lyase (SPL) can then irreversibly degrade S1P into ethanolamine phosphate and hexadecanal (Zhou and Saba 1998; Van Veldhoven 2000), or sphingosine-1-phosphate phosphatase (SPP) can remove the phosphate group from S1P by hydrolysis to convert it back to sphingosine (Le Stunff, Peterson et al. 2002). Interestingly, the activity of

ceramidase is reversible such that it can add the amide-linked fatty acid back onto sphingosine to form ceramide (Kita, Okino et al. 2000; Okino, He et al. 2003), which can then be converted back to sphinomyelin by sphingomyelin synthase (Ullman and Radin 1974; Voelker and Kennedy 1982). This pathway of sphingolipid biosynthesis, depicting the aforementioned steps, is illustrated in Fig. 1.1. The interconversion between sphingomyelin, ceramide, sphingosine and S1P which occurs at the plasma membrane is often referred to as the degradative, or sphingomyelinase, pathway and has been shown to be of significant importance in cell signaling (Kolesnick 2002).

Key Sphingolipid Metabolic Enzymes

Sphingolipids have been shown to elicit a variety of biological effects through their involvement in the activation of many cell signaling cascades (Ohanian and Ohanian 2001; Colombaioni and Garcia-Gil 2004; Ipatova, Torkhovskaya et al. 2006). In many cases, these effects can be attributed to stimulation or suppression of the enzymes involved in producing these important sphingolipids (Hannun, Luberto et al. 2001; Hanada 2003; Spiegel and Milstien 2007).

Serine palmitoyltransferase

As stated above, SPT catalyzes the initial step in *de novo* sphingolipid biosynthesis; therefore, its activity can be important in controlling the accumulation of sphingolipids, which can in turn govern their actions (Hanada 2003). SPT consists of a heterodimeric enzyme complex made up of two gene products: SPTLC1 and SPTLC2 (Dickson, Lester et al. 2000; Hanada, Hara et al. 2000). Mutations in SPTLC1, which are

thought to give dominant negative effects to SPT, are the cause of hereditary sensory neuropathy (Dawkins, Hulme et al. 2001; Bejaoui, Uchida et al. 2002; McCampbell, Truong et al. 2005). Regulation of SPT activity is primarily controlled through supply of its substrates (Merrill, Wang et al. 1988); however, it has been shown to be regulated at the transcriptional level by endotoxin and cytokines (Memon, Holleran et al. 1998). SPT can also be post-transcriptionally activated in response to etoposide, indicating a role for SPT in the control of ceramide-induced apoptosis (Perry, Carton et al. 2000). Other studies have also shown that *de novo* sphingolipid biosynthesis may be important in modulating ceramide-induced apoptosis (Lehtonen, Horiuchi et al. 1999; Dawkins, Hulme et al. 2001; Wang, Maurer et al. 2001).

Sphingosine kinases

Sphingosine kinase (SphK) can phosphorylate sphingosine (Sph) as well as the *de novo* pathway intermediate, dihydrosphingosine (dhSph), to form S1P and dihydrosphingosine-1-phosphate (dhS1P), respectively (Keenan and Haegelin 1969; Hirschberg, Kisic et al. 1970; Stoffel, Assmann et al. 1970). There are two known SphK isoforms that have been isolated, called SphK1 and SphK2 (Kohama, Olivera et al. 1998; Liu, Sugiura et al. 2000; Pitson, D'Andrea R et al. 2000). The regulation and cellular functions of SphK2 are not well established and its observed affects on cell proliferation and growth differ with regard to cellular environment (Maceyka, Sankala et al. 2005; Okada, Ding et al. 2005; Van Brocklyn, Jackson et al. 2005).

SphK1 activity can be regulated by a large number of upstream activators (Maceyka, Payne et al. 2002). For example, G-protein coupled receptors, growth factors

and immunoglobulin receptors can all activate SphK1 activity (Choi, Kim et al. 1996; Melendez, Floto et al. 1998; Meyer zu Heringdorf, Lass et al. 1998; Meyer zu Heringdorf, Lass et al. 1999; Olivera, Edsall et al. 1999). Furthermore, SphK1 activity can be regulated by subcellular localization through the phosphorylation of serine 225 as a result of ERK activity (Pitson, Moretti et al. 2003). It should be noted that regulation of the kinase in this manner only facilitates its interaction with the plasma membrane and has no effect on the enzyme's inherent activity (Pitson, Xia et al. 2005). Activity of SphK1 can also be controlled at the transcriptional level by agents such as phorbol 12-myristate 13-acetate (PMA) and transforming growth factor-beta (TGF-beta) (Buehrer, Bardes et al. 1996; Yamanaka, Shegogue et al. 2004).

The activity of SphK1 elicits a variety of biological activities, several of which implicate a role for this kinase in tumor progression. In MCF-7 breast cancer cells, SphK1 overexpression increased proliferation, suppressed apoptosis and led to the formation of larger tumors in nude mice (Nava, Hobson et al. 2002), while cells expressing a dominant-negative mutant SphK1 had the reverse affects (Sukocheva, Wang et al. 2003). Furthermore, levels of SphK1 expression may even be a prognostic indicator for human glioblastoma patients (Van Brocklyn, Jackson et al. 2005).

SphK1 is able to produce its effects on cells through its production of S1P which, through a family of G-protein coupled receptors termed $S1P_{1-5}$, can mediate a variety of biological functions (Spiegel and Milstien 2003; Young and Van Brocklyn 2006). Some of these functions include increased cell growth, prolonged survival, regulation of cell motility and invasiveness as well as the regulation of important actions in the immune system like lymphocyte trafficking (Olivera, Kohama et al. 1999; Rosen and Goetzl

2005; Young and Van Brocklyn 2007). S1P has also been shown to act as an intracellular messenger (Meyer zu Heringdorf, Liliom et al. 2003; Watterson, Sankala et al. 2003). However, key proteins relating to its specific involvement in this manner have not been found. S1P exhibits several anti-apoptotic effects, which have been revealed through studies showing its effect on activating signaling pathways involved in cell survival including ERK1/2, Akt (protein kinase B) and NFκB (Lee, Van Brocklyn et al. 1998; Gonda, Okamoto et al. 1999; Okamoto, Takuwa et al. 1999; Baudhuin, Cristina et al. 2002; Van Brocklyn, Letterle et al. 2002; Xia, Wang et al. 2002; Spiegel and Milstien 2003).

Two precursors to S1P, ceramide and sphingosine, have the opposite effects concerning cell survival because they have been shown to prevent cell growth and induce apoptosis (Hannun and Luberto 2000; Cuvillier 2002). The ability of SphK1 to promote cell growth and survival through its production of S1P is thought to arise from a dynamic balance between S1P and ceramide/sphingosine, which has led to a proposed "sphingolipid rheostat", in which the relative amounts of these sphingolipid metabolites determine cell fate (Cuvillier, Pirianov et al. 1996).

dhS1P has many of the same cellular functions as S1P, because it can also stimulate S1P receptors (Van Brocklyn, Lee et al. 1998; Tamama, Kon et al. 2001). It has also been shown that dhS1P is responsible for the upregulation of matrix metalloproteinase 1 which, at this time, is the only cellular activity that can be directly attributed to dhS1P and not S1P (Bu, Yamanaka et al. 2006). One study discovered that overexpression of SphK1 led to significant increases in dhS1P production, which did not occur with overexpression of SphK2, suggesting that SphK1 may be primarily

responsible for the phosphorylation of dhSph to dhS1P (Berdyshev, Gorshkova et al. 2006).

S1P phosphatases

As mentioned above, S1P is de-phosphorylated to sphingosine by S1P phosphatases (Le Stunff, Peterson et al. 2002). There are two known S1P phosphatases in humans: SPP1 & SPP2 (Mandala, Thornton et al. 2000; Ogawa, Kihara et al. 2003). Exogenous addition of S1P to cells overexpressing SPP1 results in higher levels of apoptosis through a marked increase in ceramide levels (Le Stunff, Galve-Roperh et al. 2002). It was recently determined that this buildup of ceramide levels is due to the ability of SPP1 to inhibit ER-to-Golgi trafficking, which prevents ceramide from being synthesized into more complex sphingolipids (Giussani, Maceyka et al. 2006). On the other hand, reducing SPP1 expression though siRNA knockdown significantly increased S1P levels, resulting in resistance to apoptosis inducers TNFα and daunorubicin (Johnson, Johnson et al. 2003). At this point, it appears as though SPP1 can regulate apoptosis through control of ceramide levels and S1P levels. Recent evidence has implicated SPP2 as having a pro-inflammatory role that is regulated by NF-κB binding (Mechtcheriakova, Wlachos et al. 2006).

S1P lyase

To reiterate, S1P is irreversibly degraded to ethanolamine phosphate and hexadecanal by S1P lyase (SPL) (Zhou and Saba 1998; Van Veldhoven 2000). The few studies that exist concerning the biological implications of mammalian SPL have all

shown a consistent role for SPL activity in supporting an apoptotic cellular environment. Overexpression of SPL renders cells more susceptible to serum starvation through enhanced production of ceramide (Reiss, Oskouian et al. 2004) and increases sensitivity to cisplatin, carboplatin and doxorubicin in a manner consistent with increased p38 activity (Min, Van Veldhoven et al. 2005). In addition, siRNA knockdown of SPL results in a subdued apoptotic response, while expression levels of this enzyme were significantly lower in colon cancer tissues when compared to normal tissues (Oskouian, Sooriyakumaran et al. 2006).

Statement of the Hypothesis

Past studies have begun to elucidate the importance of sphingolipids in sustaining normal levels of virus growth and protein expression in herpesvirus-infected cells (Steinhart, Busch et al. 1984; Allan-Yorke, Record et al. 1998). However, there are no studies that show active modulation of sphinglipid metabolism or sphingolipid induced cell signaling by HCMV.

Interestingly, there are several cell signaling pathways activated by HCMV that are necessary for its replication, which are also activated through the G proteins coupled to S1P receptors. For instance, the binding of HCMV to cell surface receptors during virus binding and entry results in subsequent activation of PI3-K and PLC γ pathways (Wang, Huong et al. 2003; Wang, Huang et al. 2005), which are critical for infection and replication (Slobbe-van Drunen, Vossen et al. 1997; Johnson, Wang et al. 2001). S1P binding to the $S1P_1$ receptor has also been shown to lead to activation of PI3-K and PLC pathways (Okamoto, Takuwa et al. 1998; Rakhit, Conway et al. 1999; Spiegel and

Milstien 2003). Also, HCMV infection results in sustained levels of activated ERK1/2, which are implicated in promoting expression of early genes (Rodems and Spector 1998). Likewise, S1P can activate ERK MAP kinases, leading to an increase in cell proliferation (Lee, Van Brocklyn et al. 1998; Gonda, Okamoto et al. 1999; Okamoto, Takuwa et al. 1999; Van Brocklyn, Letterle et al. 2002). Because the HCMV genome contains four G-protein coupled receptor sequence homologs (Stropes and Miller 2004), there may be an advantage for HCMV replication gained by the activity of G-proteins that could be ensured through the activity of either dhS1P or S1P binding to the S1P receptors. Overall, it appears that cell signaling by sphingolipids could provide stimulation of cell signaling cascades, which would assist HCMV in its replication.

With this knowledge, the following **hypothesis** was developed: **HCMV infection results in the modulation of sphingolipids, through either transcriptional or post transcriptional regulation of enzymes involved in their metabolism, in order to promote viral gene expression and/or replication.** If we are correct in our hypothesis, there exist great opportunities for the suppression of HCMV infection through therapeutic interventions directed at sphingolipid metabolites or their enzymes.

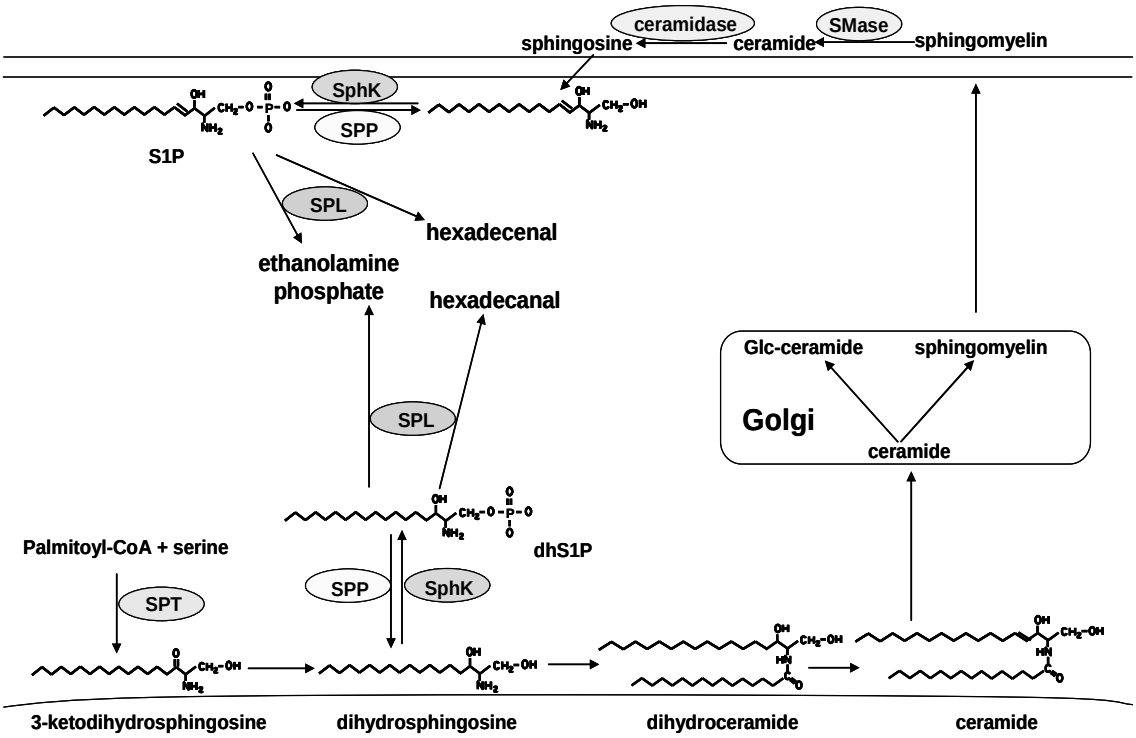

Figure 1.1. Diagram depicting sphingolipid biosynthesis occurring in the endoplasmic reticulum and Golgi complex as well as sphingolipid breakdown at the plasma membrane. Note that Glc-ceramide stands for glucosyl ceramide and represents the beginning of glycosphingolipid synthesis.

CHAPTER 2

HUMAN CYTOMEGALOVIRUS REGULATES BIOACTIVE SPHINGOLIPIDS AND REQUIRES SPHINGOSINE KINASE FOR OPTIMAL GENE EXPRESSION

ABSTRACT

Human cytomegalovirus (HCMV) exploits a variety of cellular signaling pathways in order to promote its own replication. However, whether HCMV modulates lipid signaling pathways is an essentially unexplored area of research in virus-host cell interactions. In this study, we examined the accumulation of the sphingolipids sphingosine-1-phosphate (S1P), dihydrosphingosine-1-phosphate (dhS1P) and ceramide, and the enzymes responsible for the biosynthesis and degradation of these lipids. Our results show that HCMV infection results in increased accumulation and activity of sphingosine kinase (SphK), the enzyme that generates S1P and dhS1P. This occurs at a step of the virus life cycle after virus entry but before replication of viral DNA. Furthermore, analysis of transcripts encoding other key enzymes in the sphingolipid metabolic pathway revealed that both synthetic and degradative enzymes of this pathway are dynamically regulated upon infection. We also utilized a mass spectrometry approach to generate a sphingolipidomic profile of HCMV-infected cells. We show that

HCMV infection results in increased levels of dhS1P and ceramide at 24 hrs, suggesting an enhancement of de novo sphingolipid synthesis. Subsequently dhS1P decreases at 48 hrs coincident with the upregulation of degradative enzymes. Finally, we provide evidence that virus-associated changes in sphingolipid metabolism directly impacts the virus replication cycle. Targeting expression of SphK1 with siRNA led to a specific reduction in accumulation of the immediate early (IE) transactivator, IE1. Together, these findings demonstrate that host cell sphingolipids are dynamically regulated upon infection with a herpesvirus in a manner that promotes virus gene expression.

INTRODUCTION

HCMV is a β-herpes virus that is endemic in the human population, and in healthy adults infection with this virus is relatively benign. The dramatic exceptions in which HCMV can cause serious diseases are in congenitally- and perinatally-infected infants, and in immunocompromised individuals or immunosuppressed transplant recipients. In recent years, some studies have suggested a potential link between HCMV and several chronic diseases including atherosclerosis, coronary restenosis, and possibly cancer (Speir, Modali et al. 1994; Horvath, Cerny et al. 2000; Cobbs, Harkins et al. 2002; Harkins, Volk et al. 2002; Samanta, Harkins et al. 2003). The key aspects of host cell-virus interactions responsible for HCMV persistence and pathogenesis are poorly understood. In addition to advancing fundamental aspects of virus and host cell biology,

more detailed knowledge of this virus-host cell interface may also reveal rational points of intervention that could be exploited for the treatment of HCMV-associated diseases.

Many cell processes succumb to regulation by viral gene products, including cell communication systems. For example, HCMV can attenuate or block autocrine and paracrine signaling pathways that culminate in the activation of antiviral cellular defenses. HCMV disrupts interferon signal transduction (Miller, Rahill et al. 1998; Miller, Zhang et al. 1999; Miller, Zhang et al. 2000) and also antagonizes the antiviral cell response mediated by activation of interferon regulatory factor 3 (IRF 3) in response to virion glycoprotein B-initiated signaling events (Navarro, Mowen et al. 1998; Boyle, Pietropaolo et al. 1999; Browne, Wing et al. 2001; Preston, Harman et al. 2001) .

Many cell signaling pathways are also activated by virus infection. Numerous transcriptional changes are induced by binding of the HCMV glycoprotein B (gB) to the epidermal growth factor receptor (EGFR) and the αvβ3 integrin coreceptor during virus binding and entry (Wang, Huong et al. 2003; Wang, Huang et al. 2005). Subsequent activation of phosphoinositide 3-kinase (PI3-K) and phospholipase C γ (PLC γ) pathways appear critical to successful infection by promoting disruption of cell microfilaments necessary for capsid transport to the nucleus (Jones, Lewis et al. 1986; Ogawa-Goto, Tanaka et al. 2003; Wang, Huang et al. 2005).

Very early interactions of the virus with cellular receptors also results in activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and p38 mitogen activated protein kinases (MAPK), as well as cyclooxygenase-2 (COX2) which are implicated in promoting expression of immediate early genes (Rodems and Spector 1998; Johnson, Huong et al. 2000; Johnson, Ma et al. 2001; Zhu, Cong et al. 2002). Expression

of viral genes, especially immediate early genes, also stimulates a second wave of MAP kinase activation, arachidonic acid release, and activation of E2Fs, cyclins and p53 (Jault, Jault et al. 1995; Nokta, Hassan et al. 1996; Rodems and Spector 1998; Johnson, Huong et al. 2000).

Furthermore, HCMV encodes at least one interleukin 8-like chemokine homolog (UL146) and four chemokine G-protein coupled receptor sequence homologs encoded by the US27, US28, UL33 and UL78 genes (Penfold, Dairaghi et al. 1999) and reviewed in (Stropes and Miller 2004). Both US27 and US28 gene products can bind CC chemokine ligands, and, in at least in some cell types, US28 signals constitutively resulting in sustained activation of the PI-3K pathway (Gao and Murphy 1994; Bodaghi, Jones et al. 1998; Casarosa, Bakker et al. 2001). It has been suggested that these gene products could impact pathogenesis through sequestration of CC chemokines, induction of smooth muscle cell migration, or by recruitment of HCMV-infected cells to sites of vascular injury (Bodaghi, Jones et al. 1998; Streblow, Soderberg-Naucler et al. 1999; Randolph-Habecker, Rahill et al. 2002).

However, nothing is known about whether HCMV modulates host cell signaling by sphingolipids. Sphingolipids are present in membranes of all eukaryotic cells and, in recent years, have been shown to function as signaling molecules (reviewed in (Spiegel and Milstien 2003)). Most work has focused on the bioactive sphingolipids sphingosine-1-phosphate (S1P) and ceramide. While S1P tends to promote cell survival and proliferation, ceramide has opposing effects often leading to cessation of cell proliferation and apoptosis (Maceyka, Payne et al. 2002).

S1P can signal both intracellularly and through a family of G protein-coupled receptors termed $S1P_{1-5}$ to regulate diverse biological processes (Spiegel and Milstien 2003; Anliker and Chun 2004; Young and Van Brocklyn 2006). These include increased cell growth and survival (Cuvillier, Pirianov et al. 1996; Maceyka, Payne et al. 2002), both positive and negative regulation of cell migration (Taha, Argraves et al. 2004), vascular integrity (McVerry and Garcia 2005) and angiogenesis (Lee, Thangada et al. 1999; English, Welch et al. 2000). Moreover, S1P impacts several key aspects of immunity including lymphocyte trafficking and chemotaxis, mast cell activity, and the activation and cytokine secretion profiles of T lymphocytes and dendritic cells (reviewed in (Payne, Milstien et al. 2004; Cyster 2005; Rosen and Goetzl 2005). Dihydrosphingosine-1-phosphate (dhS1P), also known as sphinganine-1-phosphate, lacks the trans double bond at the 4-position. dhS1P is an S1P receptor agonist and can therefore elicit many of the same biological processes as S1P, (Van Brocklyn, Lee et al. 1998; Tamama, Kon et al. 2001) although overall much less is known about dhS1P compared to S1P.

S1P is produced from the phosphorylation of sphingosine, which is created by the deacylation of ceramide within the sphingolipid degradatory pathway, by sphingosine kinases (SphK). Two SphK isoforms have been cloned, termed SphK1 and SphK2 (Kohama, Olivera et al. 1998; Liu, Sugiura et al. 2000; Pitson, D'Andrea R et al. 2000). dhS1P is produced from the phosphorylation of dihydrosphingosine, which is an intermediate of *de novo* sphingolipid synthesis, predominantly by the activity of SphK1 but not SphK2 (Berdyshev, Gorshkova et al. 2006). SphK1 is activated by a variety of signals including growth factors (Olivera and Spiegel 1993), immunoglobulin receptors

(Choi, Kim et al. 1996; Melendez, Floto et al. 1998), and various G protein-coupled receptors (Meyer zu Heringdorf, Lass et al. 1998), and has been shown to enhance cell proliferation, survival (Olivera, Kohama et al. 1999), transformation (Xia, Gamble et al. 2000) and tumor malignancy (Van Brocklyn, Jackson et al. 2005). The regulation and function of SphK2 is less well understood. In some cellular contexts SphK2 is anti-proliferative and pro-apoptotic (Maceyka, Sankala et al. 2005), whereas in others it may not affect growth and survival (Okada, Ding et al. 2005), or may even promote cell proliferation (Van Brocklyn, Jackson et al. 2005).

Because sphingolipid signaling impacts several fundamental physiological processes, we sought to determine if and how this system was altered upon virus infection. We therefore examined the expression and activity of SphK1 in HCMV-infected cells. Our results indicate that SphK1 transcript abundance, protein levels and activity were all elevated by HCMV infection. Induction of SphK1 activity was apparent by both laboratory adapted and clinical isolates of HCMV, and occurred in primary, immortalized and tumor cell lines. Our studies also suggest that activation of SphK1 requires expression of an immediate early or early viral gene(s). We also show that HCMV infection resulted in the dynamic regulation of both synthetic and degradative enzymes involved in sphingolipid metabolism. Analysis of the sphingolipid profile of HCMV-infected cells revealed an increase in the amount of dhS1P and ceramides at 24 hrs, coinciding with the upregulation SphK1. Subsequently there was a decrease in dhS1P at 48 hrs that coincided with the upregulation of degradative enzymes. Finally, siRNA knockdown of SphK1 results in reduced expression of the immediate early gene,

IE1. To our knowledge, this is the first report of regulation of sphingosine kinase upon infection by a member of the *herpesviridae* family.

MATERIALS AND METHODS

Cell Culture

U-373 MG glioma cell lines were obtained from ATCC. U-251 MG cells were provided by Dr. Allan Yates. Glioma cell lines were maintained in Eagle's minimum essential medium containing 10% fetal bovine serum, non-essential amino acids and sodium pyruvate (all media from Mediatech, Herndon, VA). MRC-5 human primary fibroblasts were obtained from ATCC and maintained in modified Eagle's medium supplemented with 10% fetal calf serum, 1.7 mM sodium bicarbonate, 1.4 mM sodium chloride, essential and nonessential amino acids, vitamins, and sodium pyruvate at manufacturer-recommended concentrations (Sigma). Telomerase-immortalized human foreskin fibroblasts, HFF-TEL, were the kind gift of Tom Shenk and maintained in Dulbecco's Modified Eagles Media supplemented with 10% fetal bovine serum. Human umbilical vein endothelial cells (HUVEC) were isolated from vessels as previously described (Sedmak, Roberts et al. 1990) and propagated in endothelial cell growth medium (ECGM) consisting of M-199 (GibcoBRL, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS, Hyclone, Logan, UT), 22.5 μg/ml bovine brain extract (BioWhitakker, Inc., Walkersville MD), 12 U/ml sodium heparin (Sigma, St. Louis MO), and 20 mM HEPES buffer. All growth surfaces for endothelial cells were pretreated with

human fibronectin (25 μg/ml, Upstate Biotechnology, Lake Placid, NY). Cells were passed weekly by brief trypsin digestion at a ratio of 1:4, and used in experiments at passage 5-7. Cells were maintained at 37°C in 95% air, 5% CO_2.

Viruses

HCMV strain AD169 was purchased from ATCC and were propagated in MRC 5 cells. Virus titers were determined in MRC-5 cells by standard plaque assay (Wentworth and French 1970). CMV strain VHL/E, originally isolated from duodenal biopsy material from a bone marrow transplant recipient (Waldman, Roberts et al. 1991), was propagated in HUVEC as detailed elsewhere (Waldman, Adams et al. 1992) to preserve its natural endothelial cytopathogenicity. For UV-inactivation of virus, virus stocks were subjected to 100μ/J/cm^2 UV irradiation in a Hoefer UVC 500 UV crosslinker for 5 minutes. The efficacy of UV treatment and optimal exposure time was measured by exposing virus to UV for increasing intervals of times. MRC-5 cells were then exposed to virus subjected to various irradiation times, and IE1 protein accumulation was measured in cell lysates harvested 16 hrs after infection by SDS-PAGE (see below) and immunoblot analysis (data not shown).

Antibodies

Rabbit polyclonal antibodies to SphK1 were made to a peptide (RNHARELVRSEELGRWD) near the N-terminus representing residues 57 – 73 and affinity purified from serum by Quality Controlled Biochemicals (Hopkinton, MA). Mouse monoclonal antibody to the HCMV IE1 proteins were purchased from the

Rumbaugh-Goodwin Institute**.** Antibodies to the HCMV pp150 and UL44 proteins were the gift of Bill Britt. Anti-glyceraldehyde dehydrogenase (GAPDH) was purchased from Chemicon.

Sphingosine Kinase Assay

Sphingosine kinase activity was measured by phosphorylation of sphingosine with [γ-^{32}P]ATP and thin layer chromatography as described (Van Brocklyn, Jackson et al. 2005).

RNA Interference

Predesigned small interfering RNA (siRNA) oligonucleotides to SphK1 and random control siRNA (Silencer Negative control siRNA #2, cat# 4613) were purchased from Ambion. The negative control siRNA has no significant homology to known human, mouse or rat genes. Sequence for the SphK1 specific siRNA is as follows: (pre-designed siRNA ID #1181, sense 5′-GGCUGAAAUCUCCUUCACGtt-3′, antisense 5′-CGUGAAGGAGAUUUCAGCCtc-3′). The siRNA oligonucleotides were transfected into cells using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. After four hours medium was changed to normal growth medium and cells were incubated for the times indicated in Results.

Preparation of cell lysates and immunoblotting

Cells grown in 6-well plates were solubilized at the times indicated in the Results after exposure to the AD169 strain of virus with or without treatment with interfering

RNA's. Cells were rinsed in PBS and solubilized in lysis buffer containing 1% Triton-X-100, 50 mM Tris, 150 mM NaCl, and 1% (v/v) protease inhibitor cocktail (Sigma). Lysates were incubated at 4°C for 30 min, and insoluble material was pelleted by centrifugation. Equivalent amounts of protein from each lysate were separated by electrophoresis in sodium dodecyl sulfate (SDS) polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose sheets (Amersham). Sheets were reacted with primary antibodies indicated in the Results, and subsequently to horseradish peroxidase-conjugated secondary antibody (Santa Cruz). Bound antibodies were visualized using a chemiluminescent detection system (ECL, Amersham) and exposure to film. For SphK overexpression, cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions, with a construct containing the human SphK1 open reading frame cloned in frame with a N-terminal c-myc epitope tag in the pcDNA3 (Invitrogen) expression vector.

Real Time Quantitative PCR

Real time PCR analysis was performed as previously described (Van Brocklyn, Jackson et al. 2005). Briefly, total RNA was extracted from cultured cells using Trizol (Invitrogen, Carlsbad, CA) according to manufacturers instructions, followed by treatment with DNase1 (Ambion, Austin, TX) for 20 min at 37°C. cDNA was created using the Superscript II First Strand Synthesis System (Invitrogen) according to manufacturers instructions. PCR reactions were setup using Taqman® Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Real time PCR analysis was performed using Applied Biosystems ABI PRISM® 7700 Sequence Detection System for

40 cycles. Data was obtained using Sequence Detection System 1.7a software and exported to Microsoft Excel worksheets for analysis. SphK1, SphK2, S1P phosphohydrolase 1 & 2 (SPP1 & SPP2) and S1P Lyase (SPL) expression levels were normalized to mock-infected HFF SphK1 expression at 12 hrs. S1P receptors ($S1P_{1-3}$) expression levels were normalized to mock-infected HFF $S1P_1$ expression. Primer sequences of Applied Biosystems Assays-on-Demand™ gene expression assays are proprietary, however RefSeq accession numbers for genes, location of binding sites for fluorescent probes, and amplicons, approximately 50 – 150 base pairs surrounding probe location, are provided as follows: $S1P_1$ (catalog # HS00173499 m1) based on RefSeq NM 001400. Probe was located at base 83, exon1/exon2 boundary. $S1P_2$ (cat# HS00244677 s1) based on RefSeq NM005226, probe location 968. $S1P_3$ (cat# Hs00245464 s1) based on RefSeq NM004230, probe location 623, SphK1 (cat# Hs00184211 m1) based on RefSeq NM021972, probe location at exon 5/6 boundary, base 802, SphK2 (cat# Hs00219999 m1) based on RefSeq NM020126, probe location at exon3/4 boundary, base 875, SPP1 (cat # Hs00229266_m1) based on RefSeq NM030791, probe location at exon 1/2 boundary, base 782, SPP2 (cat # Hs00544786_m1) based on RefSeq NM152386, probe location at exon 4/5 boundary, base 649, SPL (cat # Hs00187407_m1) based on RefSeq NM003901, probe location at exon 8/9 boundary, base 915.

Sphingolipid Quantitation

Quantitation of sphingolipids in mock- and CMV-infected cells was performed using liquid chromatography tandem mass spectrometry (LC MS/MS) as previously

described (Merrill, Sullards et al. 2005) using the internal standard cocktail from Avanti Polar Lipids (Alabaster, AL).

RESULTS

SphK activity is elevated in HCMV-infected cells.

We first sought to investigate the effect of HCMV infection on SphK activity. U-373 MG glioma cells were exposed to 5 PFU per cell of the AD169 strain of HCMV and harvested 24, 48 and 72 hrs after infection. Cells were solubilized and cell lysates were then assayed for SphK activity as described in Materials and Methods. As shown in the top panel of Fig. 2.1A, higher levels of S1P were generated in *in vitro* kinase reactions from infected cell lysates relative to uninfected cell lysates. Quantitation of the S1P spots detected by TLC was translated into SphK activity (middle panel Fig. 2.1A) allowing us to compare the increase in activity in infected cells relative to uninfected cells (bottom panel, Fig. 2.1A). Higher levels of SphK activity were observed in HCMV-infected U-373 MG cells at all time points and a notable increase in SphK activity was readily observable at 24 hrs after infection. We typically observe a decrease in SphK activity with respect to time in culture (Van Brocklyn, Jackson et al. 2005) as was the case in this experiment. While the overall SphK activity decreased over the 72 h time period in both infected and uninfected cells, the relative SphK activity in infected cells continued to increase over the course of infection such that by 72 hrs, it was nearly 5 fold higher than that observed in uninfected cells. To ascertain if the increased SphK activity is specific

to the laboratory-adapted AD169 strain of HCMV, we compared SphK activity in cell lysates derived from U-373 MG cells infected with AD169 or a clinical isolate strain, VHL/E, at 48 hrs after infection. SphK activity was higher in both the AD169 and the VHL/E-infected cells when compared to mock-infected U-373 MG cells (Fig. 2.1B). From these experiments we conclude that HCMV infection of U-373 MG cells results in increased SphK activity, and that maximal induction occurs 48 to 72 hrs after infection.

We next investigated whether this phenomenon was specific to HCMV infections of U-373 MG cells or if this was a general characteristic of HCMV infections in other glioma cells as well as fibroblasts. As shown in Fig. 2.2A, we compared SphK activity in U-373 MG cells to another glioma line, U-251 MG, and to immortalized human fibroblasts (HFF-TEL) as well as primary fibroblasts serum starved for 48 hrs (SS HFF). Cells were mock-infected or exposed to 5 PFU per cell of the AD169 strain of HCMV. At 48 hrs after infection, cells were harvested and analyzed for SphK activity. We observed a 4-fold and 2.5-fold increase in SphK activity in U-373 MG and U-251 cells, respectively. HFF-TEL cells and serum starved HFF cells exhibited over a 2 fold increase under the same conditions. We next measured SphK activity in endothelial cells (HUVEC), which are also known to be productively infected with clinical isolates of HCMV. Cells were left uninfected or exposed to 1 PFU per cell of the VHL/E strain of HCMV. Cells were harvested and analyzed over a 96 h time period. As shown in Fig. 2.2B, SphK activity was elevated in infected endothelial cells at all tested time points when compared to mock-infected cells. The maximal increase in SphK activity (over 2 fold) was observed at 72 hrs post infection. We concluded from this series of

experiments that activation of SphK is a common aspect of HCMV infection in primary, immortalized and transformed cell types.

HCMV infection alters the abundance of mRNA transcripts for key enzymes involved in sphingolipid metabolism.

As discussed above, both SphK1 and SphK2 phosphorylate sphingosine to produce S1P, whereas predominantly SphK1 phosphorylates dihydrosphingosine to produce dhS1P. These bioactive lipids can then be converted back to sphingosine or dihydrosphingosine through the activity of S1P phosphohydrolase 1 and 2 (SPP1 & SPP2) or irreversibly degraded by S1P lyase (SPL) (Oskouian and Saba 2004 & Fig. 2.8A). In light of the changes in SphK activity in HCMV-infected cells we wanted to investigate how virus infection impacts the levels of mRNA transcripts coding for SphK and other important sphingolipid metabolic enzymes. In order to do this we utilized real time quantitative PCR to measure the transcript levels of the aforementioned enzymes, as well as the S1P receptors, $S1P_{1\text{-}3}$. In this experiment HFF cells were left uninfected or exposed to 1 PFU per cell of the AD169 strain of HCMV for 12, 24 or 48 hrs in triplicate and then harvested for RNA extraction. mRNA was quantitated by real time PCR and the mRNA levels of all the enzymes were normalized to mock-infected HFF SphK1 levels at 12 hrs. S1P receptor ($S1P_{1\text{-}3}$) mRNA levels were normalized to mock-infected HFF $S1P_1$ levels. As shown in Fig. 2.3A, the levels of SphK1 mRNA were significantly elevated in HCMV-infected cells at 24 and 48 hrs post infection (although most prominently at 24 hrs) when compared to mock-infected cells. However, the levels of SphK2 mRNA were not significantly elevated in HCMV-infected cells at any time point (Fig. 2.3B). It should

be noted that the overall levels of SphK2 transcript abundance was approximately 100 times lower than that of SphK1 in all tested samples. The S1P receptors, measured at 24 hrs post infection, showed no significant difference in transcript abundance in HCMV-infected cells when compared to mock-infected cells (Fig. 2.3C). Interestingly, HCMV infection also resulted in increased transcript abundance of the degradative enzymes SPP1, SPP2 and SPL. Although, the levels of SPP1 mRNA were significantly elevated in HCMV-infected cells at both the 12 and 24 h time points (Fig. 2.3D), we found that SPP1 transcript levels were variable in replicate experiments (data not shown). SPP2 mRNA levels could not be detected in mock-infected cells but were detected in HCMV-infected cells. All measured SPP2 mRNA levels were significantly elevated in HCMV-infected cells at all time points when compared to mock-infected cells. Accumulation of SPP2 mRNA was maximal at 48 hrs after infection, at which time levels were over 10 fold higher than that measured at 24 hrs after infection (Fig. 2.3E). SPL levels were very similar between mock and HCMV-infected cells at the 12 h time point, but were nearly 2 fold higher in HCMV-infected cells at 24 and 48 hrs; although, only the difference at the 48 h time point was statistically significant (Fig. 2.3F). Taken together these results show that HCMV infection results in the dynamic regulation of both synthetic (SphK1) and degradative (SPP2 and SPL) enzymes in the sphingolipid pathway.

HCMV infection alters the abundance of sphingolipids in a time-dependent manner.

Altogether these studies show that virus infection leads to an increase in transcript levels and activity of SphK1 within the first 48 hrs after infection, at which time there is an increase in the transcript levels of enzymes involved in the degradation of bioactive

sphingolipids. Based on these findings, we predicted that S1P and/or dhS1P should be elevated at early times after infection but levels would wane beginning at 48 hrs after infection. To test this prediction, we employed mass spectrometry to directly measure accumulation of bioactive sphingolipids in infected cells including sphingosine (Sph), dihydrosphingosine (dhSph), S1P, dhS1P, ceramide and dihydroceramide. In order to examine the amounts of these sphingolipids in the context of HCMV infection, HFF cells were serum starved for 48 hrs and then were mock-infected or exposed to 1 PFU per cell of the AD169 strain of HCMV for 24 or 48 hrs in triplicate. Cells were then harvested for the extraction of sphingolipids which were quantified by LC MS/MS. As shown in Fig. 2.4A-D, when comparing levels of sphingoid bases and their 1-phosphate derivatives in HCMV-infected cells to mock-infected cells at 24 hrs, only the amount of dhS1P, was significantly different, as dhS1P levels were over two fold higher in HCMV-infected cells (Fig. 2.4D). However, at the 48 h time point the levels of all measured sphingoid bases were significantly lower in HCMV-infected cells compared to mock-infected cells (Fig. 2.4A-D). We also measured the levels of total ceramide and total dihydroceramide in mock- and HCMV-infected cells at 24 and 48 hours. In HCMV-infected cells at 24 hrs both the amounts of total ceramide (Fig. 2.4E) and total dihydroceramide (Fig. 2.4F) were elevated when compared to mock-infected cells at 24 hrs, however only the difference in total ceramide levels at this time point was statistically significant. There was no significant difference in the levels of either total ceramide or total dihydroceramide when comparing HCMV-infected cells to mock-infected cells at 48 hrs. We also observed a similar trend of moderately increased glucosylceramide and sphingomyelin at 24 hours, although these differences did not reach statistical

significance (data not shown). Further measurements of different ceramide and dihydroceramide species, containing fatty acids of various chain lengths and degrees of saturation, did not reveal a significant alteration in the proportions of various species in response to HCMV infection (data not shown). These experiments show that a marked increase in the levels of dhS1P, total ceramide and possibly dihydroceramide, are observed within 24 hrs of infection, whereas a significant decrease in the levels of Sph, dhSph, S1P and dhS1P are observed at 48 hrs after infection. The increase in dihydrosphingolipids suggests that *de novo* sphingolipid synthesis is activated by HCMV during the first 24 hrs of infection.

HCMV infection results in increased abundance of SphK1.

Both the real-time quantitation of transcripts and the increase in cell-associated dhS1P, but not S1P directly implicates a role for SphK1 rather than SphK2 in infected cells. The increased SphK1 activity could be due to activation of SphK expressed at basal levels, and/or to an increase in accumulation of SphK protein. To begin to address the nature of SphK1 activation in infected cells, we generated an antibody specific to human SphK1 (Fig. 2.5A). We found that this antibody recognized a protein of the correct size (45 kDa) in lysates of CHO-K1 cells transfected with a myc-tagged SphK1 gene (lane 2), but not lysates of cells transfected with vector alone (lane 1). This antibody also recognizes two bands of approximately 42 and 46 kDa representing endogenous SphK1 protein expressed in U-373 MG cells and fibroblasts (Fig. 2.5B and 2.5C). The two bands recognized by this antibody likely correspond to two of the three subtypes of SphK1, SphK1a, 1b, and 1c which differ at the N-termini (Venkataraman,

Thangada et al. 2006). We used this antibody to compare total cellular levels of SphK1 in lysates from infected and uninfected cells. U-373 MG and HFF-TEL cells were mock-infected or were exposed to 5 PFU per cell of the AD169 strain of HCMV, and accumulation of SphK1 protein was measured at 48 hrs after infection. We observed an increased accumulation of SphK1 protein levels in HCMV-infected glioblastoma and fibroblast cells (Fig. 2.5B and 2.5C). Although this does not preclude a role for regulation of basal SphK1 activity, we conclude that the increased SphK activity is at least in part accounted for by increased accumulation of SphK1 protein in HCMV-infected cells.

HCMV-mediated induction of SphK activity occurs after virus entry but prior to viral DNA replication.

We next sought to characterize the stage of the virus life cycle associated with increased SphK activity. U-373 MG cells were mock-infected, exposed to 10 PFU per cell of the AD169 strain of HCMV, or exposed to the equivalent amount of UV-inactivated HCMV and harvested 48 h later for SphK activity. SphK activity in cells exposed to UV-inactivated virus was nearly identical to levels in uninfected cells, whereas three-fold higher levels of SphK activity was observed in cells infected with the AD169 strain of HCMV (Fig. 2.6A). This finding suggests that elevated SphK activity cannot be accounted for by activation of signaling pathways caused by virus binding to cell surface receptors.

We next measured SphK activity in mock- and HCMV-infected cells exposed to 1 mM phosphonoformic acid (PFA), a drug inhibitor of viral DNA replication. As shown

in Fig. 2.6B, blockade of viral DNA synthesis and subsequently, expression of late genes dependent on DNA synthesis, had no impact on the HCMV-mediated induction of SphK activity. SphK activity in mock-infected U-373 MG cells and cells treated with PFA were below 10 pmol/min/mg protein. In contrast SphK activity levels were approximately 25 pmol/min/mg protein in HCMV-infected U-373 MG cells both with and without PFA treatment. The identical pattern of SphK activity was observed in fibroblasts, though the overall magnitude of basal and induced SphK activity was greater in these cells relative to the U-373 MG cells. From these studies we conclude that induction of SphK activity in HCMV-infected cells occurs at step of the virus life cycle subsequent to virion binding to surface receptors but prior to onset of viral DNA replication.

RNAi-mediated knockdown of SphK1 expression alters the accumulation of HCMV immediate early gene product IE1.

The emerging picture from these studies is that early events in HCMV infection activate the *de novo* pathway of sphingolipid synthesis and especially the activity of SphK1. To test whether activation of SphK1 impacts the virus life cycle, we used RNAi-mediated silencing to block accumulation of SphK1, and then measured accumulation of virus gene products in productively infected cells. The efficacy and specificity of the siRNA treatment is shown in Fig. 2.7A. In this experiment, primary human foreskin fibroblasts (HFF) were transfected with silencing (si)RNA oligonucleotides specific for SphK1 and subsequently fed with serum free DMEM. We have previously shown that this siRNA oligonucleotide has no effect on expression of SphK2 (Van Brocklyn,

Jackson et al. 2005). After 72 hrs of culture under serum-free conditions, cells were then harvested for a sphingosine kinase assay. As shown in Fig. 2.7A, siRNA treatment diminished SphK activity nearly 3 fold as measured by *in vitro* kinase assays relative to untreated cells and to cells treated with a control siRNA that does not target human genes. We also measured SphK1 knockdown in infected cells. Fibroblasts grown in 6-well plates were transfected with control siRNA, siRNA specific for SphK1 or were left untreated. After transfection, the cells were serum starved for 48hrs and then were exposed to 0.5 PFU per cell of AD169. Cells were then harvested 24, 48 and 72 h after infection. Protein lysates were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with antibodies specific for SphK1. The SphK1-specific siRNA led to dramatic reduction in accumulation of this protein (Fig 2.7B) and a specific reduction in the accumulation of IE1 protein but not early or late proteins (not shown). In a replicate experiment (Fig 2.7C), we examined the levels of HCMV immediate early protein 1 (IE1), the early protein UL44, the late protein pp150. Although SphK1 knockdown was not as complete in the first experiment, we observed the identical pattern of viral gene product accumulation: Cells treated with the siRNA to SphK1 exhibited a decrease in accumulation of IE1 at 48 and 72 h after infection when compared to cells treated with control siRNA (lanes 3-6). No decrease was observed for UL44 at 48 or 72 h after infection and only a slight decrease in pp150 was seen at 72 h post infection. Replicate samples were taken from this experiment for a virus growth curve analysis. As seen in Fig. 2.7D, there was no difference between the number of infectious virus progeny released from control or SphK1 siRNA transfected cells at either 0 or 80 hours post

infection. These findings indicate that the induction of SphK1 activity by HCMV functions to promote a sustained accumulation of the immediate early viral protein IE1.

DISCUSSION

Sphingolipids are important constituents of viral and cellular membranes and act as signaling mediators that regulate several fundamental physiological processes. Herein we provide evidence that cellular sphingolipids and the enzymes that regulate their accumulation are modulated during infection with a herpesvirus. We also provide evidence that alteration of the sphingolipid profile is a strategy HCMV uses to optimize the cellular environment for virus replication. The major findings of this study are as follows: (1) HCMV infection leads to an increase in the accumulation of dhS1P and ceramides within the first 24 hours of infection, consistent with a stimulation of the *de novo* pathway of sphingolipid metabolism; (2) By 48 hrs after infection, levels of S1P, dhS1P and their precursors are diminished and this coincides with the upregulation of enzymes responsible for the degradation of S1P and dhS1P; (3) Protein levels of SphK1, the key enzyme in the generation of S1P and dhS1P, are elevated in HCMV-infected cells; (4) The activity of SphK1 is elevated beginning as early as 12 hours after infection, and increased activity of this enzyme is observed through 72 hrs after infection; and (5) Sustained accumulation of the IE1 protein, the product of the *UL123* gene, is substantially diminished under conditions where SphK1 protein levels were reduced by siRNA treatment.

Earlier studies suggest that alteration of sphingolipid levels may have profound consequences on the life cycle of herpes viruses. In Niemann-Pick Disease cells, which are deficient in sphingomyelinase, production of infectious herpes simplex virus (HSV) is dramatically reduced, and an inhibitor of glycosphingolipid synthesis decreases HSV production to 0.1% of its normal level (Steinhart, Busch et al. 1984). Also, addition of exogenous ceramides or stimulation of ceramide synthesis by treatment of cells with sphingomyelinase have been shown to reduce biosynthesis of HCMV glycoprotein B (gB), although treatment of cells with C6-ceramide led to increased accumulation of gB (Allan-Yorke, Record et al. 1998).

In the present study, we show for the first time that HCMV infection causes accumulation of SphK1 protein and increased activity of SphK1. Induction was observed after infection with laboratory-adapted and clinical isolates in primary fibroblasts and endothelial cells, immortalized fibroblasts, and in two distinct glioma cell lines. Together, these data suggest that induction of SphK1 activity is a common consequence of HCMV infection regardless of the virus strain or cell type.

We have not identified the precise trigger that causes upregulation and/or activation of SphK1, but our studies show that exposure of cells to UV-inactivated virus is not sufficient to induce SphK1 activity. We also found that inhibition of DNA synthesis and consequent expression of strict late genes did not interfere with upregulation of SphK1 activity. We conclude that one or more immediate early or early gene products is required to elicit the increased accumulation and activity of this enzyme.

SphK1 catalyzes the phosphorylation of sphingosine and dihydrosphingosine to produce S1P and dhS1P respectively (Kohama, Olivera et al. 1998). S1P can function

extracellularly through a group of cell surface G protein-coupled receptors (Hla 2003). Moreover, S1P produced within cells can act in an autocrine or paracrine manner through its G protein-coupled receptors (Hobson, Rosenfeldt et al. 2001; Jolly, Bektas et al. 2004; Goparaju, Jolly et al. 2005). dhS1P has been shown to be an S1P receptor agonist (Tamama, Kon et al. 2001) and can presumably act in much the same way. Our results show a preferential increase in dhS1P rather than S1P in HCMV-infected cells. An extensive literature has emerged documenting the biological activities of S1P, while fewer studies have focused on elucidating the biological effects dhS1P. One recent study showed that dhS1P but not S1P mediated SphK-induced upregulation of matrix metalloproteinase 1 gene expression in a manner consistent with G-protein-coupled receptor activity (Bu, Yamanaka et al. 2006). However, the extent of both the overlap and differences in the biological activities of these two very similar sphingolipids have yet to be defined.

Our findings demonstrate that increased SphK1 activity is required for sustained accumulation of the IE1 gene product. Although these studies do not exclude the possibility that SphK1 has activity towards another substrate in virus-infected cells, a likely explanation for this result is that SphK1 activity stimulates formation of dhS1P and activation of S1P receptors. Subsequent transduction of the signals regulated by these receptors may act to promote IE1 biosynthesis or stability.

dhS1P released in this scenario could function in a paracrine fashion via its G-protein coupled receptors to increase the survival and proliferation of target cells in order to maintain an ideal environment for infection, and may also affect the extracellular matrix. Evidence in support of a pro-survival activity of sphingolipid signaling comes

from studies showing that that respiratory syncytial virus (RSV) infection of lung epithelial cells results increased levels of dhS1P (Berdyshev, Gorshkova et al. 2006) and SphK1-dependent activation of two survival pathways mediated by Erk and Akt (Monick, Cameron et al. 2004). Interestingly, HCMV encodes several G protein-coupled receptors (Stropes and Miller 2004), one of which, US28, has been shown to signal through the G_i pathway (Billstrom, Johnson et al. 1998), which is utilized by all 5 S1P receptors. It seems possible that HCMV has employed two ways of activating G_i-coupled receptors. By encoding these receptors HCMV can activate related pathways within an infected cell, and by boosting the activity of SphK and subsequently dhS1P levels, the virus may facilitate the priming of these pathways in infected or neighboring cells.

It is interesting to note that the increased dhS1P does not come at the expense of its precursor dihydrosphingosine or later derivatives ceramide and dihydroceramide, which are actually increased at 24 hrs. These data suggest that HCMV causes an upregulation of *de novo* sphingolipid synthesis occurring at the endoplasmic reticulum. This finding substantially extends an earlier study linking HCMV infection with glycosphingolipid synthesis (Radsak and Wiegandt 1984). A schematic depicting *de novo* sphingolipid synthesis is shown in Fig 2.8A (for reviews see (Merrill 2002; Le Stunff, Milstien et al. 2004), and a summary of our data illustrating how HCMV infection interfaces with this pathway is presented in Fig. 2.8B, left panel. Our results also indicate that at 48 hrs post infection there is a significant decrease in the levels of sphingosine, dihydrosphingosine, S1P and dhS1P that coincides with higher levels of degradative enzymes (SPP2 and SPL) (Fig. 2.8B, right panel). The observation that decreased dhS1P

levels do not correlate with increased dhSph suggest that SPL and not SPP2 may have the predominant effect on sphingolipid levels at this time point.

The increase in enzymes involved in turnover of dhS1P and S1P may represent an attempt of the cell to combat virus infection by lowering the levels of these sphingolipids. Alternatively, upregulation of degradative enzymes may also be regulated by viral gene products in order to prevent signaling through S1P receptors at this time. Another possibility is that these changes promote the accumulation of other sphingolipids needed at later stages of infection to facilitate virus maturation or envelopment. Along these lines, a recent study showed that the lipid envelope of HIV-1 is highly enriched in sphingolipids, particularly dihydrosphingomyelin, and that inhibition of sphingolipid synthesis decreases HIV-1 infectivity (Brügger, Glass et al. 2006).

In summary, our findings suggest that dynamic regulation of bioactive sphingolipids is a general feature of HCMV infection which is required for sustained accumulation of immediate early viral gene products, presumably through a mechanism involving dhS1P signaling. These findings warrant further studies to ascertain if and how virus infection impacts the activities of dhS1P through S1P receptor-regulated cellular processes such as cell survival, proliferation, motility. Also, considering the important role of S1P receptor-mediated regulation of lymphocyte trafficking and immune responses, further studies to investigate how HCMV and other viruses interface with this signaling system may ultimately shed light on virus replication, spread and pathogenesis.

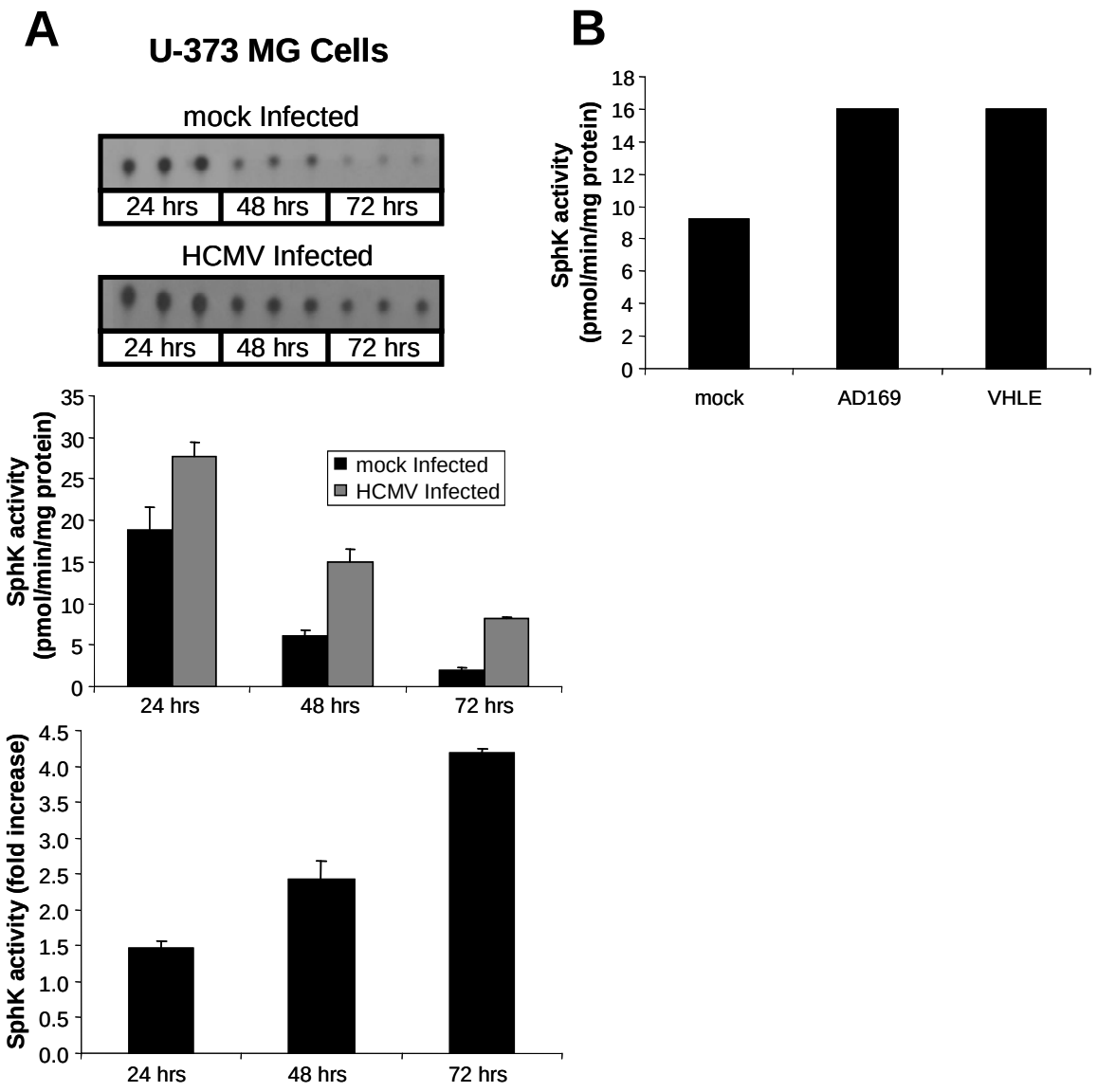

Figure 2.1. Elevated SphK activity in HCMV-infected U-373 MG cells. A) Time course of SphK activity of U-373 MG cells infected with HCMV. Cell lysates from mock-infected U-373 MG cells, or cells exposed to 5 PFU per cell of the AD169 strain of HCMV, were analyzed for SphK activity as described in Materials and Methods (Top). Data are means ± standard deviations of three independent determinations (Middle). Fold increase of SphK activity of mock-infected to virus-infected cells is shown (Bottom). B) SphK activity of U-373 MG cells exposed to 1 PFU/cell of the AD169 or VHLE strain of CMV.

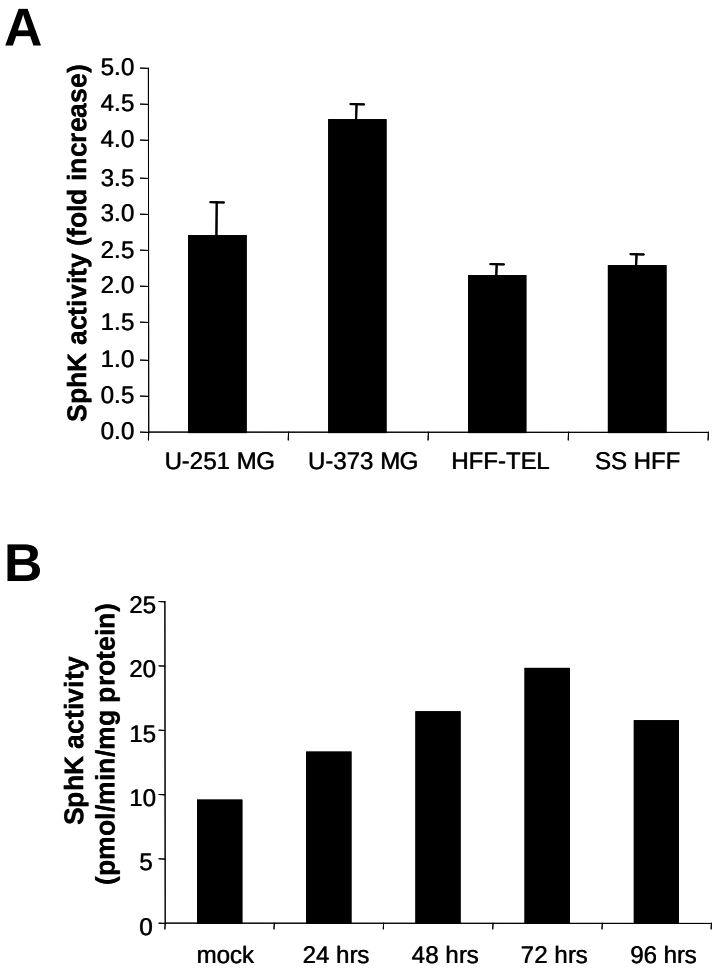

Figure 2.2. Induction of SphK1 activity is independent of cell type. A) Fold increase of SphK activity in HCMV-infected U-251 MG, U-373 MG, HFF telomerase immortalized cells (HFF-TEL) and primary HFF cells which were serum starved (SS HFF) for 48 hrs prior to infection. Cell lysates from mock-infected or AD169-infected (5PFU per cell) cells were analyzed for SphK activity as described in Materials and Methods. Data represent the fold difference between HCMV-infected cells and mock-infected cells for each cell type and are given as means ± standard deviations of three independent determinations. B) SphK activity is elevated in endothelial cells. Endothelial cells were either mock-infected or infected with 1 PFU per cell of the AD169 strain of HCMV. At the specified times after infection, cell lysates were analyzed for SphK activity.

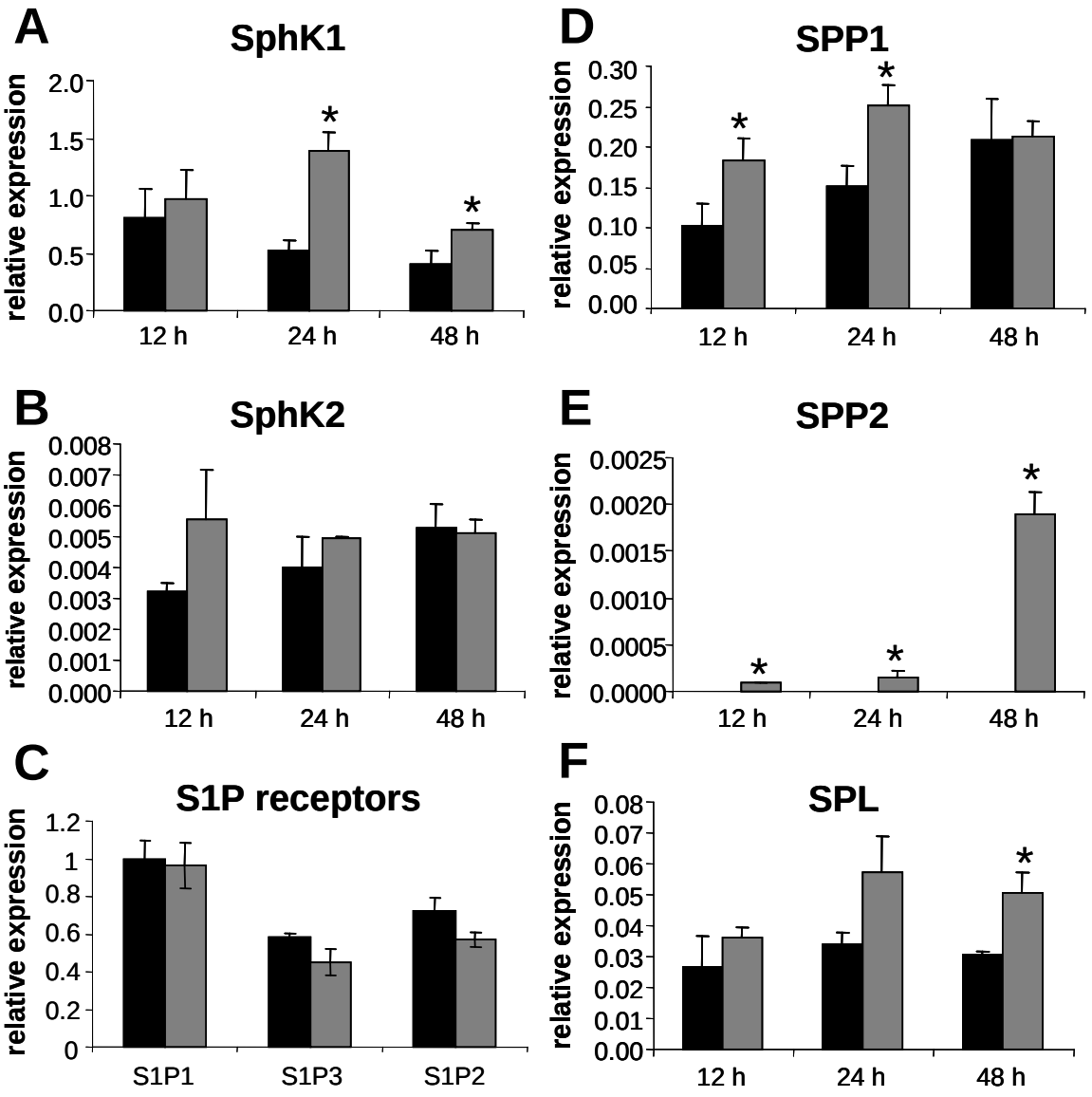

Figure 2.3. Quantitation of transcripts specifying enzymes that regulate sphingolipid metabolism. HFF cells were mock-infected or exposed to 1 PFU per cell of the AD169 strain of HCMV for 12, 24 or 48 hrs and then harvested for RNA extraction. Relative transcript levels were quantitated by real time PCR. Transcript levels in A-E are all relative to SK-1 levels mock-infected HFF cells harvested at the 12 hour time point. The black bars correspond to values derived from mock-infected cells and the grey bars correspond to values derived from HCMV-infected cells for A) SphK1, B) SphK2, C) S1P receptors (S1P1-3) at 24 hours post infection, D) SPP1, E) SPP2 and F) SPL. P values of less than 0.05 are indicated with a asterisk.

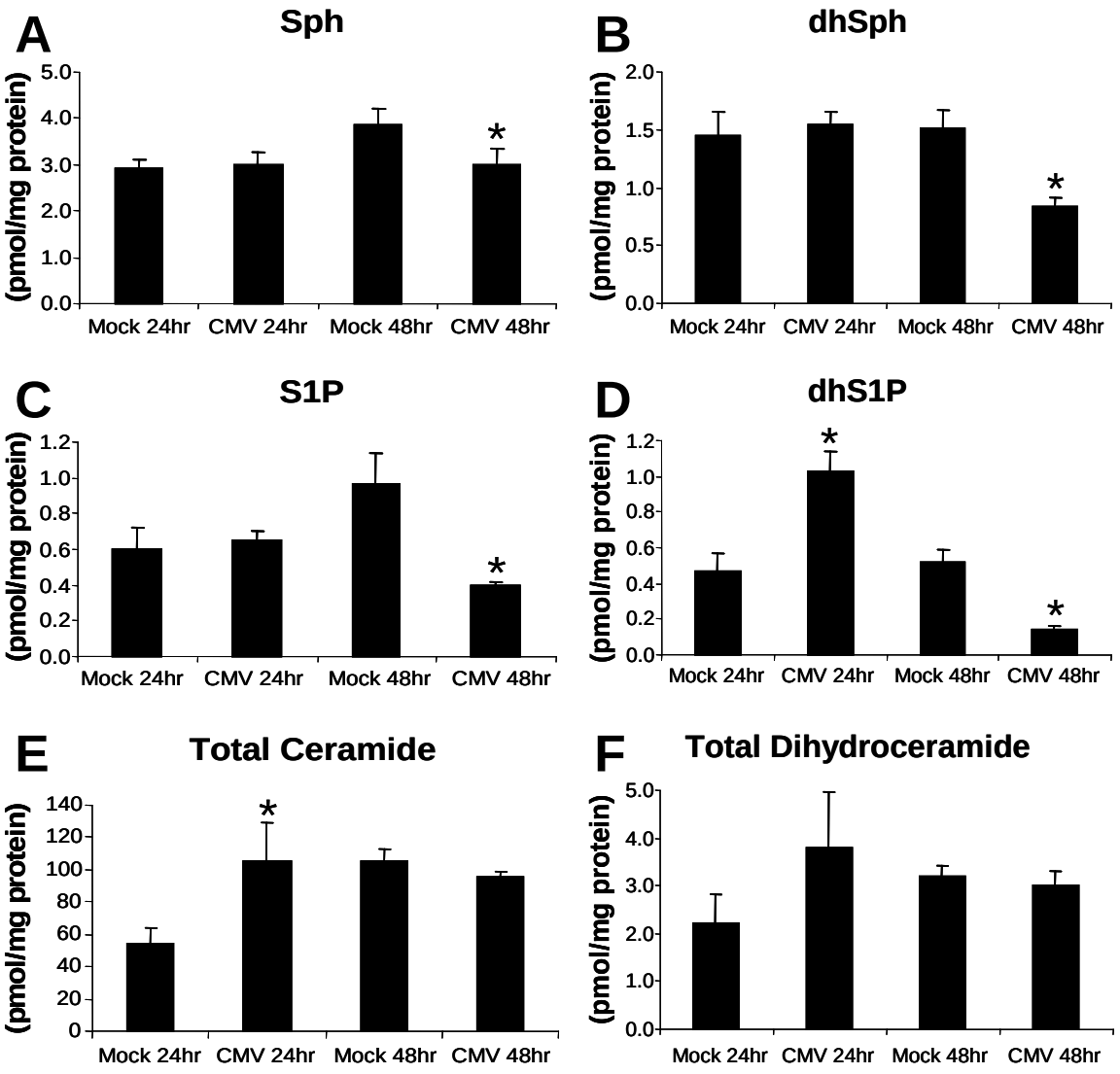

Figure 2.4. The sphingolipid profile of cells is altered by virus infection. HFF cells were serum starved for 48 hrs and then left uninfected or exposed to 1 PFU per cell of the AD169 strain of HCMV for 24 or 48 hrs in triplicate. Cells were then harvested for the extraction of sphingolipids which were later quantified by tandem liquid chromatography/mass spectrometry. The data are given as the amount of each sphingolipid in picomoles per milligram of total protein and represent the means ± standard deviations of three independent determinations. A) Quantitation of sphingosine (Sph), B) dihydrosphingosine (dhSph), C) sphingosine-1-phosphate (S1P) and D) dihydrosphingosine-1-phosphate (dhS1P) at 24 and 48 hours of mock or HCMV infection. E) Quantitation of total ceramide and F) total dihydroceramide at 24 and 48 hours of mock or HCMV infection. An asterisk indicates a P value of less than 0.05.

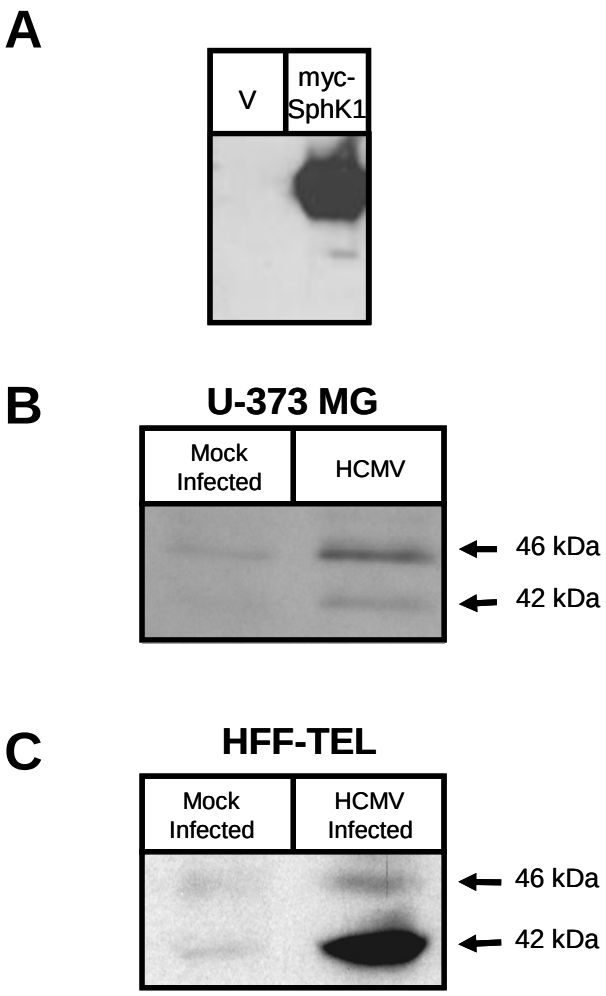

Figure 2.5. SphK1 protein levels are elevated upon infection. A) Film image of an immunoblot of SphK1 using an antibody that was generated against human SphK1. Cell lysates generated from CHO-K1 cells transfected with a myc-tagged SphK1 gene (lane 2) or transfected with vector alone (lane 1) were subjected to electrophoresis in an SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose sheet and probed with rabbit anti SphK1 in order to detect the levels SphK1 at approximately 45 kDa. B) Film image of an immunoblot of SphK1 in U-373 MG cells infected with HCMV. U-373 MG cells were mock-infected or exposed to 5 PFU per cell of the AD169 strain of HCMV for 48 hours. SphK1 levels were analyzed by immunoblot as described above. C) HFF-TEL cells were either mock-infected or exposed to 5 PFU per cell of the AD169 strain of HCMV for 48 hours. SphK1 levels were analyzed by immunoblot as described above.

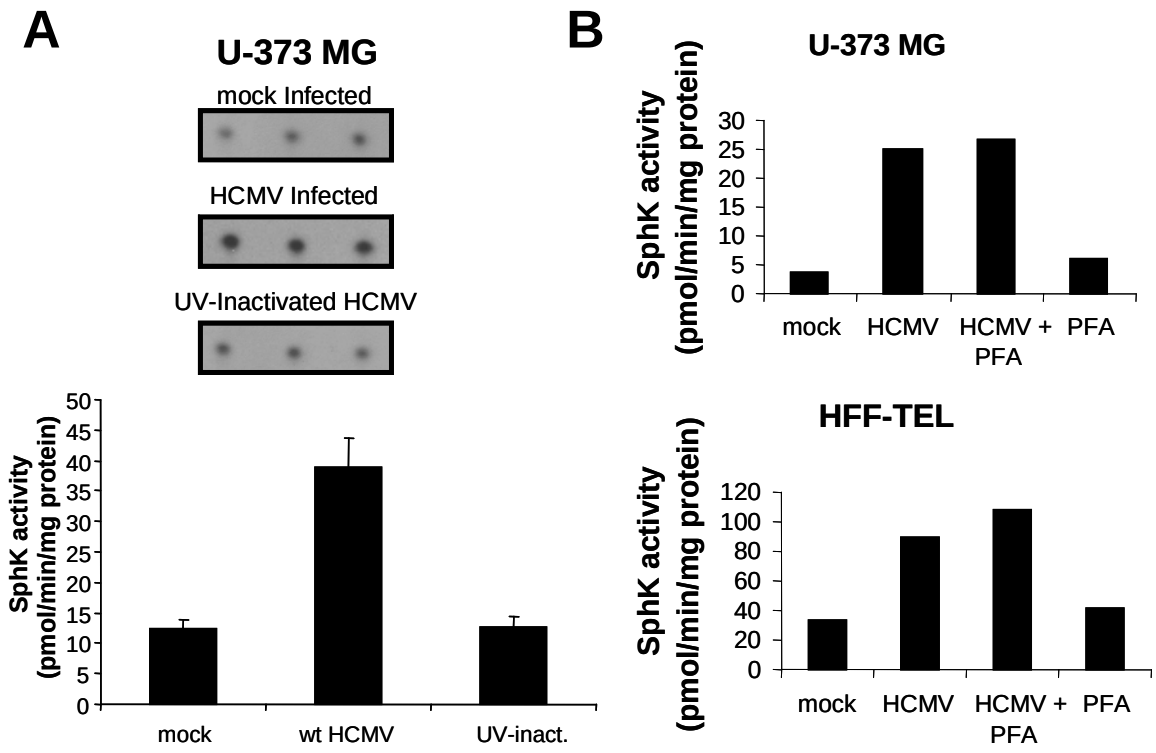

Figure 2.6. HCMV-induced increase in SphK activity does not occur with UV-inactivated HCMV and is not affected by phosphonoformic acid. A) U-373 MG cells were mock-infected, exposed to 10 PFU per cell of HCMV, or exposed to 10 PFU per cell of UV-inactivated HCMV and analyzed for SphK activity 48 hrs after exposure to virus as described in Materials and Methods. Data are means ± standard deviations of three independent determinations (bottom). B) SphK activity of U-373 MG cells and HFF-TEL cells infected with HCMV in PFA-containing media. U-373 MG cells (top) and HFF telomerase immortalized cells (bottom) were mock–infected or exposed to 10 PFU per cell of the AD169 strain of HCMV. Cells were cultured in the regular media or media supplemented with PFA. At 48 hrs after infection cell lysates were then analyzed for SphK activity as described in Materials and Methods.

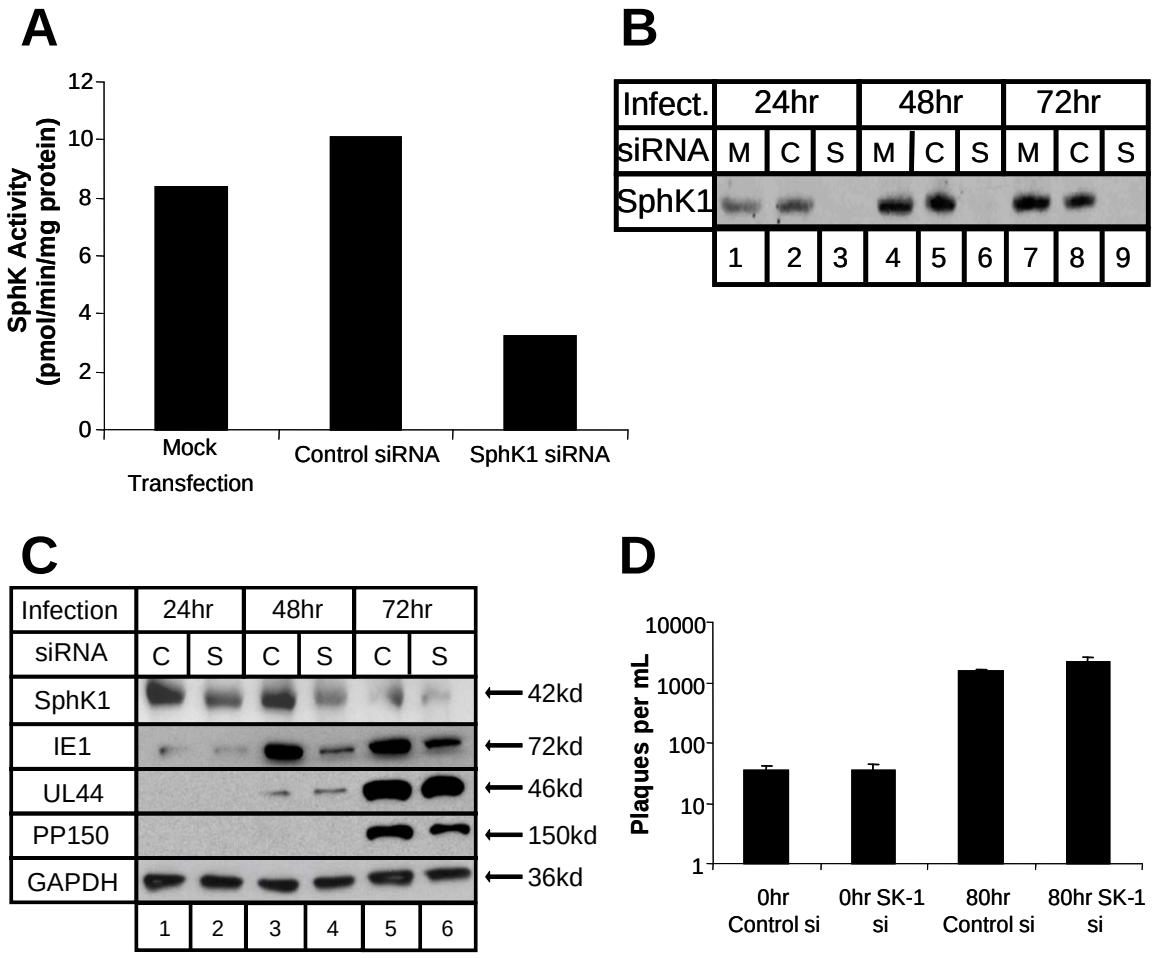

Figure 2.7. SphK knockdown with siRNA reduces accumulation of HCMV immediate early protein. A) SphK activity is suppressed by siRNA specific for SphK1. HFF cells were left untransfected, transfected with control siRNA or SphK1 siRNA and then serum starved for 72 hrs. The cells lysates were then analyzed for SphK activity as described in Materials and Methods. B and C) HFF cells were transfected with control siRNA (C) or SphK1 siRNA (S) and subsequently serum starved for 48 hrs. Then cells were infected with HCMV at 0.5 PFU per cell for 24, 48 or 72 hrs prior to harvesting cell lysates. Immunoblots were performed as described in Materials and Methods using antibodies to SphK1 and viral proteins. To control for loading of lysates, blots were also reacted to antibodies to cellular GAPDH proteins. D) Replicate samples were taken from the experiment in panel C at 0 and 80 hours post infection for a virus growth curve analysis.

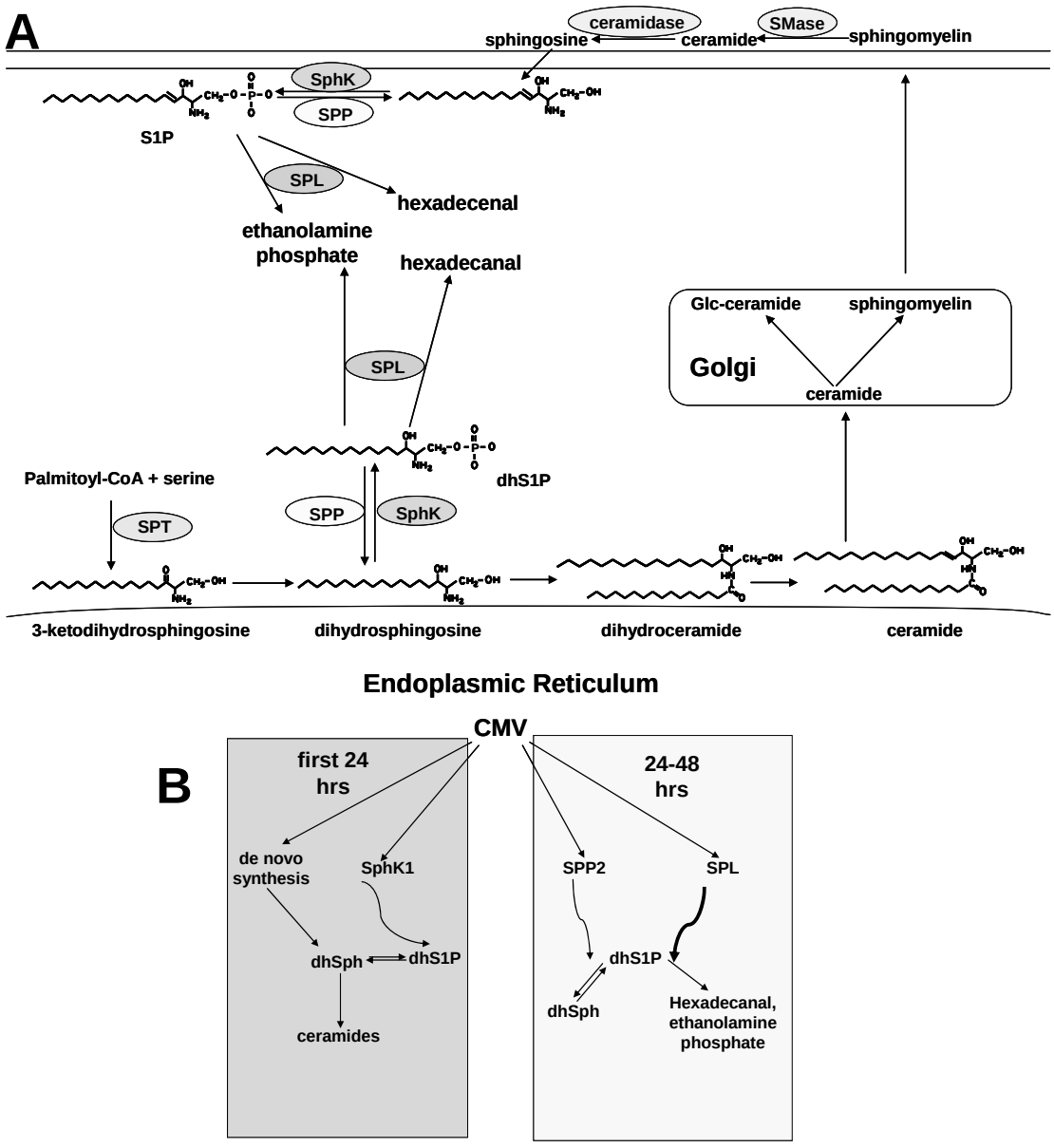

Figure 2.8. A) Diagram depicting sphingolipid biosynthesis occurring in the endoplasmic reticulum and Golgi complex as well as sphingolipid breakdown at the plasma membrane. Note that *de novo* synthesis produces dihydrosphingosine, while sphingosine is only created through sphingolipid breakdown. Enzymes and sphingolipids not previously defined include serine palmitoyltransferase (SPT) and glucosyl ceramide (Glc-ceramide). B) Diagram depicting effects of CMV infection on sphingolipid metabolism. Although actual time of induction of SphK and S1P hydrolyzing enzymes overlaps somewhat, the earlier phase appears to favor sphingolipid synthesis as shown by increases in dhS1P and ceramides, while the later phase appears to favor breakdown. The bold arrows indicate enzymatic steps especially impacted by infection. We predict a preference for SPL activity over SPP2 activity as indicated by decreased levels of both long chain bases and their phosphates.

CHAPTER 3

TEMPORAL REGULATION OF DIHYDROSPHINGOSINE AND DIHYDROSPHINGOSINE-1-PHOSPHATE LEVELS CAN DETERMINE EXPRESSION LEVELS OF HUMAN CYTOMEGALOVIRUS GENE PRODUCTS

ABSTRACT

Human cytomegalovirus (HCMV) promotes its replication through stimulation of a variety of cell signaling pathways. The sphingolipids, dihydrosphingosine-1-phosphate (dhS1P) and sphingosine-1-phosphate (S1P), are produced from the phosphorylation of dihydrosphingosine (dhSph) and sphingosine (Sph), respectively, by the activity of sphingosine kinase (SphK). dhS1P and S1P can activate many signaling pathways through binding to G-protein coupled S1P receptors, named $S1P_{1-5}$. Our past results have shown that HCMV dynamically regulates sphingolipid metabolism and that SphK is needed for optimal accumulation of the immediate early gene product, IE1. In the following study we show that siRNA-mediated knockdown of serine palmitoyltransferase, the enzyme responsible for the initial step in *de novo* sphingolipid synthesis, reduces the number of infectious virus progeny released within HCMV-infected HFF cells. This knockdown also results in increased accumulation of IE1, suggesting that although HCMV needs the *de novo* sphingolipid synthesis pathway for

optimal virus growth, an intermediate of this pathway, such as dihydrosphingosine (dhSph), may inhibit protein accumulation. We then provide evidence that dhSph inhibits HCMV protein accumulation whereas dhS1P promotes it, although only at early times during infection. Furthermore, pretreatment of cells with dhS1P or S1P prior to infection results in reduced accumulation of HCMV early and late gene products. These results imply that HCMV can only benefit from increased levels of dhS1P at early times of infection. Lastly, an experiment using a drug inhibitor to suppress SphK activity during HCMV infection indicates that activation of SphK may act to promote virus replication. The results of this study indicate that HCMV modulates sphingolipid metabolism to optimize virus growth and control the levels of dhSph and dhS1P in a time-dependent manner to optimize viral protein accumulation.

INTRODUCTION

The β-herpes virus HCMV is endemic in the human population and can cause serious disease and even death in congenitally-infected infants and in immunocompromised individuals or immunosuppressed transplant recipients. This virus can utilize disparate means by which to activate many cell signaling pathways in order to promote its replication (Jones, Lewis et al. 1986; Jault, Jault et al. 1995; Nokta, Hassan et al. 1996; Rodems and Spector 1998; Johnson, Huong et al. 2000; Ogawa-Goto, Tanaka et al. 2003; Wang, Huang et al. 2005). Sphingolipids can activate a variety of cell signaling pathways; yet, the ability of HCMV to control levels of these lipids by modulating

enzymes involved in their synthesis has only been exemplified through our most recent investigation.

Sphingolipids are constituents of cell membranes within all mammalian cells, and lately it has been shown that they have a significant role in cell signaling (Spiegel and Milstien 2003). In particular, sphingosine-1-phosphate (S1P) has been studied rather extensively in recent years and has been shown to exhibit a variety of biological activities through activation of signaling cascades coupled to its G-protein coupled receptors named $S1P_{1-5}$ (Spiegel and Milstien 2003; Rosen and Goetzl 2005; Young and Van Brocklyn 2006). S1P is formed through the phosphorylation of sphingosine (Sph) by sphingosine kinases, which can also phosphorylate dihydrosphingosine (dhSph) to form dihydrosphingosine-1-phosphate (dhS1P) (Berdyshev, Gorshkova et al. 2006). The only structural difference between Sph and dhSph, as well as S1P and dhS1P, is that both dhSph and dhS1P do not have the trans double bond at the 4-position. Many studies have implicated a role for Sph in the induction of apoptosis and inhibition of cell proliferation (Cuvillier 2002). However, very little is known about dhSph, but because it is structurally similar to Sph, it is thought to have similar biological effects to that of Sph (Ahn, Chang et al. 2006). This scenario is true of S1P and dhS1P in that they can both stimulate S1P receptors and therefore have many of the same effects on cell signaling resulting in these sphingolipids having the opposite effects of Sph, such as promoting cell proliferation and suppressing apoptosis (Cuvillier, Pirianov et al. 1996; Van Brocklyn, Lee et al. 1998; Tamama, Kon et al. 2001; Maceyka, Payne et al. 2002).

Our previous studies have shown for the first time that sphingolipids are dynamically regulated upon infection with HCMV. This dynamic regulation appears to

result in an increase in *de novo* sphingolipid metabolism at early times of infection, resulting in an increase in ceramide and dhS1P levels. On the other hand, at later times of infection, there is an increase in the levels of mRNA transcripts for degradative enzymes corresponding to a decrease in the levels of several sphingolipids, including dhS1P and dhSph. In addition, we gave relevance to this regulation through evidence suggesting that SphK1 activity and expression is increased during HCMV infection and functions to sustain levels of the IE1 gene product. The data from this study implicated a role for dhS1P in promoting the accumulation of this viral protein.

In the following investigation, we attempt to further resolve the importance of dhS1P and the dynamic regulation of sphingolipids to HCMV replication. Our results suggest that *de novo* sphingolipid biosynthesis is required for the production of optimal levels of infectious virus progeny, but products from this pathway may suppress the levels of IE1 protein accumulation. We have also shown, through exogenous addition of dhSph and dhS1P, that dhSph suppressed viral protein accumulation whereas dhS1P can stimulate and possibly suppress this accumulation depending on when during infection its exogenous addition takes place. Moreover, our results indicate that pretreatment with either dhS1P or S1P suppresses the accumulation of both early and late HCMV gene products. Finally, the inhibition of SphK1 activity with the drug inhibitor 2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole reduces virus growth nearly 100 fold at 48 hrs post infection. Together these data reveal a potential temporal role for dhSph and dhS1P in HCMV gene expression and growth, which helps to explain the temporal regulation of enzymes involved in regulating the levels of these sphingolipids.

MATERIALS AND METHODS

Cell Culture

MRC-5 human primary fibroblasts were obtained from ATCC and maintained in modified Eagle's medium (all media from Mediatech, Herndon, VA) supplemented with 10% fetal calf serum, 1.7 mM sodium bicarbonate, 1.4 mM sodium chloride, essential and nonessential amino acids, vitamins and sodium pyruvate at manufacturer-recommended concentrations (Sigma). Human foreskin fibroblasts, HFF, were maintained in Dulbecco's Modified Eagles Media supplemented with 10% fetal bovine serum and nonessential amino acids. Cells were passed weekly by brief trypsin digestion at a ratio of 1:4 and used in experiments at passage 5-7. Cells were maintained at 37°C in 95% air, 5% CO_2.

Viruses

HCMV strain AD169 was purchased from ATCC and were propagated in MRC 5 cells. Virus titers were determined in MRC-5 cells by standard plaque assay (Wentworth and French 1970).

Antibodies

Rabbit polyclonal antibodies to SphK1 were made to a peptide (RNHARELVRSEELGRWD) near the N-terminus representing residues 57 – 73 and affinity purified from serum by Quality Controlled Biochemicals (Hopkinton, MA).

Rabbit polyconal antibodies to serine palmitoyltransferase long chain base subunit 1 (SPT1 or SPTLC1) were purchased from Santa Cruz Biotechnology, Inc.. Mouse monoclonal antibody to the HCMV IE1 proteins were purchased from the Rumbaugh-Goodwin Institute**.** Antibodies to the HCMV pp150 and UL44 proteins were the gift of Bill Britt. Anti-glyceraldehyde dehydrogenase (GAPDH) was purchased from Chemicon.

Drug Inhibitor

The SphK inhibitor 2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole was purchased from Calbiochem and stored diluted in DMSO. The inhibitor was used at a concentration of 1μg/ml for 24 hrs unless noted otherwise.

Sphingolipids

S1P, dhS1P and dhSph were all purchased from Avanti polar lipids. Treatment of cells with dhSph and dhS1P were at concentrations of 500nM and 3uM respectively. Pre-treatment of cells (prior to exposure to AD169) with S1P and dhS1P were at concentrations of 1μM.

Sphingosine Kinase Assay

Sphingosine kinase activity was measured by phosphorylation of sphingosine with [γ-^{32}P]ATP and thin layer chromatography as described (Van Brocklyn, Jackson et al. 2005).

RNA Interference

Predesigned small interfering RNA (siRNA) oligonucleotides to SphK1, SPTLC1 and random control siRNA (Silencer Negative control siRNA #2, cat# 4613) were purchased from Ambion. The negative control siRNA has no significant homology to known human, mouse or rat genes. Sequence for the SphK1 specific siRNA is as follows: (pre-designed siRNA ID #1181) sense 5′-GGCUGAAAUCUCCUUCACGtt-3′, antisense 5′-CGUGAAGGAGAUUUCAGCCtc-3′. Sequence for the SPTLC1 specific siRNA is as follows: (pre-designed siRNA ID #111681) sense 5'-GCCACAAAACUGUGGUGAAtt-3', antisense 5'-UUCACCACAGUUUUGUGGCtt-3'. The siRNA oligonucleotides were transfected into cells using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. After four hours, medium was changed to normal growth medium and cells were incubated for the times indicated in Results.

Preparation of cell lysates and immunoblotting

Cells grown in 6-well plates were solubilized at the times indicated in the Results after exposure to the AD169 strain of virus with or without treatment with interfering RNA's. Cells were rinsed in PBS and solubilized in lysis buffer containing 1% Triton-X-100, 50 mM Tris, 150 mM NaCl and 1% (v/v) protease inhibitor cocktail (Sigma). Lysates were incubated at 4°C for 30 min, and insoluble material was pelleted by centrifugation. Equivalent amounts of protein from each lysate were separated by electrophoresis in sodium dodecyl sulfate (SDS) polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose sheets (Amersham). Sheets were reacted with primary

antibodies indicated in the Results, and subsequently to horseradish peroxidase-conjugated secondary antibody (Santa Cruz). Bound antibodies were visualized using a chemiluminescent detection system (ECL, Amersham) and exposure to film.

Viral Growth Curve Analysis

Quantification of infectious progeny virus was done by standard plaque assay as described (Wentworth and French 1970).

RESULTS

Knockdown of SPT using siRNA increased HCMV IE1 gene product accumulation but decreased the number of infectious virus progeny released.

De novo sphingolipid synthesis begins by condensation of serine and palmitoylCoA by SPT (Stoffel and Bister 1974; Mandon, van Echten et al. 1991). SPT consists of two gene products, SPTLC1 (or SPT1) and SPTLC2, which form a heterodimeric enzyme complex (Dickson, Lester et al. 2000; Hanada, Hara et al. 2000). Our past results suggesting that HCMV infection stimulates *de novo* spingolipid synthesis led us to investigate the effect of suppressing the accumulation of SPT on viral gene expression and growth. We hypothesized that by inhibiting the initial step in sphingolipid synthesis in this manner, during HCMV infection, there would be a decrease in viral gene expression and growth. To address this question, we used RNAi-mediated silencing of SPTLC1 to block its accumulation and then measured accumulation of virus

gene products in productively infected cells. First, primary human foreskin fibroblasts (HFF) grown in 6-well plates were transfected with silencing (si)RNA oligonucleotides specific for SPTLC1 and subsequently fed with serum free DMEM. As a control, cells were transfected with control siRNA generated against a random sequence. After 48 hrs of serum starvation, cells were exposed to 0.5 PFU per cell of AD169 and then harvested at 24, 48 and 72 h after infection. Protein lysates were separated by SDS-PAGE, transferred to nitrocellulose and reacted with antibodies specific for SPT1 and SphK1 as well as the HCMV immediate early protein 1 (IE1), the early protein UL44, the late protein pp150 and GAPDH as a loading control. As shown in Fig. 3.1A, the knockdown of SPT with siRNA resulted in a moderate decrease in the accumulation of SPT at all time points, while having no effect on the accumulation of SphK1. (compare lanes 1&2, 3&4 and 5&6 in the SPT and SphK1 rows). The decrease in SPT accumulation was quantified by densitometry using the LabWorks program (UVP, Inc.) and normalized to corresponding GAPDH levels. These numerical values corresponding to the levels of SPT were then displayed in arbitrary units in Fig. 3.1B. This figure shows a moderate decrease in SPT accumulation in cells treated with siRNA to SPTLC1 at 24 hours post infection, while at 48 and 72 hours post infection this decrease was less pronounced. Contrary to our hypothesis, the siRNA knockdown of SPT resulted in a considerable increase in the accumulation of the immediate early gene product IE1 at 24, 48 and 72 hours post infection (Fig. 3.1A, compare lanes 1,3 & 5 to 2,4 & 6 in the IE1 row). As expected, we could not detect UL44 or pp150 until 48 and 72 hours post infection, and at these times, there was no change in the levels of accumulation for either of these gene products (lanes 3&4 and 5&6 in the UL44 and PP150 rows). These results indicate that

there may be some part of *de novo* sphingolipid synthesis, most likely one of the intermediate sphingolipids, which inhibits the accumulation of the IE1 viral gene product.

We next wanted to understand what effect knocking down SPT would have on the levels of infectious virus progeny. In order to examine this, we first transfected HFFs with siRNA oligonucleotides specific for SPTLC1 or control siRNA generated against a random sequence. After transfection, the cells were serum starved for 48 hours by feeding with serum free DMEM and then exposed to 0.5 PFU per cell of AD169. Infectious virus, both cell associated and within media, was harvested at 0 and 80 hours post infection for viral growth curve analysis. At 80 hours post infection, there was over a 3.5 fold decrease in the number of infectious virus progeny from cells transfected with SPTLC1 siRNA when compared to cells transfected with control siRNA (Fig. 3.1C). Specifically, there was an average of approximately 4900 plaque forming units per mL counted from cells transfected with control siRNA, whereas there was an average of 1300 plaque forming units per mL counted from cells transfected with SPTLC1 siRNA. These findings indicate that the induction of *de novo* sphingolipid synthesis by HCMV, which was suggested by data in our previous investigation, functions to produce higher levels of infectious progeny virus despite having a product which may inhibit IE1 protein accumulation.

Exogenous addition of dhSph and dhS1P to HCMV infected cells affects viral protein accumulation.

Data from our previous studies indicate that HCMV infection elevates SphK1 activity, which is required for sustained accumulation of IE1 and that dhS1P signaling

may be responsible for this. SphK1 is primarily responsible for the phosporylation of dhSph to form dhS1P (Berdyshev, Gorshkova et al. 2006). dhSph is an intermediate of the *de novo* sphingolipid synthesis pathway, and the results mentioned above imply that an intermediate such as this may suppress the levels of IE1 accumulation. Furthermore, our last study showed that enzymes involved in the degradation of dhS1P are upregulated at later times during infection, implying that dhS1P may hinder some aspect of HCMV replication at these times. Naturally, we wanted to better understand the influence that dhSph and dhS1P could have on viral protein accumulation at both early and late times of infection. We hypothesized that treatment of cells with dhSph at any time during infection would result in decreased accumulation of IE1. Concerning dhS1P, our hypothesis was that treatment at early times during infection would result in increased accumulation of IE1, whereas treatment with this sphingolipid at late times would result in a decrease in the accumulation of one or more viral gene products. In order to evaluate these sphingolipids in this manner, we first serum starved HFF cells grown in 6-well plates for 48 hours by feeding with serum free DMEM. Then cells were exposed to 0.5 PFU per cell of AD169, and at 0 hours post infection or 24 hours post infection, the cells were treated with either nothing, 3μM of dhSph or 500nM of dhS1P all within serum free DMEM containing 0.4% fatty acid free bovine serum albumen. These cells were then all harvested at 48 hours post infection in order to measure the accumulation of viral gene products IE1, UL44 and pp150. The film image of these viral proteins as well as GAPDH as a loading control can be seen in fig. 3.2A. The bands from the viral proteins in this image were then quantified by densitometry using LabWorks (UVP, Inc.) and normalized to corresponding GAPDH levels. These numerical values corresponding to

the levels of each viral protein were then displayed in arbitrary units in fig. 3.2B. As seen in fig. 3.2B, cells treated with dhSph at 0 hours post infection (0h DHSph) appeared to have slightly lower levels of accumulation of the viral proteins IE1, UL44 and pp150 when compared to cells left untreated at this time point (0h No Treatment). There was a more pronounced decrease in the accumulation of these viral proteins in cells treated with dhSph at 24 hours post infection (24h DHSph). In contrast to the effects of dhSph at 0 hours post infection, cells treated with dhS1P at 0 hours post infection (0h DHS1P) displayed a marked increase in the accumulation of IE1 and a slight increase in levels of UL44 and pp150 when compared to untreated cells (0h No Treatment). Interestingly, cells treated with dhS1P at 24 hours post infection showed a noticeable decrease in the accumulation of IE1 and a slight decrease in levels of pp150 when compared to cells left untreated at 24 hours post infection (24h No Treatment). Altogether, these results indicate that increased levels of dhS1P at early times of infection can promote HCMV protein accumulation, while increased levels dhS1P at later times of infection or increased levels of dhSph at any time during infection can hinder viral gene product accumulation.

Pretreatment of cells with dhS1P and S1P prior to infection with HCMV reduces accumulation of viral gene products.

An important scenario to consider when evaluating the importance of SphK1 activity to HCMV infection would be one in which S1P or dhS1P (either of which SphK1 can produce) was released from infected cells and elicited their effects on neighboring cells prior to infection. We sought to simulate this scenario through pretreatment of cells

with S1P or dhS1P prior to infection with HCMV. In order to accomplish this, we fed HFF cells with serum free DMEM to serum starve for 48 hours. 24 hours into serum starvation, we re-fed cells with 1μM of either dhS1P or S1P in serum free DMEM containing 0.4% fatty acid free bovine serum albumen. As a control, cells were fed with only serum free DMEM containing 0.4% fatty acid free bovine serum albumen. After 24 hours, when cells had been serum starved for 48 hours and pretreated with sphingolipids for 24 hours, we exposed cells to 0.5 PFU per cell of AD169. Cells were then harvested for measuring the accumulation of the HCMV gene products IE1, UL44 and pp150 at 24, 48 and 72 hours post infection. As shown in fig. 3.3, at 24 hours post infection, the pretreatment of cells with dhS1P or S1P, lanes 2 and 3 respectively, appears to have resulted in a slight increase in IE1 gene product accumulation. However, this increase was not seen at 48 (lanes 5 & 6) or 72 hours post infection (lanes 8 & 9). The accumulation of the UL44 gene product could not be detected until 48 hours post infection, while the pp150 gene product was only detected at 72 hours post infection. Control treated cells had barely detectable levels of UL44 at 48 hours post infection (lane 4), while cells pretreated with dhS1P or S1P had no detectable levels (lanes 5 & 6). At 72 hours post infection, there was a marked decrease in the accumulation of both the UL44 and pp150 gene products within cells pretreated with dhS1P or S1P (lanes 8 & 9) when compared to control treated cells (lane 7). These data suggest that elevated extracellular levels dhS1P or S1P, prior to infection, can inhibit the accumulation of HCMV early and late gene products.

Drug inhibition of SphK activity results in reduced HCMV growth.

SphK is responsible for the phosphorylation of dhSph to dhS1P, and this enzyme's activity and expression are upregulated upon infection with HCMV. We have now shown that exogenous addition of dhSph and dhS1P, immediately after infection, have opposite effects on viral protein accumulation. A central question arising from these observations is whether HCMV-mediated induction of SphK activity impacts virus growth. To address this question, we examined virus growth in serum starved quiescent cells to establish a more biologically relevant quiescent state, used a drug inhibitor of SphK to block its activity and then measured virus growth using a standard plaque assay. In the first experiment, primary human foreskin fibroblasts (HFF) were serum starved for 48 hrs and then exposed to 5 PFU per cell of AD169 for 48 h. Immediately after infection, fibroblasts were exposed to drug vehicle only (DMSO) or to 1 μg/mL of the SphK inhibitor, 2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole. As shown in Fig. 3.4A, SphK activity was still elevated by HCMV infection of serum starved quiescent cells, where the time in culture and cell density was equal. Furthermore, the drug inhibitor reduced SphK activity in infected cells by nearly 3 fold. Next, to establish whether the treatment with the drug inhibitor would affect cell growth, we serum starved HFF cells for 48 hrs and then exposed them to 5 PFU per cell of AD169 for 48 hrs. Immediately after infection, cells were treated with DMSO or 1μg/mL of the SphK inhibitor in triplicate. Cells were then harvested for counting at 24, 48 and 72 hrs post infection. As shown in Fig. 3.4B, there are no substantial differences between the number of cells in DMSO and SphK inhibitor treated cells at any time point, showing that the SphK inhibitor does not affect the viability of cells in these conditions. Finally, to

measure virus growth, HFF cells were plated at a density of $2x10^5$ cells per well in 6-well plates and then serum starved for 48 hrs. These cells were then exposed to 5 PFU per cell of AD169, and immediately after infection were treated with DMSO or 1μg/mL of the SphK inhibitor in triplicate. Infectious virus was harvested from cells and media at 0, 24, 48, 72 and 96 hrs for viral growth curve analysis. As shown in Fig. 3.4C, we observed a profound 94.7 fold decrease in the production of infectious progeny virus at 48 hrs post infection in cells treated with the SphK inhibitor. A continued decrease was seen at 72 hrs and 96 hrs post infection of 4.6 fold and 1.7 fold, respectively. From these results, we conclude that SphK is used by HCMV to favor increased levels of dhS1P over dhSph in order to promote viral gene expression and possibly growth at early stages of infection.

DISCUSSION

In the past, studies have shown that sphingolipids mediate various cellular signaling pathways (Ipatova, Torkhovskaya et al. 2006), but very little is known about what role these lipids may have within cells infected with HCMV. Our results from a previous study revealed that HCMV infection upregulates SphK1 activity and expression, which promotes the accumulation of the IE1 gene product. This study also suggested that *de novo* sphingolipid biosynthesis is increased during early phases of HCMV infection, whereas an increase in the levels of mRNA transcripts for degradative enzymes correlated with a decrease in the levels of several sphingolipids at later phases. The

results within the present study further resolve the temporal importance of sphingolipids to HCMV protein accumulation and growth.

Data from our previous investigation showed a significant increase in dihydrosphingolipids and ceramides, which indicated that *de novo* pathway of sphingolipid synthesis was stimulated upon infection. RNAi mediated knockdown of a gene responsible for the primary step in *de novo* sphingolipid synthesis, SPTLC1, prior to HCMV infection resulted in decreased production of infectious virus progeny. These results suggest that HCMV requires increased levels of newly synthesized sphingolipids beyond S1P and dhS1P to maintain optimal levels of virus growth or infectivity of virus progeny. A study involving human immunodeficiency virus (HIV-1) supports the latter of these possibilities. In this study, it was shown that inhibition of sphingolipid synthesis had no effect on the number of viruses released, but resulted in the production of viruses that were substantially less infectious (Brügger, Glass et al. 2006). Thus, it is possible that, in order to maintain optimal levels of infectivity, HCMV requires a specific sphingolipid composition for its envelope which it obtains from the production of sphingolipids from the *de novo* pathway.

The results from the SPTLC1 siRNA experiment also showed that cells transfected with siRNA to SPTLC1 prior to infection with HCMV had higher levels of IE1 protein accumulation when compared to cells transfected with control siRNA. This indicates that there may be a sphingolipid within this pathway that inhibits the accumulation of IE1. Supporting this idea is our data showing that when dhSph (an intermediate of *de novo* sphingolipid biosynthesis and substrate of SphK) is added to the media during HCMV infection, there is a decrease in the accumulation of IE1 as well as

UL44 and pp150. Conversely, our findings show that exogenous addition of dhS1P (a product of SphK) immediately following infection, increases the accumulation of the viral gene product IE1. This helps to verify a role for SphK1 activity in supporting the accumulation of IE1 protein levels through the production of dhS1P. These results imply that the elevation of SphK1 activity by HCMV infection has a dual purpose. One is to favor an increase in levels of dhS1P at early times to sustain accumulation of IE1, presumably though the activation of signaling pathways coupled to S1P receptors. The other purpose is to simultaneously decrease the levels of dhSph, which would naturally increase due to the stimulation of *de novo* sphingolipid synthesis, in order to prevent the inhibition of viral protein accumulation.

Illustration of the need for HCMV to temporally regulate sphingolipids lies in our results showing that exogenous addition of dhS1P at 24 hours post infection has a negative effect on the accumulation of the IE1 and, to a lesser extent, the pp150 gene products. This is the opposite of what was seen when dhS1P was exogenously added at 0 hours post infection. This data lends significance to the results of our prior study, indicating that enzymes responsible for the degradation of sphingolipids are upregulated at later times of infection, which correspond to a decrease in the levels of Sph, dhSph, S1P and dhS1P. It seems that HCMV only benefits from dhS1P at early times of infection and must decrease the amount of this sphingolipid within cells at later times of infection in order to prevent its negative effects on viral gene expression. This may indicate that HCMV utilizes different strategies involving sphingolipids, for promoting the accumulation of IE1. dhS1P signaling at early times of infection may stimulate signaling cascades needed to support the accumulation of IE1 by means of promoting its

transcription. At later times of infection, however, HCMV may promote IE1 accumulation by preventing its degradation, and the signaling cascades stimulated by dhS1P may counteract this.

Further evidence revealing the purpose behind the temporal regulation of sphingolipids by HCMV is shown in our experiment demonstrating that pretreatment with dhS1P or S1P prior to infection results in reduced accumulation of early and late gene products. This experiment simulates an environment in which dhS1P or S1P is released from infected cells and affects neighboring cells prior to their infection. The effects of these sphingolipids observed in this experiment were identical, and so we presume that they occur through activation of S1P receptors, as both dhS1P and S1P can function in this way (Van Brocklyn, Lee et al. 1998; Tamama, Kon et al. 2001). Consequently, the release of either dhS1P or S1P from infected cells could activate S1P receptors on neighboring cells, which would then prevent the accumulation of early and late gene products in cells subsequently infected with the virus. This provides another rationale for the results from our previous study showing a decrease in levels of dhS1P and S1P at later times of infection. The degradation of these lipids could help to prevent their paracrine actions and allow for optimal viral gene expression within neighboring cells which have yet to be infected.

Our past study showed that HCMV infection resulted in an increase in SphK activity at times ranging from 12 to 72 hours post infection. The biological relevance of SphK1 was also illustrated in this previous study, showing that cells transfected with siRNA prior to infection with HCMV had decreased levels of IE1 gene product accumulation. However, when virus growth was examined in cells subjected to these

same conditions, there was no observed effect. In this context, only transcriptionally induced upregulation of SphK1 expression is suppressed, while basal levels of this enzyme are left to function normally. Therefore, we sought to investigate whether inhibition of SphK activity using the inhibitor 2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole, would have an effect on virus growth. In this study, we demonstrated that treatment of cells with this SphK inhibitor, immediately after infection with HCMV, resulted in a substantial decrease in levels of infectious virus progeny at 48 hours post infection. However, these studies do not exclude the possibility that the inhibition of HCMV growth is due to a nonspecific effect of the SphK inhibitor. Yet, this may indicate that HCMV induced stimulation of SphK activity plays a role in promoting virus growth. It should be noted that this scenario is fairly speculative and would require further studies to support this possibility.

In conclusion, this study sheds light on why HCMV dynamically alters the levels of sphingolipids during infection. This is shown through evidence which demonstrates the impact of *de novo* sphingolipid synthesis as well as dhSph and dhS1P on HCMV protein accumulation and growth. Specifically, the control of the dhSph/dhS1P axis by SphK1 and the degradation of dhS1P at later times of infection both appear to play a major role in the regulation of viral protein accumulation. The evidence provided within these studies could help to create a unique means by which to combat HCMV infection.

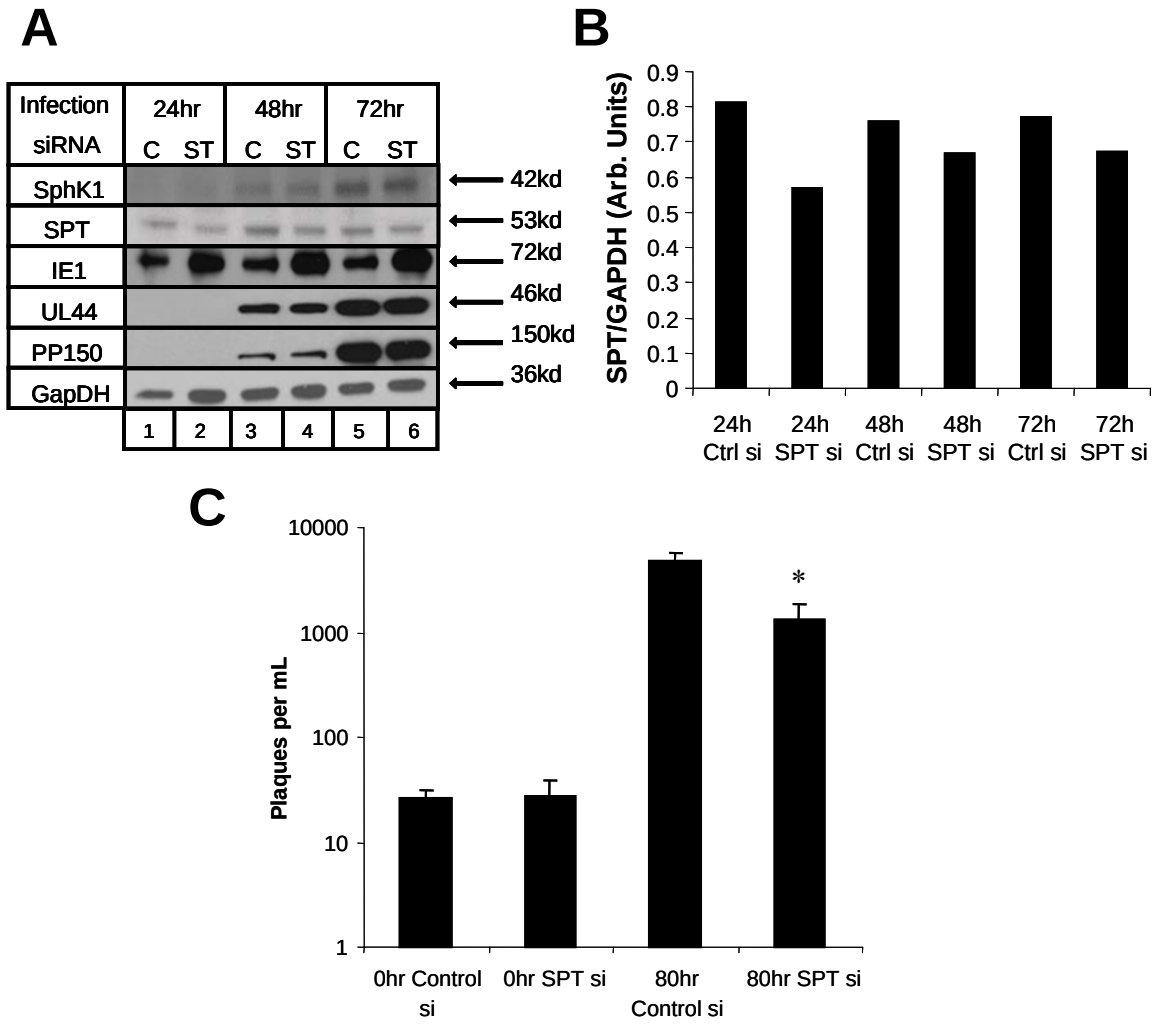

Figure 3.1. HCMV protein accumulation and growth within cells transfected with siRNA specific for SPTLC1. A) HFF cells were transfected with control siRNA (C) or SPTCL1 siRNA (ST) and subsequently serum starved for 48 hrs. Then cells were infected with the AD169 strain of HCMV at 0.5 PFU per cell for 24, 48 or 72 hrs prior to harvesting cell lysates. Immunoblotting was performed as described in Materials and Methods using antibodies to SphK1, SPT1 and viral proteins. Blots were also reacted to antibodies to cellular GAPDH proteins to control for loading of lysates. B) The bands corresponding SPT in the film image (A) were quantified by densitometry using the LabWorks program (version 4.0.0.8 from UVP, Inc.), normalized to corresponding GAPDH levels and displayed in arbitrary units. C) HFF cells were transfected with control siRNA or SPTLC1 siRNA and then serum starved for 48 hrs. Cells were then exposed to 0.5 PFU/cell of AD169, and at 0 or 80 hours post infection, they were harvested, sonicated and infectious virus was measured by plaque assay in MRC-5 cells. MRC-5 cells were then overlaid with B-media containing 0.5% agarose and incubated for 7 days. Afterwards, cells were fixed with 10% neutral buffered formalin and then stained with 0.1% crystal violet. The data are means ± standard deviations of three independent determinations of the number of counted plaques per mL within each experimental group. An asterisk indicates a P-value of less than 0.005.

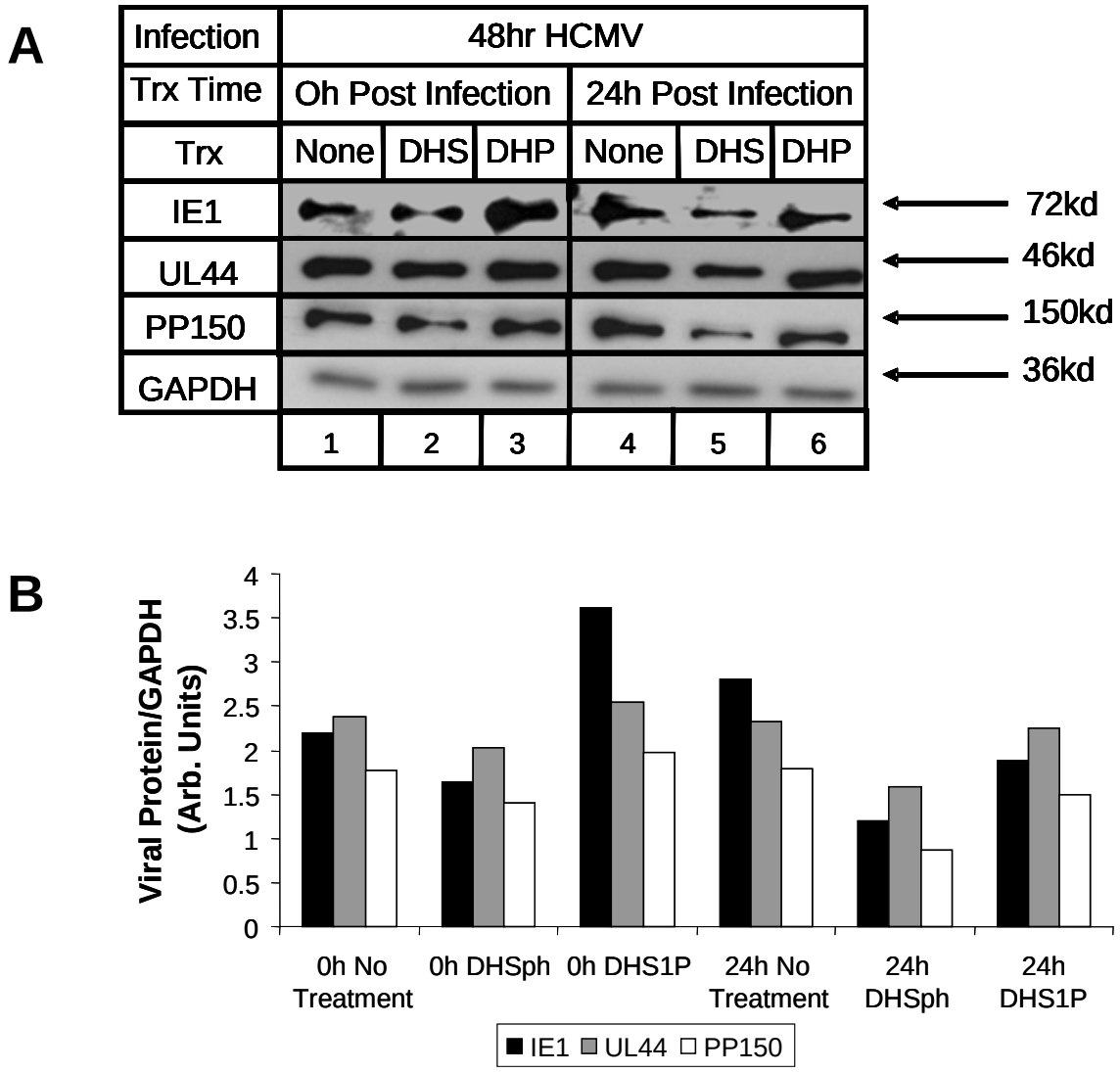

Figure 3.2. HCMV gene product accumulation within cells treated with dhSph or dhS1P during infection. HFF cells were serum starved for 48 hours and then exposed to 0.5 PFU per cell of AD169. At 0 or 24 hours post infection, the infected cells were treated with nothing, 3μM of dhSph or 500nM of dhS1P all within serum free DMEM containing 0.4% fatty acid free bovine serum albumen. Then all cells were harvested at 48 hours post infection in order to measure the accumulation of viral gene products. The bands corresponding to the viral proteins in the film image (A) were quantified by densitometry using the LabWorks program (version 4.0.0.8 from UVP, Inc.) and normalized to corresponding GAPDH levels. B) The values of the normalized levels of each viral protein were then displayed in arbitrary units. Black bars represent levels of IE1, while the grey and white bars represent levels of UL44 and pp150, respectively.

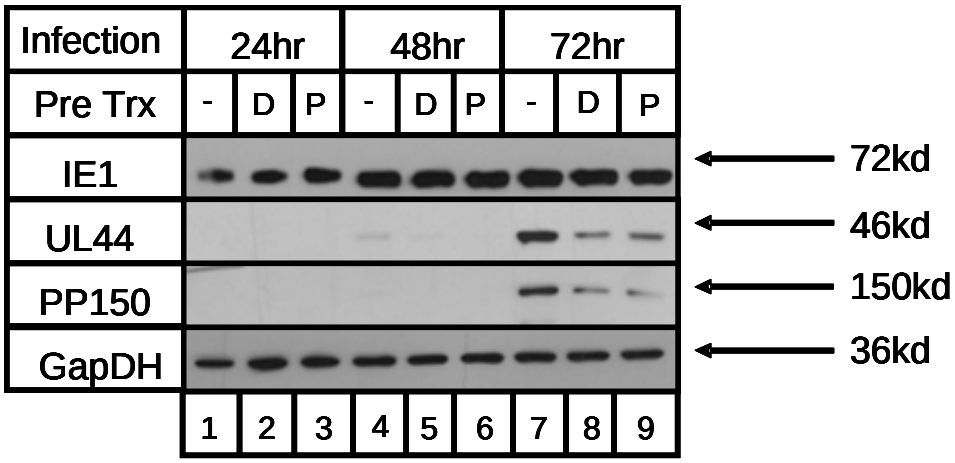

Figure 3.3. The accumulation of viral gene products in cells pretreated with dhS1P or S1P prior to HCMV infection. HFF cells were serum starved for a total of 48 hours prior to infection. 24 hours prior to infection, cells were pretreated with either nothing, 1μM of dhS1P or 1μM S1P all within serum free DMEM containing 0.4% fatty acid free bovine serum albumen. Cells were then exposed 0.5 PFU per cell of AD169 for 24, 48 or 72 hrs prior to harvesting cell lysates. Immunoblotting was performed as described in Materials and Methods using antibodies to viral proteins and GAPDH as a loading control.

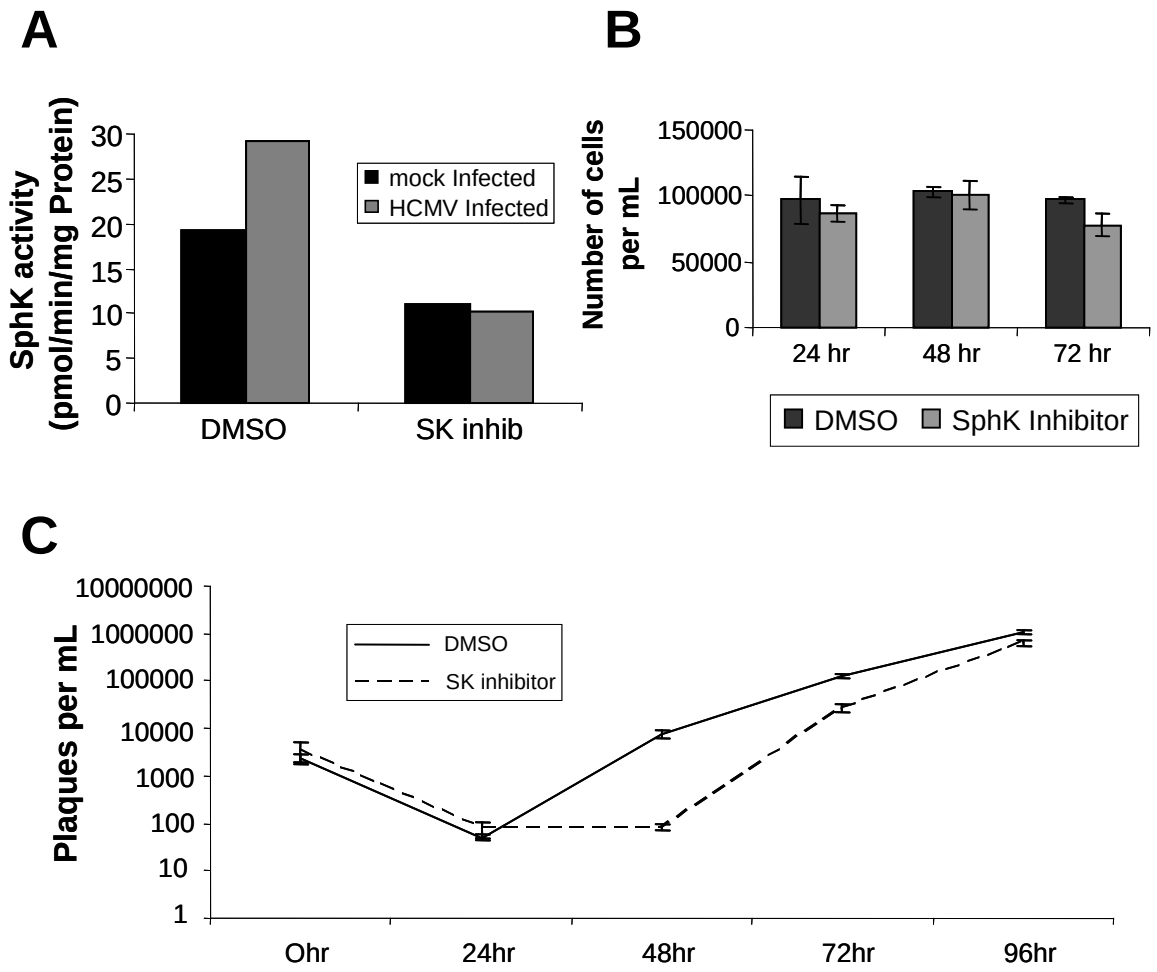

Figure 3.4. Virus growth in serum starved HFF cells treated with the SphK inhibitor (2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole). A) HFF cells were serum starved for 48 hrs and then left uninfected (black bars) or exposed to 5 PFU/cell of the AD169 strain of HCMV for 48 hrs (grey bars). Immediately afterward, cells were either treated with DMSO (vehicle control) or 1μg/ml of SphK inhibitor. Cells lysates were then analyzed for SphK activity as described in Materials and Methods. Radioactive spots were scraped from TLC plates and quantitated by scintillation counting. B) HFF cells were serum starved for 48 hrs and then exposed to 5 PFU/cell of the AD169 strain of HCMV. Immediately following infection (time 0), cells were treated with 1μg/ml of SphK inhibitor or DMSO. Cells were harvested for counting at intervals of 24 hrs ranging from 24 to 72 hrs. The data are means ± standard deviations of three independent determinations. C) HFF cells were serum starved for 48 hrs and then exposed to 5 PFU/cell of AD169. Immediately following infection (time 0), cells were treated with 1μg/ml of SphK inhibitor or DMSO. Cells were later harvested at intervals of 24 hrs ranging from 0 to 96 hrs, then sonicated, and infectious virus was measured by plaque assay in MRC-5 cells. MRC-5 cells were then overlaid with B-media containing 0.5% agarose and incubated for 7 days. Afterwards cells were fixed with 10% neutral buffered formalin and then stained with 0.1% crystal violet. The data are means ± standard deviations of three independent determinations of the number of counted plaques per mL within each experimental group.

CHAPTER 4

FINAL DISCUSSION AND FUTURE STUDIES

The studies within this dissertation have examined the ability of HCMV to alter sphingolipid metabolism in order to promote viral protein accumulation and growth. These investigations have also looked at the effects of several sphingolipids on viral protein accumulation. Our results have led us to the following conclusions, from which we developed a schematic shown in Fig. 4.1. This figure illustrates the means by which HCMV temporally regulates enzymes controlling sphinglipid metabolism in order to control the levels of sphingolipids. In the first 24 hours of infection (left panel of Fig. 4.1), HCMV appears to stimulate the *de novo* sphingolipid synthesis pathway resulting in higher levels of dhSph, ceramides and dihydroceramides. At this time, HCMV also stimulates SphK1 activity and expression which converts dhSph to dhS1P, resulting in higher levels of dhS1P. This action of SphK1 seems to function to both favor levels of dhS1P, which can enhance HCMV IE1 protein accumulation, and prevent an increase in the levels of dhSph, which can suppress HCMV IE1 protein accumulation. However, the mechanism behind the dhS1P and dhSph mediated effects on HCMV gene product accumulation are not understood. We speculate that dhS1P functions by binding to S1P receptors in order to stimulate signaling pathways, which then influence the accumulation

of viral proteins; however, there could be a novel intracellular function of dhS1P which serves this function. The right panel of Fig. 4.1 illustrates the HCMV induced regulation of sphingolipid metabolic enzymes observed between 24 and 48 hours post infection. At this time SphK1 activity and the accumulation of mRNA transcripts coding for enzymes involved in the degradation of dhS1P are elevated. Because there is a decrease in the levels of dhS1P and S1P as well as dhSph and Sph observed at this time, it appears that SPL has the more prominent effect over that of SPP2; otherwise one would expect to see higher levels of dhSph and Sph. This allows for the reduction of both dhSph and dhS1P levels, which in this time frame both suppress the accumulation of viral gene products. The degradation of dhS1P also prevents high levels of this sphingolipid from being released from the infected cell and having a negative effect on early and late gene product accumulation in cells subsequently infected with HCMV.

The importance of sphingolipids to herpesvirus growth and protein expression has been illustrated in a few studies (Steinhart, Busch et al. 1984; Allan-Yorke, Record et al. 1998), but our understanding of how sphingolipids are regulated during infection and the mechanisms behind their actions are not well established. Within these studies, we provide evidence which indicates that increased *de novo* sphingolipid synthesis, during HCMV infection, is needed to sustain optimal levels of infectious virus progeny. Recent studies involving other viruses have also demonstrated a similar need for *de novo* sphingolipid synthesis. A study involving HIV-1 showed that inhibition of sphingolipid synthesis resulted in reduced infectivity of virus progeny (Brügger, Glass et al. 2006). Furthermore, suppression of *de novo* synthesis of sphingolipids during infection can inhibit hepatitis C virus and influenza virus replication (Hidari, Suzuki et al. 2006;

Umehara, Sudoh et al. 2006). These studies begin to reveal the possibility that viruses may have a universal need for sphingolipids during their replication.

Sphingolipids could be of particular importance to HCMV not only in terms of promoting viral protein accumulation and growth as discussed above, but also by their influence on immune cells. Activation of the $S1P_1$ receptor, has been shown to affect trafficking of several different immune cells, including macrophages and hematopoietic progenitor cells (Seitz, Boehmler et al. 2005; Singer, Tian et al. 2005). HCMV has been shown to infect these cell types, which have been proposed to facilitate dissemination and latency of the virus (Ibanez, Schrier et al. 1991; Maciejewski, Bruening et al. 1992). dhS1P is a $S1P_1$ agonist, and thus may elicit the same chemotactic effects on these immune cells (Tamama, Kon et al. 2001). This would suggest that dhS1P released from HCMV infected cells could attract macrophages and hematopoietic progenitor cells to the site of infection by stimulating $S1P_1$ receptors on their surface. The virus could then infect these cells in order to spread through the host and establish subsequent sites of infection. Our results regarding the decreased levels of dhS1P and S1P at later times of infection appear to contradict this. However, the $S1P_1$ receptor's effects on the chemotaxis of immune cells are, in many cases, concentration dependent, such that low concentrations of S1P (100nM) stimulate this effect whereas high concentrations (1μM) do not (Goetzl and Graler 2004; Rosen and Goetzl 2005). This would explain how HCMV could maintain the ability to attract immune cells through paracrine signaling of dhS1P by reducing the levels of this sphingolipid at later times of infection. The need for this virus to reduce the levels of dhS1P at these times is given further support by our data showing that pretreatment of cells with 1μM concentrations of dhS1P prior to infection

results in decreased accumulation of early and late gene products. This would imply that HCMV may not be able to obtain optimal levels of viral protein accumulation in cells attracted to the site of infection, and later infected with the virus, if the extracellular environment contains a high concentration of dhS1P.

Another means by which HCMV could facilitate its dissemination is through upregulating the expression of SPP2. We have shown that HFF cells exhibit a considerable increase in the levels of SPP2 mRNA transcripts 48 hours after infection with HCMV. A recent study has proposed a pro-inflammatory role for SPP2 since it facilitates the induction of the chemoattractants interleukin 1β and interleukin 8 by tumor necrosis factor alpha (Mechtcheriakova, Wlachos et al. 2006). Taken together, these results suggest that HCMV could augment the inflammatory response through activation of SPP2 at the transcriptional level in order to attract and then infect immune cells.

Finally, this dissertation has illustrated that sphingolipids are regulated by HCMV and promote this virus's protein accumulation and growth. Studies involving other viruses have begun to establish a similar need for sphingolipids in their replication. It appears as though HCMV may benefit from the many signaling capabilities of sphingolipids by controlling their metabolism. These signaling events could even attract immune cells to sites of infection, which could aid the virus's spread within its host. Therapies targeting sphinolipids in order to suppress HCMV protein accumulation, growth and possibly pathogenesis could arise from advancing our understanding of the importance of sphingolipids to this virus.

Future Studies

The results within this dissertation have illustrated the novel finding that HCMV modulates sphingolipid metabolism in order to promote viral gene product accumulation and growth. These findings suggest further investigations in order to better define the mechanism behind this alteration of sphingolipid metabolism and the presumed actions of the sphingolipids that have increased levels during infection.

One such study would involve the use of siRNA-mediated knockdown of different S1P receptors within fibroblasts infected with HCMV. This, along with the stimulation of the remaining receptors by exogenous addition of dhS1P and western blotting to detect viral proteins, could isolate which receptor(s) may be responsible for supporting the accumulation of the IE1 gene product. However, it would be very interesting if knocking out any or all of the S1P receptors had no effect. If the exogenous addition of dhS1P immediately following HCMV infection resulted in an increase in the accumulation of the IE1 gene product regardless of which S1P receptors were knocked out, it would suggest that either an intracellular activity of dhS1P or an unknown receptor for dhS1P is responsible for this effect. Both of these possibilities would be exciting avenues to explore.

It would also be interesting to better resolve the importance of the activation SphK activity to HCMV growth. In order to accomplish this, HFF cells overexpressing a dominant negative mutant form of SphK could be infected with HCMV and analyzed for the production of infectious virus progeny. A dominant negative SphK has already been developed and has been shown block the activation of SphK activity without any effects on basal SphK activity (Pitson, Moretti et al. 2000). The use of this dominant negative

SphK would specifically prevent only the increased SphK activity induced by HCMV infection and would elucidate the importance of this induction to HCMV growth without the nonspecific effects associated with drug inhibitors of SphK.

The kinetics of the upregulation of SphK activity and expression induced by HCMV suggest that an immediate early or early gene(s) may be responsible for this. Because of their ability to manipulate transcriptional activity through interaction with other transcription factors and binding to DNA sequences, the immediate early proteins IE1 and IE2 would be good candidates to examine for this activity (Castillo and Kowalik 2002). Transfecting HFF cells with these genes, either individually or in combination, and subjecting the cells to a SphK assay could ascertain if these gene products are responsible for the HCMV induced increase in SphK activity. Furthermore, RNA can be extracted from duplicate samples of this experiment to analyze if the gene products may be responsible for the increase in accumulation of mRNA transcripts for SphK1.

It would also be interesting to see if a similar pattern of sphingolipid modulation could be observed in other herpes viruses, namely HSV. This could be accomplished by subjecting cells infected with this virus to the same experiments shown in this dissertation used to examine HCMV-infected cells. For example, test mock-infected and HSV-infected fibroblasts for differences in the levels of mRNA transcripts for various enzymes involved in sphingolipid metabolism using real time quantitative PCR. Also, use mass spectrometry to analyze any potential differences between the levels of various sphingolipids within HSV-infected fibroblast and the levels in mock-infected cells. This would allow for a profile of how sphingolipids could be altered within HSV infected cells, and furthermore could be compared to what is shown within this dissertation in

order to ascertain whether HSV alters sphingolipid metabolism in a similar way. If this were true, it would suggest that the alteration of sphinolipid metabolism is a general means by which herpesviruses promote their replication.

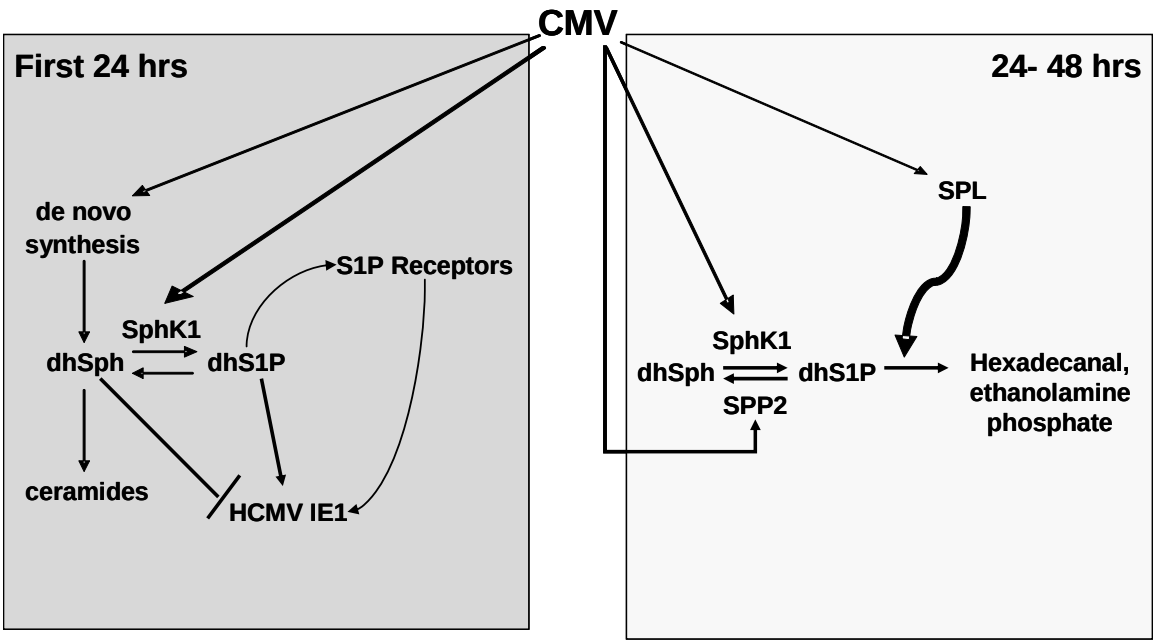

Figure 4.1. Diagram depicting the effects of HCMV infection on sphingolipid metabolism as well as the effects of sphingolipids on IE1 gene product accumulation.

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