이학박사학위논문

인간 거대세포 바이러스 (Human Cytomegalovirus) 감염이 숙주 스트레스반응 시스템에 미치는 영향

Effects of Human Cytomegalovirus Infection on Host Stress Response System

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ABSTRACT

Activation of cellular stress responses (CSRs) is necessary for maintaining the integrity of macromolecules against various stresses. It is well-known that viral infection induces stresses to host cell and the host system responds differentially to viral stresses, depending on the type of virus. In this thesis research, interaction between human cytomegalovirus (HCMV or HHV-5) and the stress response systems of host cell was investigated.

Nrf2 (NF-E2-related factor 2) is one of the key transcription factors that regulate the expression of various genes involved in the control of oxidative stress. When the Nrf2 signaling pathway is activated, the expression level of Nrf2 is up-regulated. Increased Nrf2 translocates to the nucleus and regulates the expression of downstream target genes that encode heme oxygenase-1 (HO-1) and glutamate-cysteine ligase catalytic subunit (GCLC). In this thesis, it was demonstrated that HCMV infection up-regulates the level of Nrf2, HO-1, and GCLC via a mechanism that involves viral gene expression and casein kinase 2 (CK2). Host cells infected by HCMV had higher survival rates following oxidative stress induced by buthionine sulfoximine (BSO), which is an inhibitor of GCLC, than the uninfected control cells. This cell-protective effect was abolished by the use of Nrf2-specific shRNA. These data suggest that Nrf2 activation may be necessary to protect host cells from oxidative stress.

It had previously been reported that HCMV infection activates mTOR (mammalian target of rapamycin) pathway even under stressed conditions, producing

positive effects on viral DNA replication. However, the underlying mechanism has been unclear. It was revealed in this thesis that the downstream target of mTOR that can support viral replication was C/EBPα (CCAAT/enhancer binding protein α). It appears that virus-mediated activation of mTOR enhances HCMV genome replication by up-regulating translation process of C/EBPα. Indeed, when the function of C/EBPα was inhibited by overexpressing CHOP (C/EBP homologous protein), viral DNA replication and late gene expression were suppressed, lowering the level of viral production.

Viral infection results in the activation of host immune and inflammatory responses, generating a significant level of stress in the host. NF-κB (nuclear factor-κB) is one of the major regulators in this process. There are two separate pathways for NF-κB activation, canonical and non-canonical. It had been reported that glycogen synthase kinase 3β (GSK3β) is involved in the regulation of both pathways. It was demonstrated that in this thesis that HCMV infection activates GSK3β. When the HCMV-mediated activation of GSK3β was suppressed by using GSK3β-specific shRNA, canonical NF-κB was still induced by HCMV infection. However, it abolished the HCMV-mediated induction of the expressions of non-canonical NF-κB subunit p52 and B-cell activating factor (BAFF), a downstream target gene of p52-RelB. These data suggest that virally activated GSK3β may be involved in the induction of non-canonical NF-κB signaling. The HCMV-mediated activation of non-canonical NF-κB signaling may explain the pathologic features associated with HCMV such as polyclonal B-cell activation.

Through this thesis work, novel ways of the interaction between HCMV and host cell were demonstrated. Virus could increase the level of Nrf2 and C/EBPα to make the host survive better and viral genome to replicate more efficiently. It was also revealed that HCMV infection activated the non-canonical NF-κB pathway, which could have serious

consequences on the status and progression of diseases. Together, these data may contribute to clarifying the mechanism behind the pathogenic effects of HCMV, especially under the stressed conditions, such as chronic inflammation.

Keywords

Cellular stress response (CSR) / HCMV / Nrf2 / Cell survival / mTOR / C/EBPα / Viral DNA replication/ GSK3β / non-canonical NF-κB/ Chronic inflammation

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ABBREVIATIONS

ATF6	activating transcription factor 6
ATM	ataxia-telangiectasia mutated
ATR	ATM and Rad3-related
BAFF	B-cell activating factor
BSO	buthionine sulfoximine
C/EBPα	CCAAT/enhancer binding protein α
CHOP	C/EBP homologous protein
CHX	cycloheximide
CK2	casein kinase 2
CSR	cellular stress responses
DDR	DNA damage response
ER	endoplasmic reticulum
eIF2α	eukaryotic initiation factor 2α
GCLC	glutamate-cysteine ligase catalytic subunit
GCV	ganciclovir
GSH	glutathione
GSK3β	glycogen synthase kinase 3β
HCMV	human cytomegalovirus
HBV	hepatitis B virus

HCV	hepatitis C virus
HFFs	primary human foreskin fibroblasts
HIV	human immunodeficiency virus
HO-1	heme oxygenase – 1
HSF1	heat shock factor 1
HSR	heat shock response
HSV	herpes simplex virus
IFN	Inteferon
IκBα	inhibitor of κB α
IKK	IκB kinase
IR	ionizing radiation
IRE1	inositol-requiring enzyme 1
ISG54	interferon stimulated gene 54
Keap1	kelch-like ECH-associated protein 1
Luc	Luciferase
LY	LY294002
MRN	MRE11–RAD50–NBS1
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor-κB, nuclear factor κ-light-chain-enhancer of activated B cells
NIK	NF-κB inducing kinase
Nrf2	NF-E2 related factor 2
oriLyt	lytic origin of DNA replication
p70S6K	p70S6 kinase
PERK	PKR-like endoplasmic reticulum kinase

PI3K	phosphatidylinositol 3-kinase
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
shRNA	small hairpin RNA
SV40	simian virus 40
TAg	large T antigen
TBB	4,5,6,7-tetrabromobenzotriazole
tBHQ	tert-butyl hydroxyquinone
TERT	telomerase catalytic subunit
TSC1/2	tuberous sclerosis protein 1/2
Wort	Wortmannin
UPR	unfolded protein response
UV	ultraviolet

CHAPTER I

Introduction

1. Viral infection and the cellular stress responses

At the cellular level, ‘stress’ generally refers to an agent that can cause damage to macromolecules, including proteins, nucleic acids, and carbohydrates (Kultz, 2005). Cells have several adaptive responses against stresses, collectively known as the cellular stress responses (CSR), which serve to reduce stress-induced damage and maintain cellular homeostasis (Fulda *et al.*, 2010). In response to a severe stress that exceeds a specific threshold, some stress responses can induce cell death pathways (Table. I-1) (Fulda *et al.*, 2010).

Viral infection induces stress in the host cell. Indeed, activation of the CSR is observed in virus-infected cells. However, viral infection has several features that distinguishe it from other stress inducers, suggesting that activation of the CSR is the result of complex virus–host interactions, rather than a simple adaptive response to virus-induced stress.

1.1 Cellular stress responses (CSR)

Several stimuli induce macromolecular damage; these include mild temperature change (heat shock), accumulation of unfolded proteins in the ER (ER stress), exposure to ionizing (IR) or ultraviolet (UV) radiation, and elevation of intracellular ROS levels (oxidative stress). For example, heat shock and ER stress can lead to the misfolding of proteins, which can severely impair cellular functions. In addition, IR and UV induce DNA damage, and various macromolecules are susceptible to damage induced by oxidative stress (Fulda *et al.*, 2010; Kultz, 2005).

To survive under these conditions, cells activate the CSR. The heat shock response (HSR) and unfolded protein response (UPR) are activated in response to heat shock and ER stress, respectively. The induction of heat shock proteins is transcriptionally regulated by the action of heat shock factor 1 (HSF1), which is activated by accumulation of unfolded proteins in the cytosol. The UPR is activated by the recognition of unfolded proteins in the secretory pathway, especially in the ER, and is composed of three distinct pathways mediated by PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). Upon activation, the HSR and UPR regulate folding capacity, translation initiation, and the ubiquitin–proteasome pathway. This pathway plays an important role in the process called the ER-associated degradation (ERAD) by which ER-localized proteins are targeted for degradation. DNA damage caused by IR or UV exposure induces the DNA damage response (DDR), which is mediated by two structurally related kinases, ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR). The consequences of DDR activation are cell-cycle arrest, the induction of DNA-repair mechanisms, and apoptosis (Fulda *et al.*, 2010).

The antioxidative stress response protects against oxidative stress mediated by the redox-sensitive transcription factor NF-E2-related factor 2 (Nrf2). When activated, Nrf2 induces the expression of antioxidant enzymes, such as the catalytic subunit of glutamylcysteine ligase (GCLC) and heme oxygenase-1 (HO-1), which counteract the pathological effects of oxidative stress (Niture *et al.*, 2010). In addition, one common cellular response to several types of stress is activation of the nuclear factor κB (NF-κB). ROS induces NF-κB activation through the classical IκB kinase (IKK) complex (Gloire *et al.*, 2006). UV and IR also activate this transcription factor through separate mechanisms mediated by activation of the casein kinase 2 (CK2) and ATM, respectively (Miyamoto,

2011; Piva *et al.*, 2006). NF-κB activation is involved in the survival of stressed cells and the induction of inflammatory responses (Piva *et al.*, 2006).

Dysregulation of the CSR can contribute to the development of disease (Fulda *et al.*, 2010). For example, knockout mice lacking PERK, one of the main regulators of the UPR, suffer from diabetes mellitus due to the loss of pancreatic β-cells (Harding *et al.*, 2001). UPR activation is especially important in protecting these cells because they secrete large amounts of insulin and are therefore more sensitive to ER stress (Fulda *et al.*, 2010). In addition, neuronal death caused by oxidative stress plays a major role in the pathogenesis of neurodegenerative diseases, such as Parkinson's disease (PD) (Uttara *et al.*, 2009). Indeed, Nrf2-knockout mice exhibit a more severe phenotype in the MPTP-induced model of PD, suggesting that Nrf2 is a therapeutic target of PD (Chen *et al.*, 2009). In addition, Nrf2 activation protects against myocardial infarction: fumarate, a metabolite that induces Nrf2 activation, exerts cardioprotective effects in a mouse model (Ashrafian *et al.*, 2012).

The CSR may also be associated with the tumor progression. A tumor microenvironment characterized by low oxygen and nutrient supply induces the UPR in tumor cells, although the role of this induction is not yet clear (Fulda *et al.*, 2010). The Nrf2-mediated response plays contradictory roles in tumor development, with the specific effects most likely depending on tumor stage (Lau *et al.*, 2008). Nrf2 activation promotes the survival of tumor cells by inducing chemoresistance. On the other hand, Nrf2 also suppresses tumor development by reducing levels ROS, a well-known mutagen. The latter effect seems to be especially important during the early stages of tumor development. Some heat shock proteins have been reported to aid in tumor development, and the DDR is also involved in this process (Fulda *et al.*, 2010).

	Inducer	Key regulators	Downstream effects	Relationship with diseases
Heat shock response (HSR)	Mild temperature change	HSF1	Induction of heat shock proteins	Neurodegenerative diseases
Antioxidative stress response	Increase in intracellular ROS	Nrf2	Induction of antioxidant and detoxifying enzymes	Neurodegenerative diseases, myocardial infarction, cancer
Unfolded protein response (UPR)	Accumulation of unfolded protein in the ER	PERK, IRE1, and ATF6	Induction of ER chaperone, Translation attenuation, ERAD	Diabetes mellitus
DNA-damage response (DDR)	DNA damage	ATM and ATR	Cell cycle arrest, DNA-repair, apoptosis	Cancer

Table I-1. Examples of the cellular stress responses (CSR). Some examples of the CSR are shown. The HSR is activated by a variety of stresses in addition to temperature change, however, other responses such as antioxidative stress response, the UPR, and the DDR are known to be activated in response to a certain type of stress. Key regulators of the CSR are also shown. Downstream effects of CSR activation are known to reduce stress-induced damage and maintain cellular homeostasis. The dysregulation of the CSR is often associated with human diseases such as neurodegenerative diseases, cancer, and metabolic diseases (from Fulda *et al.*, 2010).

1.2 The interaction between viral infection and the CSR

Activation of the CSR by viral infection

Viral infection induces stress in the host cell. For instance, virus infection can induce oxidative stress in a variety of ways. HIV gp120 and influenza A virus cause oxidative stress by the action of cellular oxygenases such as cytochrome P450 and Nox2-containing NADPH oxidase, respectively (Shah *et al.*, 2013; Vlahos *et al.*, 2011). HCV NS5A protein induces oxidative stress by the disturbance of ER calcium homeostasis, resulting in the activation of calcuim signaling (Gong *et al.*, 2001). RSV infection indirectly induces the level of ROS through down-regulating the expression of antioxidant enzymes (Hosakote *et al.*, 2009). Decreased activity of antioxidant enzymes is also observed in children suffering from HIV infection (Reshi *et al.*, 2014). These data suggest that cellular factors, well-known to be involved in the regulation of ROS levels, may play major roles in inducing oxidative stress during viral infection although the exact component involved in each viral infection seems to vary depending on the type of virus.

Furthermore, viral infection induces the ER stress due to massive production of virally encoded secretory proteins (He, 2006). Viral infection causes damage to cellular DNA, and the genomes of some DNA viruses may be recognized as DNA-damaging agents. For instance, infection with E4 mutant adenovirus leads to the recruitment of cellular DNA repair proteins to the exposed viral DNA end (Boyer *et al.*, 1999; Stracker *et al.*, 2002). However, in most viral infections, it is not clear how viral structure can play a role as a DNA-damaging agent (Weitzman *et al.*, 2010).

The CSR is activated in virus-infected cells (Fig. I-1). In some cases, the cellular stress imposed by viral infection plays an important role in CSR activation; however, the

CSR is often directly activated by the effects of viral gene products. The activation of NF-κB and the HSR is well-known to be induced by several virus infections (Kim & Oglesbee, 2012; Santoro *et al.*, 2003). The activation of Nrf2 is reported during infections by hepatitis B virus (HBV) or HCV (Burdette *et al.*, 2010; Schaedler *et al.*, 2010). The UPR is activated by several viruses, including RNA viruses, such as simian virus 5, and DNA viruses, such as herpes simplex virus 1 (HSV-1) and HCMV (He, 2006). The DDR is also activated following infection with some DNA viruses, such as simian virus 40 (SV40), adenovirus, and HCMV, as evidenced by the activation of ATM and phosphorylation of histone H2AX (Turnell & Grand, 2012).

Possible roles of the CSR during viral infection

CSR activation plays several roles during viral infection (Table I-2). For example, the activation of NF-κB by oxidative stress imposed by HIV infection induces the expression of viral genes, cytokines, and chemokines. Treatment with an antioxidant, such as NAC, reduces the expression levels of those genes (Schwarz, 1996). The HSR is involved in infections by viruses such as adenovirus, HIV, and vaccinia virus. Suppression of HSF1, the major transcriptional regulator of the HSR, or treatment with inhibitors that target individual heat shock proteins suppresses viral infection by reducing the level of viral gene expression (Filone *et al.*, 2014; Glotzer *et al.*, 2000; Kumar & Mitra, 2005).

In general, viral infection induces oxidative stress in host cells with different consequences depending on its severity. Low level of oxidative stress can activate cellular signaling pathways, exemplified by the activation of NF-κB and STAT3 via viral infection (Gong *et al.*, 2001; Speir *et al.*, 1996). Virus-induced ROS often plays important roles during the early stage of infection through facilitating viral entry and regulating anti-viral

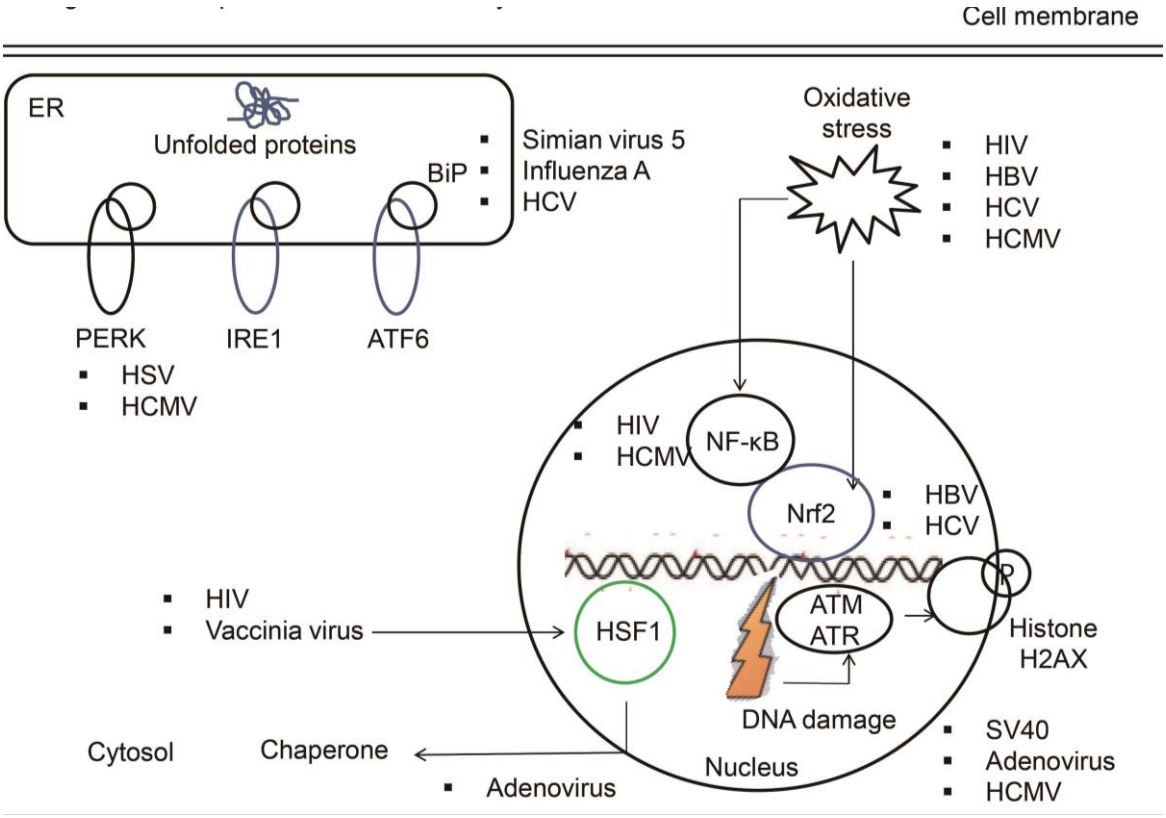


Figure I-1. CSR activation by viral infection. Viral infection induces stress in the host cell. Indeed, activation of the CSR is observed in virus-infected cells. Oxidative stress induced by viral infections including HIV and HBV causes activation of NF-κB and Nrf2. The ER stress induced by several viruses leads to the up-regulation of expressions of ER-resident chaperones including BiP and the activation of PERK. Viral infection is well-known to activate the HSR. Infection with DNA viruses activates DDR of the host cell as evidenced by the activation of ATM, which phosphorylates histone H2AX.

signaling downstream of pattern recognition receptors (Bottero *et al.*, 2013; Gonzalez-Dosal *et al.*, 2011).

However, in some cases, infections by HIV, HSV, HBV, and HCV lead to oxidative damage in host cells, which can limit viral replication. (Joyce *et al.*, 2009; Liu *et al.*, 2008; Valyi-Nagy & Dermody, 2005). Activation of the Nrf2 pathway by HBV and HCV protects infected cells from oxidative insults and thus Nrf2 activation may contribute to the development of chronic viral infection (Burdette *et al.*, 2010; Schaedler *et al.*, 2010). On the other hand, a protective effect of Nrf2 signaling has been observed in Nrf2-knockout mice. When infected with respiratory syncytial virus (RSV) or influenza virus, which causes oxidative stress and tissue damage in the respiratory tract, mice lacking Nrf2 exhibit more severe pathologic features (Cho *et al.*, 2009; Yageta *et al.*, 2011). Thus, although it is not yet known whether viruses use Nrf2 signaling for their own benefit, it is clear that oxidative stress imposed by viral infection activates Nrf2 and promotes the survival of host cells.

Multiple components of the UPR and the DDR play important role(s) during viral infection (He, 2006). GRP78/BiP, which is induced by virus-induced ER stress, binds to the HN glycoprotein of simian virus 5 and facilitates proper folding of this protein (Ng *et al.*, 1989). GRP78/BiP is also involved in the folding of the glycoproteins of vesicular stomatitis virus (VSV), influenza virus, and HCV (Choukhi *et al.*, 1998; Hurtley *et al.*, 1989; Machamer *et al.*, 1990). SV40 infection or the expression of large T antigen (TAg) activates ATM, which in turn phosphorylates serine 120 residue of TAg. Mutation of this serine to alanine or knockdown of ATM expression impairs viral replication (Shi *et al.*, 2005), suggesting that proteins involved in the UPR and the DDR can be used by viruses to promote their own replication.

	Identified Roles
Heat shock response	Regulation of viral gene expression
Antioxidative stress response	Protection of host cells against oxidative stress induced directly or indirectly by viral infection
Unfolded protein response	Proper folding of the viral proteins (mainly glycoproteins) Often inhibited by viral infection
DNA-damage response	Enhancement of Viral replication Often inhibited by viral infection

Table I-2. Roles of the CSR during viral infection. CSR activation plays several roles during viral infection. It is well-reported that the HSR is beneficial to the virus through regulating the level of viral gene expression. The activation of the antioxidative stress response reduces oxidative damage induced by the direct effect of viral infection and/or indirect consequence of the host inflammatory response. It is not yet clear that this protective effect of Nrf2 is ultimately an advantage to the virus or not. The interaction between viral infection and the UPR or the DDR is more complex. For example, the virus utilizes a component of these pathways to increase the folding of the viral protein and the synthesis of the viral genome. While, viral infection inhibits some downstream effects of UPR and DDR activation probably because they are harmful to the virus.

Modulation of the CSR by viral infection

In the context of stress responses, viral infection has several features distinct from other stress inducers. For examples, viral infection can directly activate the CSR without inducing cellular stress by exploiting the regulatory mechanisms of the CSR. Furthermore, only a subset of the downstream effects of CSR activation are activated in virus-infected cells. Although the exact role of this modulation of the CSR is not fully understood, several examples suggest that viruses may inhibit the potential harmful effects of CSR activation for their own benefit (Fig. I-2).

The VP24 protein of Marburg virus, which belongs to the Filoviridae family, activates the Nrf2 pathway (Edwards *et al.*, 2014). VP24 directly interacts with Keap1, which binds and inhibits Nrf2 under unstressed conditions. This Keap1–Nrf2 interaction leads to ubiquitination and degradation of Nrf2, thereby lowering the basal level of Nrf2 expression in unstressed cells. However, Nrf2 inducers such as oxidative stress disrupt this interaction and subsequently induce expression and transcriptional activation of Nrf2 (Niture *et al.*, 2010). Through its interaction with Keap1, VP24 inhibits the Keap1–Nrf2 interaction and thereby activates the Nrf2 pathway independently of oxidative stress (Edwards *et al.*, 2014). The study that revealed this interaction was the first report to demonstrate that a viral protein can induce Nrf2 activation directly, rather than through the induction of oxidative stress and/or cellular signaling pathways normally involved in the upstream regulation of Nrf2 (Fig. I-2, left panel).

Because viruses depend completely on the synthetic machinery of the host cell, several viral infections are associated with the induction of the UPR. However, some effects of UPR activation (such as attenuation of translation initiation) would be

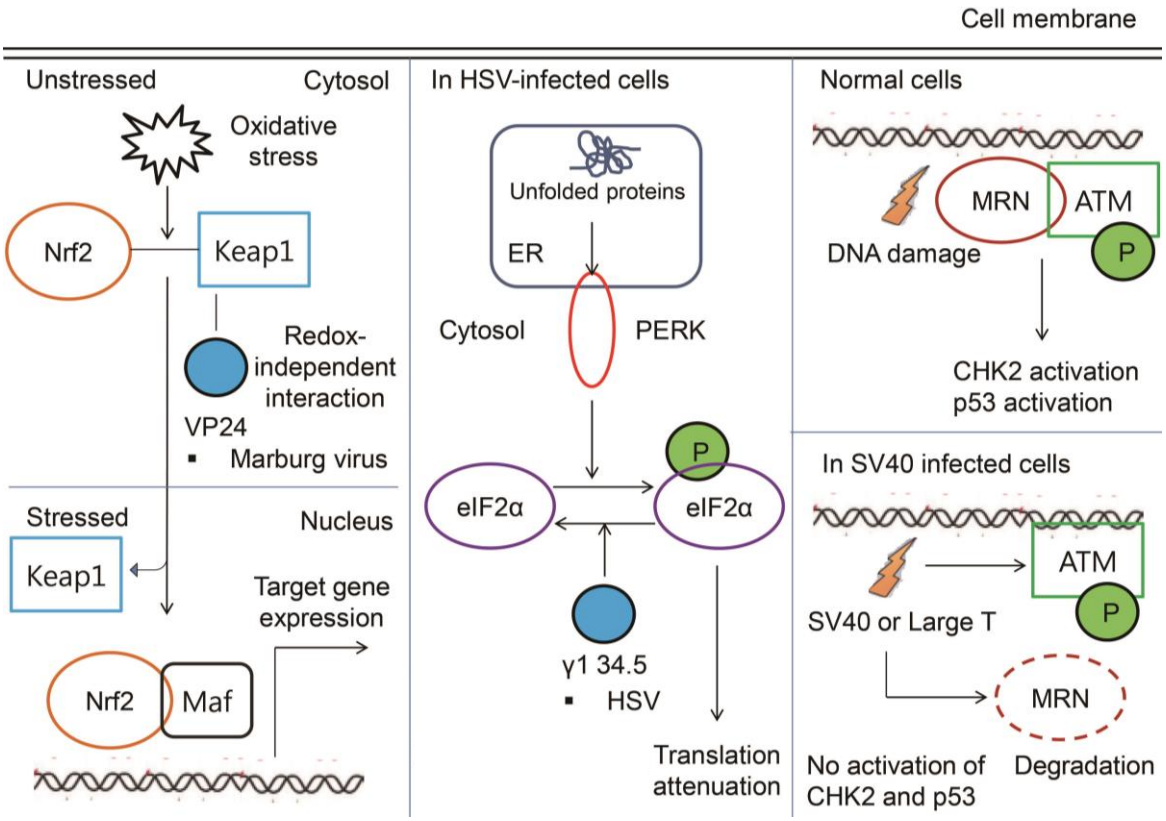


Figure I-2. Viral modulation of the CSR. Examples of CSR modulation by viral infection are shown. The VP24 protein of Marburg virus activates the Nrf2 signaling pathway through the direct interaction with Keap1, a negative regulator of this pathway. Therefore, oxidative stress may not be involved in the VP24-mediated activation of Nrf2. HSV activates PERK, while it prevents the phosphorylation of eIF2α, a downstream event of PERK activation. This is because the γ1 34.5 protein of HSV dephopsphorylates eIF2α. Because the phosphorylation of eIF2α can lead to attenuation of protein translation, the inhibition of eIF2α phosphorylation may be beneficial to the virus. The inhibition of some downstream events of CSR activation is also observed in HSV-infected cells, in which the MRN complex is degraded by the action of SV40 large T-antigen.

disadvantageous for the virus, suggesting that viruses need to modulate the UPR modulation during infection. One example is provided by HSV-1, a member of the Herpesviridae family. HSV-1 infection induces ER stress and activates PERK (Cheng *et al.*, 2005), resulting in phosphorylation of eukaryotic initiation factor 2α (eIF2α), which inhibits translation initiation. However, the viral protein γ1 34.5 dephosphorylates eIF2α by activating a cellular phosphatase (He *et al.*, 1997). As a result, the overall phosphorylation level of eIF2α is not elevated, and translation initiation is not inhibited, in HSV-1 infected cells (Cheng *et al.*, 2005). Other viruses have specific mechanisms for targeting and inhibiting the PERK-eIF2α axis (Fig. I-2, middle panel) (He, 2006).

Viral infection also inhibits some of the outcomes of DDR activation. For example, SV40 infection induces two markers of DDR activation, ATM activation and H2AX phosphorylation (Shi *et al.*, 2005). However, SV40 virus infection or the expression of TAg leads to degradation of the MRE11–RAD50–NBS1 (MRN) complex (Zhao *et al.*, 2008). During the initial stage of the DDR, the MRN complex detects DNA double-strand breaks (DSBs); therefore, the function of the MRN complex is important for activation of downstream effectors such as checkpoint kinase 2 (CHK2) and p53 (Sulli *et al.*, 2012). As a result of MRN degradation, progression of the DDR is inhibited in SV40-infected cells, benefiting the virus by contributing to uncontrolled replication of viral DNA (Fig. I-2, right panel) (Wu *et al.*, 2004b).

2. Human cytomegalovirus

Human cytomegalovirus (HCMV, also known as HHV-5), a member of the β-herpesvirus family, has a linear double-stranded DNA genome approximately 230 kb in

length. The virion consists of the nucleocapsid, composed of the genome and the capsid proteins, surrounded by an amorphous layer called the tegument and the lipid bilayer envelope (Fig. I-3). HCMV transmission can occur via contact with any body fluid. Primary infection is controlled by the immune system; thereafter, the virus establishes a life-long latency with periodic reactivation. In immunologically compromised hosts, HCMV infection can cause serious diseases (Gandhi & Khanna, 2004).

Life cycle

Receptor-mediated entry of HCMV into target cells occurs via a two-stage process. First, the virion attaches to the plasma membrane of the host cell through an interaction between the viral gB and gM proteins and heparin sulfate proteoglycans (HSPGs) on the cell surface (Kari & Gehrz, 1992; 1993) (Compton *et al.*, 1993). Next, at least in some cases, endocytosis of the virion is mediated via binding of gB and gM to cellular integrins (Feire *et al.*, 2004) (Wang & Shenk, 2005). It is not yet clear whether HCMV entry is mediated by an essential cellular receptor; some data suggest that the mechanism of HCMV entry may vary depending on target cell type (Isaacson *et al.*, 2008).

After initial entry, molecules that reside in the tegument layer are released to the cytoplasm. This layer mostly consists of viral proteins, but also contains some cellular proteins and viral and cellular RNAs (Varnum *et al.*, 2004) (Varani & Landini, 2011). One major component of the tegument is the pp65 protein, encoded by the UL83 gene of HCMV (Irmiere & Gibson, 1983). pp65 is not required for viral replication (Schmolke *et al.*, 1995), but pp65-deficient mutant viruses induce much greater interferon (IFN) and pro-inflammatory responses of the host cell (Browne & Shenk, 2003), indicating that this

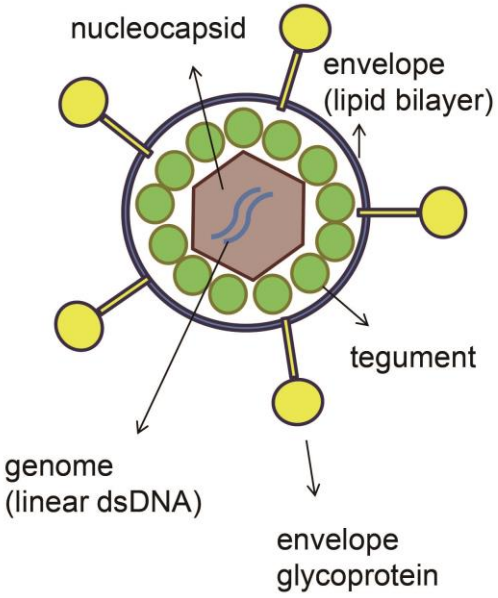


Figure I-3. The structure of the HCMV virion. The structure of the HCMV virion is simplified to include four distinct components: the envelope, the tegument, the nucleocapsid, and the genome of linear double-stranded DNA.

protein is involved in viral mechanisms of evasion of the primary immune defense system. Other tegument proteins are involved in processes such as the delivery of the viral genome to the nucleus or the initiation of viral gene expression (Kalejta, 2008).

During the lytic infection stage, most viral genes are expressed in three different temporal classes: immediate-early (IE), delayed-early (E), and late (L) (Sinclair & Sissons, 2006). The functions of the IE genes IE72 and IE86 include induction of the expression of viral and cellular genes, disruption of PML-associated nuclear bodies, and modulation of the cell cycle (Ishov *et al.*, 1997) (Ahn *et al.*, 1998) (Stinski & Petrik, 2008). Collectively, these functions make the cellular environment more favorable to viral replication (Stinski & Petrik, 2008). Many of the E genes encode proteins required for viral DNA replication, e.g., the viral DNA polymerase. The components of the HCMV virion, such as the capsid proteins and membrane glycoproteins, are expressed with late kinetics (Chambers *et al.*, 1999). The onset of viral DNA replication is required for expression of the L genes. In cultured fibroblasts, replication of the HCMV initiates approximately 24 h post-infection, starting from a cis-acting element of the viral genome called the lytic origin of DNA replication (Pari, 2008). The viral DNA is encapsidated in the nucleus, and then exported to the cytosol, where it acquires tegument proteins and the envelope to form the mature virion (Gibson, 2008).

Pathogenesis of acute and chronic diseases

HCMV infection can cause acute and chronic diseases in immunologically compromised individuals. Acute HCMV disease develops in infants, transplant recipients treated with potent immune-suppressive drugs, and AIDS patients. Uncontrolled HCMV

replication, frequently observed in such cases, leads to the lysis of host cells and end-organ damage mediated by both direct consequences of viral infection and the attack of the immune system against HCMV. Consistent with this, antiviral agents that block HCMV genome replication are effective in limiting the severity of symptoms associated with acute HCMV infection (Britt, 2008).

Chronic HCMV infection in transplant recipients can contribute to graft rejection, which is manifested by chronic inflammation (Fig. I-4) (Britt, 2008). HCMV infection can trigger the activation of the immune system, which can then attack a transplanted organ, e.g., by stimulating the production of autoantibodies (Fig. I-4, boxes 1 and 2) (Britt, 2008) (Varani & Landini, 2011). HCMV infection is a polyclonal B-cell activator *in vitro* (Fig. I-4, box 2) (Hutt-Fletcher et al., 1983). Viral infection of plasmacytoid DCs can also induce expression of IFN-α (Fig. I-4, box 1) (Varani *et al.*, 2007), which subsequently activates B-cells. In addition, HCMV infection can indirectly contribute to graft rejection by amplifying the inflammatory response of the host (Fig. I-4, box 3) (Britt, 2008) (Varani & Landini, 2011). Consistent with these *in vivo* observations, HCMV infection induces expression of inflammatory mediators *in vitro* and systemic inflammatory responses *in vivo* (Zhu et al., 2002) (van de Berg *et al.*, 2010). Because the activation of NF-κB by inflammatory mediators can re-activate latently infected HCMV (Kline *et al.*, 1998), an auto-stimulatory loop may exist between HCMV infection and the host inflammation, further increasing the pro-inflammatory capacity of this virus (Varani & Landini, 2011).

The possible contribution of HCMV to the development of cancer has been debated for many years. Recently, HCMV infection has been accepted as an onco-modulatory agent that can accelerate cancer progression and/or promote malignant phenotypes, at least in the case of glioma (Dziurzynski *et al.*, 2012). HCMV DNA is

present in many glioma samples (Cobbs *et al.*, 2002), and HCMV modulates cellular signaling pathways involved in the progression of glioma. For example, HCMV infection inhibits the functions of tumor suppressor genes and apoptosis, and also induces entry into S-phase, genome instability, and angiogenesis (Dziurzynski *et al.*, 2012). In a genetically modified mouse that spontaneously develops glioma due to a mutation in a tumor-suppressor gene, infection with MCMV leads to a more aggressive phenotype and a reduction in mean survival time (Price *et al.*, 2013). Together, these data suggest that HCMV promotes cancer.

Interactions between HCMV and CSR

HCMV infection induces the activation of NF-κB, in part by inducing cellular stresses such as oxidative stress (Speir *et al.*, 1996). In addition, the UL76 protein of HCMV induces NF-κB activation through the ATM pathway, one of the key components of the DDR (Costa *et al.*, 2013). Activated NF-κB induces the expression of many viral genes and inflammatory cytokines (Speir *et al.*, 1996) (Costa *et al.*, 2013) (Taylor & Bresnahan, 2006). Interestingly, HCMV infection can also inhibit NF-κB. For example, the IE86 protein of HCMV inhibits virus- and TNF-α–induced activation of NF-κB, and thereby suppresses expression of IL-6 and IL-8 (Taylor & Bresnahan, 2006). These data suggest that the effects of HCMV infection on the NF-κB pathway are quite complex.

HCMV infection disrupts the tuberous sclerosis protein (TSC1/2)-mediated stress response, which inhibits translation initiation under stressed conditions (Fig. I-5, box 1) (Moorman *et al.*, 2008). TSC1/2 inhibits translation initiation when levels of amino acids, ATP, and oxygen are low by suppressing the activity of the mammalian target of

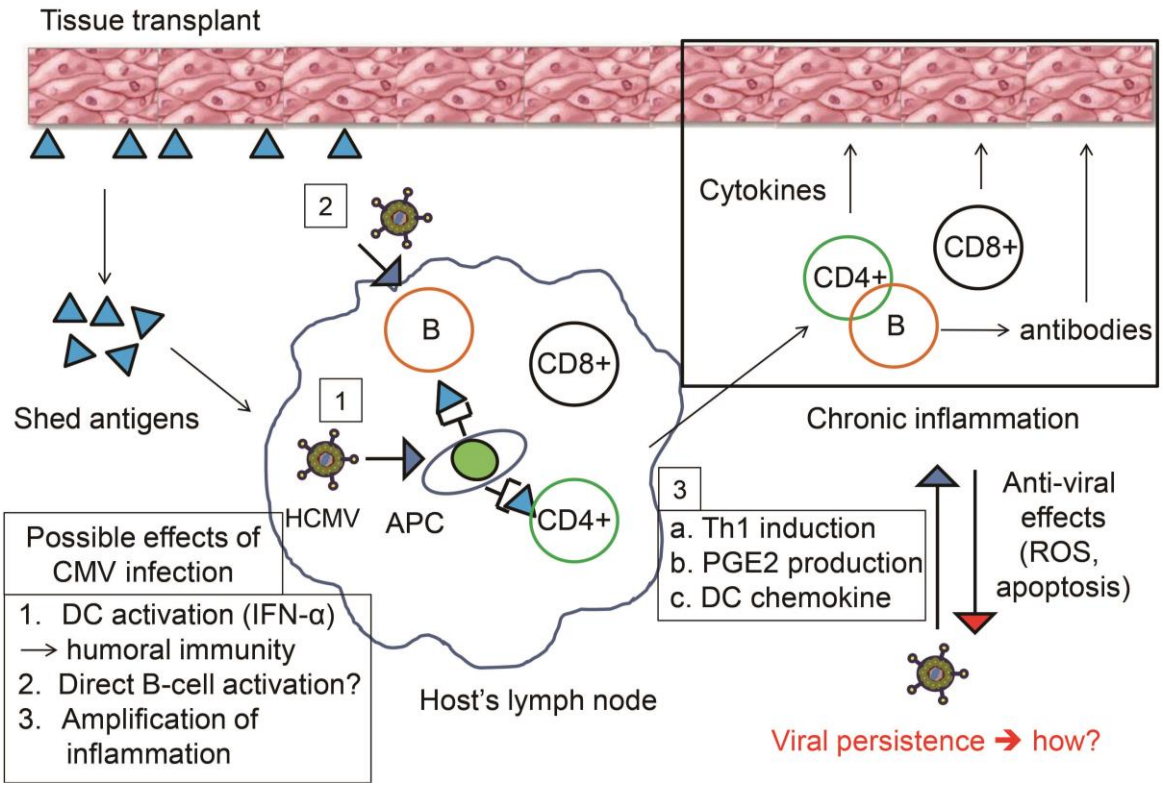


Figure I-4. Possible mechanisms of the effects of HCMV on the pathogenesis of chronic graft rejection. HCMV infection can be a risk factor for chronic graft rejection through triggering the activation of the immune system against the host such as the production of anto-antibodies (boxes 1 and 2). In addition, HCMV infection can indirectly contribute to graft rejection by amplifying the inflammatory response (box 3). Through these mechanisms, persistence of HCMV infection in the inflamed tissue of patients may have pathogenic effects. However, it is not well-known how HCMV can not only survive but also thrive under inflammatory condition which imposes severe stress to the host cell, producing anti-viral effects (Reproduced from Manzoor *et al.* 2008).

rapamycin (mTOR), one of the main regulators of translation initiation (Laplante & Sabatini, 2009). In HCMV-infected cells, however, the UL38 protein of HCMV binds and antagonizes the function of TSC1/2; consequently, mTOR is not inhibited under stressed conditions (Moorman *et al.*, 2008). HCMV infection also protects mTOR from oxidative stress, which suppresses the activity of mTOR in a TSC1/2-independent manner (Fig. I-5, box 2) (Tilton *et al.*, 2011). Because mTOR is required for many steps of HCMV infection, such as viral DNA replication (Moorman & Shenk, 2010), the inhibition of these stress responses and maintenance of mTOR activation may be beneficial to the virus, especially under stressed conditions (Fig. I-5).

HCMV infection also regulates the UPR, as evidenced by the activation of PERK; however, the other pathways of the UPR, mediated by IRE1 and ATF6, are not activated in HCMV-infected cells (Isler *et al.*, 2005). Furthermore, as noted above for HSV-1, the activation of PERK by HCMV infection does not induce phosphorylation of eIF2α or the attenuation of translation initiation (Isler *et al.*, 2005) (Xuan *et al.*, 2009). It is interesting to note that PERK promotes lipid synthesis in the host cell by activating sterol regulatory element-binding protein-1c (SREBP-1c), which is required for efficient production of the lipid bilayer envelope of the virion (Yu *et al.*, 2013). These data suggest that HCMV infection modulates the UPR and utilizes at least one component of this pathway to increase the production of viral progeny.

HCMV infection also interacts with the DDR of the host cell, causing both activation of ATM and phosphorylation of H2AX. ATM and phosphorylated H2AX are localized in the viral replication compartments, the sub-nuclear structure in which the viral genome is replicated (E *et al.*, 2011). However, some DDR proteins are not recruited to this compartment, suggesting that the localization of DDR proteins is differentially

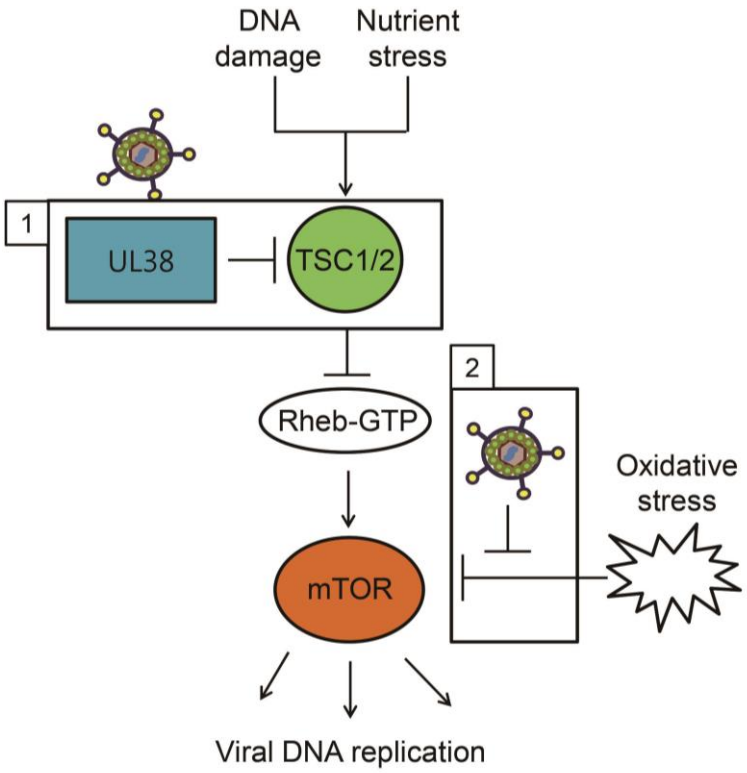


Figure I-5. Interaction between HCMV and mTOR. mTOR is the major regulator of protein translation in eukaryotic cells. In the context of HCMV infection, the activity mTOR is involved in the regulation of multiple steps of HCMV infection including viral DNA replication. In uninfected cells, the activity of mTOR is inhibited by stresses such as the DNA damage and nutrient deprivation by the action of TSC1/2. Also, in response to oxidative stress, mTOR activity is suppressed in a TSC1/2-indepenedent mechanism. HCMV infection disrupts both of these stress responses to constitutively activate mTOR even under stressed conditions (boxes 1 and 2). This modulation may be beneficial to the virus, e.g. to establish persistent infection in the stress environment such as chronic inflammation (Reproduced from Moorman *et al.* 2008).

regulated by HCMV infection (Luo *et al.*, 2007). It is possible that normal DDR processes are interrupted by HCMV infection, as indicated by data showing that HCMV-infected cells are more susceptible to IR-induced DNA damage (Gaspar & Shenk, 2006). Although one study showed that ATM is not required for HCMV replication (Luo *et al.*, 2007), another study reported that ATM activation promotes viral genome replication (E *et al.*, 2011). ATM is also involved in the activation of NF-κB by the UL76 protein of HCMV (Costa *et al.*, 2013). Taken together, these observations indicate that at least one mediator of the DDR plays an important role during HCMV infection.

3. Purpose of this study

Different viruses seem to have different ways in affecting the stress response system, and indeed the consequences of viral infection vary among different viruses. For example, stress responses of the host cell to viral infection can be beneficial or harmful to the virus itself.

HCMV is a ubiquitous herpesvirus known to induce stresses of the host cell, through the direct effects of viral infection and/or the indirect consequences of host immune responses. HCMV infection has been reported to adopt several different ways of regulating the CSR for its own benefit. However, the interaction between HCMV infection and the CSR was unclear at the time of starting this thesis research, especially in the context of oxidative stress. In this work, HCMV was used as a model virus to understand the interaction between viral infection and the stress response system of the host cell. Primary human foreskin fibroblasts were employed as host cells because it is

permissive to HCMV and has been widely used for the work on this herpesvirus.

One of the interesting characteristics of HCMV infection is that it establishes persistent infection in the organs or tissues of patients with chronic inflammation (Britt, 2008), which is closely associated with the generation of oxidative stress. Increased level of ROS can have multiple effects on the host cell. For instance, oxidative stress induces the damage to the host cell and inhibits cellular factors such as mTOR that are essential for viral replication (Tilton *et al.*, 2011), which may be harmful to the virus. ROS also activates NF-κB (Speir *et al.*, 1996), which can lead to exaggerated inflammation and reactivation of latently-infected HCMV (Varani & Landini, 2011). It is not yet clear, however, that how HCMV infection modulates these stress responses to not only survive but also thrive under the inflammatory condition, which is directly tested in this thesis. Because the presence of HCMV in this environment is known to augment the progression or seriousness of HCMV-related disease (Britt, 2008; Varani & Landini, 2011), the aim of this thesis also has clinical importance.

Because premature cell damage, prior to the completion of viral life cycle, can limit viral replication (Valyi-Nagy & Dermody, 2005), increasing survival of host cells under oxidative stress may be necessary for persistent HCMV infection in this condition. Nrf2 is a key transcription factor regulating the expression of various antioxidative stress genes and its activation is well-known to promote the survival of host cells in several virus infections (Burdette *et al.*, 2010; Cho *et al.*, 2009; Niture *et al.*, 2010; Yageta *et al.*, 2011). However, the possible role of Nrf2 in HCMV infection is not yet elucidated. Therefore, I investigated the effect of HCMV infection on Nrf2, especially with a question of whether HCMV infection could protect the host cell from oxidative damage by activating Nrf2.

In addition to promoting host cell survival, ensuring efficient viral DNA replication may also contribute to the presence of HCMV in stressed condition. Previous reports suggest that HCMV-mediated activation of mTOR may be one mechanism for this (Moorman *et al.*, 2008; Moorman & Shenk, 2010; Tilton *et al.*, 2011). However, it had not been clear how mTOR activation leads to enhanced viral DNA replication. CCAAT/enhancer binding protein α (C/EBPα) is a transcription factor involved in the genome replication of two gamma-herpes viruses (Huang *et al.*, 2006; Wang *et al.*, 2004). The expression of C/EBPα is reported to be regulated via translational regulation involving mTOR in other experimental settings (Calkhoven *et al.*, 2000). Therefore, I hypothesized that HCMV may utilize this transcription factor for its genome replication and mTOR may be involved in this process.

The activation of canonical NF-κB is well-known response to oxidative stress and mediates various aspect of HCMV infection including the regulation of immune and inflammatory responses. However, the regulation of NF-κB by HCMV infection is not fully understood. Therefore, the possible involvement of GSK3β, one of the known regulators of this pathway, was tested in the context of HCMV infection. A different pathway called the non-canonical NF-κB pathway had also been identified (Razani *et al.*, 2011). This pathway regulates the expression of target genes involved in the regulation of B-cell responses and its overexpression is associated with the production of autoantibodies (Razani *et al.*, 2011), in which HCMV had been suspected to act as a cofactor (Varani & Landini, 2011). Therefore, I also investigated the effects of HCMV infection on non-canonical NF-κB and the possible involvement of GSK3β in this process.

In summary, the major theme of thesis research has been how HCMV infection modulates the oxidative stress responses of the host cell and what could be the biological

consequences of this interaction(s). During this study, I made several novel observations which might help understand the role(s) or impact(s) of HCMV infection under pathologic conditions such as chronic inflammation, for example, how HCMV infection could persist in the body and abnormally activate the host immune system.

CHAPTER II

Materials & Methods

1. Cell culture and reagents

For HFFs, cells of passage 12-20 were used. HEK293T cells were obtained from the American Type Culture Collection. HFFs and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin solution (Invitrogen-Gibco) at 37℃ in a humidified atmosphere of 5% CO_2. Human neuroblastoma cell line SK-N-SH was obtained from Korean Cell Line Bank, and cultured in Minimum Essential Medium Eagle (Sigma) supplemented with Penicillin-Streptomycin-Glutamine (Invitrogen-Gibco). LY294002 and Wortmannin were from Cayman chemicals, and all other chemicals were from Sigma.

2. HCMV and infections

For preparation of HCMV strain Towne, HFFs were infected at a multiplicity of infection (m.o.i.) of 0.01, and incubated for 10-14 days until visible cytopathic effects were observed. Culture supernatants were collected, filtered through a 0.45 μm membrane, and concentrated by ultracentrifugation at 92600 g for 1.5 h at 4℃. Viral stocks were aliquoted and stored at -70℃. The titers of viral stocks were determined as an infectious center unit (ICU) after the measurement of the IE1/IE2-positive cells by infectious center assay (Huh *et al.*, 2008). Clinical strain JHC (passage 3), originally isolated from patients undergoing bone marrow transplantation, was provided by Dr. Chan Hee Lee, and passaged one more time in HFFs (Chungbuk National University, Cheongju, Korea) (Jung *et al.*, 2011). To prepare UV-treated HCMV, viral stocks were placed in 1.5-ml tubes and

exposed to 254-nm UV light (40W) for 1 h. For HCMV infection, cells seeded on the previous day were incubated with viral inoculums for 2 h at 37℃ in the CO_2 incubator. Unless otherwise mentioned, all infections were performed at an m.o.i. of 3.

3. Plamids

The plasmid expressing the catalytic subunit of human telomerase (pCS2-hTERT) was provided by Han-Woong Lee (Yonsei University, Seoul, Korea). The hTERT gene was PCR amplified from this plasmid using the following primers:
Forward: 5'- AGATCTATGCCGCGCGCTCCCCGCTGCCGA - 3',
Reverse: 5' - AGATCTTCAGTCCAGGATGGTCTTGAAGTC - 3'
The PCR product was cloned into the BamHI site of the retroviral vector MSIG (Jang *et al*., 2012). This vector contains the long terminal repeat originally from a murine stem cell virus (MSCV), and eGFP is expressed as a bicistronic message using the internal ribosome entry site (IRES) from encephalomyocarditis virus (EMCV).

The CHOP and the HCMV UL38 gene were amplified from the cDNA of HFFs using the following PCR primers:
CHOP, forward, 5'-ACGCGTATGGAGCTTGTTCCAGCC-3', and reverse, 5'-GGATCCTCATGCTTGGTGCAGATT-3', UL38, forward, 5'-AGATCTATGACTACGACCACGCA-3', reverse, 5' AGATCTCTACAGATCCTCTTCTGAGATGAGTTTTTGTTCGACCACGACCACCATCT-3'
The PCR products were cloned into the pGEM ® -T Easy vector (Promega) to generate

the plasmids pGTZ-hCHOP, which were then sequenced. pGTZ-hCHOP was digested with *Mlu*I and *Bam*HI. The digested products were purified and cloned into the retroviral vector MSIG, which had been digested with the same pairs of enzymes, generating the plasmids MSIG-hCHOP.

The shRNA expression vector was constructed using the plasmid pSUPER.retro.puro (OligoEngine) as a backbone. Optimal shRNA sequences targeting human Nrf2 were designed using web-based software (siDESIGN® Center), and shRNA sequences targeting human GSK3β was previously reported by others (Guo *et al.*, 2008). A 19-nucleotide target sequence and its corresponding position were designed as follows:
Nrf2 (human): 5'- TGACAGAAGTTGACAATTA -3', nucleotides 659 to 677
GSK3β (human): 5' - GGACAAGAGATTTAAGAAT - 3', nucleotides 1250 to 1268
The shRNA sequence targeting Luciferase (5'- GTGCGTTGCTAGTACCAAC -3') was used as the control. After annealing complementary single-stranded oligonucleotides, double-stranded fragments were inserted into the BglII and HindIII sites of the vector pSUPER.retro.puro (OligoEngine). The H1 promoter-driven shRNA expression cassette was excised using the EcoRI and XhoI sites from the vector expressing Nrf2-specific shRNA and subcloned into MSIG for increasing retroviral titer along with the control plasmid expressing Luciferase-specific shRNA.

4. Generation of transduced cell line

Retroviral vectors were prepared by the three-plasmid transfection method using

Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer's protocols. The packaging constructs include pVM-gp for the Gag-pol genes from MLV and pCA-VSVG for the Env gene (Yu *et al.*, 2003). Two days later, supernatants were collected and concentrated as described in the HCMV infections section. The retroviral vector titers were determined by measuring the percentage of eGFP-positive HFFs transduced with different dilutions of the viral stock. To generate gene transferred HFFs, 10^5 cells were seeded in six-well plates on the previous day and transduced with the retroviral vectors at an m.o.i. of 5. Cells were expanded, and the transduction efficiency was measured as described above. Polybrene (8 μg ml^{-1}) was included to increase the transduction efficiency. To obtain the transduced HFFs, 10^5 cells were transduced at an m.o.i. of 3; polybrene (8 μg/ml) was included to increase the transduction efficiency (over 80%). Cells expressing CHOP were sorted by FACS.

5. Immunoblotting

Cells were lysed with Cytobuster (Novagen) supplemented with protease and phosphatase inhibitor cocktail (Roche Diagnostics Ltd.) for 15 min at 4 ℃. Protein concentration was determined by a Bradford assay (Bio-Rad, Hercules). Equal amounts of protein were resolved by 9-12% (w/v) SDS-PAGE and transferred to PVDF membranes (GE Healthcare). The membranes were blocked for 1 h at room temperature (RT) with TBST (150 mM NaCl, 10 mM Tris-HCl, 0.1% (v/v) Tween 20, pH 8.0) containing 1% (w/v) bovine serum albumin (Invitrogen-Gibco). The membrane was incubated with primary antibodies diluted in block overnight at 4 ℃. After being washed with TBST for 20 min three times, the membranes were incubated with horseradish peroxidase-conjugated goat

anti-mouse or rabbit IgG secondary antibody (A0168 or A0545, Sigma) for 1 h at RT. The blots were then washed three times with TBST, and the protein bands were visualized with the enhanced chemiluminescence system (Millipore).

Immunoblotting was performed using the following primary antibodies: anti-HO-1 (SPA-894, Assay Designs-Stressgen), anti-GCLC (sc-22755, Santa Cruz Biotechnology), anti-IE (MAb810, Millipore), anti-pp28 (CA004, Virusys), anti-β-actin (A5441, Sigma), anti-phospho Akt (Ser473) (4060, Cell Signaling Technology), anti-Akt (610876, BD Pharmingen), anti-C/EBPα (SC-61, Santa Cruz Biotechnology), anti-phospho p70S6 Kinase (Thr389) (9205, Cell Signaling Technology), anti-p70S6 Kinase (2708, Cell Signaling Technology), anti-IE (Mab810, Millipore), anti-UL44 (CA006, Virusys), anti-pp28 (CA004, Virusys), anti-CHOP (2895, Cell Signaling Technology), anti-phospho GSK3β (Tyr216) (612313, BD Transduction Laboratories), anti-phospho GSK3α/β (Ser21/9) (9331, Cell Signaling Technology), anti β-catenin (610153, BD Transduction Laboratories), anti-IκBα (SC-371, Santa Cruz Biotechnology), anti-p65 (SC-372, Santa Cruz Biotechnology), anti-α-tubulin (SC-5286, Santa Cruz Biotechnology), anti-TFIIB (SC-274, Santa Cruz Biotechnology), anti-p100/p52 (4882, Cell Signaling Technology), anti-p105/p50 (SC-114, Santa Cruz Biotechnology), and anti-NIK (SC-7211, Santa Cruz Biotechnology).

6. Immunofluorescence

Cells grown on coverslips were were fixed in PBS containing 4% (w/v) paraformaldehyde, permeabilized with 0.5% (v/v) Triton X-100 in PBS, and blocked for 1 h with 10% (v/v)

human serum (S1; Millipore) and 5% (w/v) glycine in PBS. The primary antibodies that were diluted in blocking buffer were incubated with the cell monolayers overnight at 4℃. After washing with PBS, the samples were incubated with Alexa Fluor® 488- or Alexa Fluor® 555-conjugated secondary antibodies (Invitrogen) diluted in PBS for 1 h at RT. Hoechst 33258 (0.1 mM in PBS) was used to stain the nuclear DNA. When indicated, cells grown on coverslips were extracted using the CSK buffer before fixation and permeabilization, as previously described (Luo *et al.*, 2007). Immunofluorescence was performed using the following primary antibodies: anti-IE (Mab810, Millipore), anti-HO-1 (SPA-894, Assay Designs-Stressgen), anti-Nrf2 (ab62352, Abcam), anti-hTERT (600-401-252; Rockland), anti-C/EBPα (SC-61, Santa Cruz Biotechnology), UL44 (CA006, Virusys), and GFP (A11122, Invitrogen). The immunoglobulin (IgG) from normal rabbit serum was used for the negative control (I5006, Sigma).

7. Quantitative reverse transcription polymerase chain reaction (quantitative RT-PCR)

Total RNA (0.5μg) extracted using Trizol reagent was used to cDNA synthesis with AMV reverse transcriptase (Life Science) and oligo(dT) primers (Qiagen). Real time quantitative PCR was performed in triplicate with a LightCycler SYBR ® Green I technology (Takara Bio Inc.) according to the manufacturer's protocol using following primers to detect human B-cell activating factor (BAFF), glutamate-cysteine ligase catalytic subunit (GCLC), and GADPH for internal control:
BAFF, forward, 5'-TGAAACACCAACTATACAAAAAG-3', and reverse, 5'-

TCAATTCATCCCCAAAGACAT-3’ (Roescher *et al.*, 2014), GCLC, forward, 5’-TCTCTAATAAAGAGATGAGCAACATGC-3’, and reverse, 5’-TTGACGATAGATAAAGAGATCTACGAA-3’ (Devling *et al.*, 2005), and GAPDH, forward, 5’-GTCGGAGTCAACGGATTTGGTCGT-3’, and reverse, 5’-GACGGTGCCATGGAATTTGCCATG-3' (Natarajan *et al.*, 2003).
Data was analyzed by 2-ΔΔCt method (Livak & Schmittgen, 2001).

8. Northern blot hybridization

The total RNAs were isolated using TRIzol® reagent (Invitrogen). A 10-μg RNA aliquot was separated by electrophoresis on a 1% (w/v) formaldehyde-agarose gel and transferred to a nylon membrane (Hybond-N; GE Healthcare-Amersham) by overnight capillary blotting. Specific cDNA probes for the human HO-1 and C/EBPα gene were labeled using the Prime-It® II Random Primer Labeling Kit (Stratagene) and [α-^{32}P] dCTP (GE Healthcare). The membranes were hybridized with the labeled probes (10^6 c.p.m./ml) for 1 h at 68 ℃, washed with 0.2X SSC buffer and 0.1% (w/v) SDS at 55 ℃ for 30 min, and exposed to X-ray films (Agfa-Gaevert). The PCR primer sequences used to generate probes are as follows:
HO-1, forward, 5’-CACGCATATACCCGCTACCT-3’, and reverse, 5’-GTTCCTCTGTCAGCATCACC-3’, C/EBPα, forward, 5’-TGGGCGGCATCTGCGAGCAC-3’, reverse, 5’- GTGCATGGTGGTCTGGCCGC-3’.

9. Transient transfection and luciferase assay

A reporter plasmid (0.4 μg) was transfected into cells using the Lipofectamine® 2000 Reagent (Invitrogen) according to the manufacturer's protocol. A NF-κB-driven luciferase reporter plasmid along with the control plasmid was obtained from Agilent Technologies (219077 and 219090, respectively). An antioxidant response element (ARE) reporter plasmid was provided by Dr. Hun-Taeg Chung Cell (Wonkwang University, Iksan, Korea) (Lee *et al.*, 2006). Cell lysates were obtained using Reporter Lysis Buffer (Promega), and the protein concentration was determined as described above. Equal amounts of protein (5 μg) were used in a luciferase assay (Luciferase Assay System, Promega).

10. Measurement of intracellular ROS

The level of intracellular ROS was determined using fluorescence-based ROS indicator, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Invitrogen). Cells were washed in pre-warmed PBS, and incubated with 1μM of CM-H2DCFDA diluted in HBSS (Sigma) for 5 min. After incubation, cells were washed in PBS and further incubated in HBSS for 30 min at 37℃. Then, cells were rapidly trypsinized and resuspended in HBSS. The fluorescence intensity of cells was measured using FACS with the aid of CellQuest software (BD, Los Angeles, CA).

11. Chromatin immunoprecipitation

The sonicated DNA (1 μg) prepared from 2 $\times 10^6$ cells was subjected to chromatin immunoprecipitation using the ChIP-IT ® Express Chromatin Immunoprecipitation Kit (Active Motif), according to the manufacturer's protocol. The primers used for PCR amplification were as follows:

oriLyt R1, forward, 5'-AAAGATCCGAACTTTAAAATTGTGTGTTTTT-3, and reverse, 5'- TGCTCACCGCCTCGCCGGCCACGGGGTTGA-3', and oriLyt R5, forward, 5'-ACGACACACACCCAGTGGAATTTTACCG-3', and reverse, 5'-CTCCGGAACCGGGGGGGGCAAATTTTTA -3'.

Chromatin immunoprecipitation was performed using the primary antibodies to C/EBPα (SC-61) and UL44 (CA006). The IgG from normal rabbit or mouse serum was included as a control.

12. Genomic DNA extraction and real-time quantitative PCR

The genomic DNA was isolated from 2 $\times 10^6$ cells using the Gentra® Puregene® Kit (Qiagen), according to the manufacturer's protocol. The genomic DNA (1-2 ng) was subjected to real-time quantitative PCR using the LightCycler SYBR ® Green I technology (Takara Bio Inc.), according to the manufacturer's protocol. The PCR was performed using the following primers:

HCMV genome, forward, 5'-AAAGATCCGAACTTTAAAATTGTGTGTTTTT-3', and reverse, 5'-TGCTCACCGCCTCGCCGGCCACGGGGTTGA-3', and cellular DNA, forward, 5'-GCTGCCCAAACCACTTCTGT-3', and reverse, 5'-GCCCTTTCACCTCCCACCTA-3'.

13. Measurement of intracellular glutathione and cell viability

Intracellular GSH concentration and cell viability were measured using the Glutathione Assay Kit (Cayman) and the Cell Proliferation Kit I (Roche), respectively, according to the manufacturer's protocols.

14. Statistical analysis

The data are presented as the mean ± SD of triplicate samples. The significance of the difference between values was determined by an unpaired *t*-test or a one-way ANOVA, followed by Tukey's multiple comparison test or Bonferroni multiple comparisons tests, using the GraphPad Prism software (Version 5). P values less than 0.05 were considered to represent significant differences (ns, non-significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

CHAPTER III

Activation of Nrf2 signaling pathway by HCMV infection and its role under oxidative stress

1. Backgrounds

Nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) belongs to the cap 'n' collar (CNC) subfamily of basic region leucine zipper (bZIP) transcription factors that include NF-E2, Nrf1–3, and Bach1–2 in vertebrates (Sykiotis & Bohmann, 2010). Nrf2 plays a crucial role in regulating cellular responses to a variety of oxidative and xenobiotic stresses. Under non-stressed conditions, Nrf2 is expressed at low levels because of its interaction with Keap1, resulting in the ubiquitination and degradation of Nrf2. When activated, Nrf2 is stabilized by its dissociation from Keap1. Free Nrf2 binds to DNA sequences termed antioxidant-responsive elements (ARE) as a heterodimer with Maf, which subsequently activates the transcription of downstream target genes including HO-1, which protects cells against the detrimental effects of different stresses (Niture *et al.*, 2010). The oxidative modification of Keap1 and protein kinase C (PKC)-mediated phosphorylation of Nrf2 are known to disrupt the Keap1-Nrf2 interaction, thereby activating the Nrf2 pathway (Niture *et al.*, 2009). The involvement of other kinases including the phosphatidylinositol 3-kinase (PI3K) and casein kinase 2 (CK2) in the activation of Nrf2 has also been reported (Afonyushkin *et al.*, 2011; Kim *et al.*, 2012; Nakaso *et al.*, 2003; Wang *et al.*, 2008).

The Nrf2 pathway has been implicated in various viral infections. The down-regulation of Nrf2 increased the entry and replication of influenza virus in human nasal epithelial cells. However, treatment with Nrf2 activators had the opposite effect(s), thus suggesting an antiviral role for Nrf2 (Kesic *et al.*, 2011). HBV infection also activated the Nrf2 pathway, leading to increased host cell protection against oxidative stress-induced protein modification (Schaedler *et al.*, 2010). The effect of HCV infection on the Nrf2

pathway is still controversial. HCV infection has been reported to prevent host cell apoptosis through the activation of Nrf2, but it has also been claimed that HCV inhibits the Nrf2 pathway by the delocalization of small Maf proteins (Burdette *et al.*, 2010) (Carvajal-Yepes *et al.*, 2011).

It has recently been reported that HCMV infection does not activate the Nrf2 pathway in life-extended fibroblasts, which have been engineered to constitutively express the catalytic subunit of telomerase (Tilton *et al.*, 2011). HCMV infection prevented the nuclear translocation of Nrf2 and failed to activate HO-1expression. However, other investigations have shown that HO-1 expression is increased upon HCMV infection at the RNA or protein level when primary fibroblasts are used (Browne *et al.*, 2001; Stanton *et al.*, 2007). Therefore, the effect of HCMV infection on the Nrf2 pathway needs to be further clarified.

The goal of this study was to investigate the regulation and biological significance of Nrf2 during HCMV infection of primary fibroblasts. These studies indicate that HCMV infection influences Nrf2 expression and activates HO-1 expression through Nrf2. The induction of Nrf2 expression appears to be dependent on viral gene expression and the activity of the cellular kinase CK2. Our data involving buthionine sulfoximine (BSO) suggest that HCMV infection might protect host cells against oxidative stress-induced cell death through the activation of Nrf2.

2. Results

2.1 HCMV activates the transcription factor Nrf2

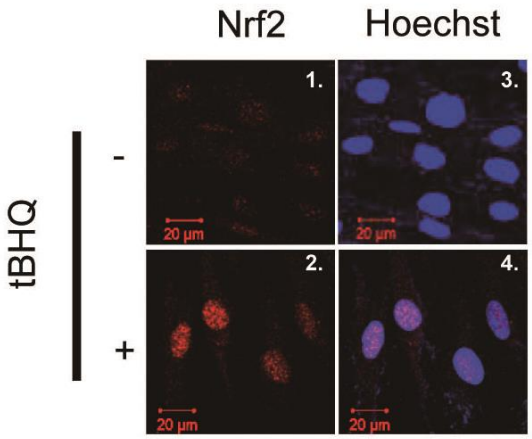
To test the effect of HCMV infection on Nrf2, human foreskin fibroblasts (HFFs)

were infected with HCMV (Towne strain), and cell monolayers were immunostained using IE1/IE2- and Nrf2-specific antibodies. As a positive control, HFFs were treated with 100 μM tert-butylhydroquinone (tBHQ), a well-known Nrf2 inducer, for 24 hours (h). Nrf2 protein levels were undetectable in normal HFFs (Fig. III-1a, panel 1), but in cells treated with tBHQ, Nrf2 protein levels increased significantly in the nucleus (Fig. 1a, compare panels 1 and 2). In the HCMV-infected cells, Nrf2 protein levels (red) started to increase at 24 h post-infection (p.i.), and reached their highest levels at 48 h p.i. (Fig. III-1b, panels 5 to 8). Nrf2 induction was observed in both the nuclear and cytoplasmic compartments (Fig. III-1b, panels 13 to 16). These results indicate that HCMV infection can activate Nrf2. To corroborate these results, a clinical HCMV strain (JHC) was also used. HFFs were infected with either the Towne or JHC strain under the same experimental conditions and analyzed using immunofluorescence using IE1/IE2- and Nrf2-specific antibodies. This clinical strain also increased Nrf2 expression in both compartments (Fig. III-1c, compare panels 4 and 6).

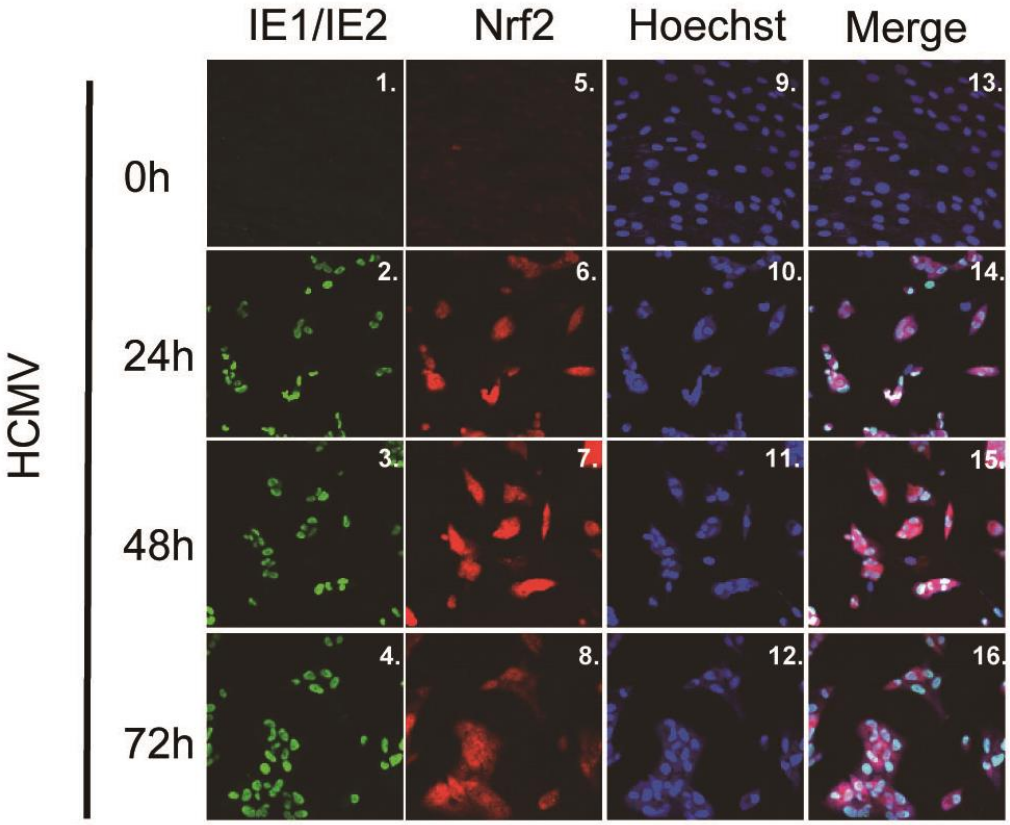
Previously, in studies using HFFs immortalized by the constitutive expression of the telomerase catalytic subunit (TERT), it was reported that HCMV infection increased Nrf2 expression but prevented its nuclear translocation, which is inconsistent with our results generated with the use of normal primary HFFs (Tilton *et al.,* 2011). To directly compare these two types of HFFs, we also constructed HFFs that express TERT using retroviral vectors expressing TERT and GFP as a bicistronic message or GFP alone as a control. Transduced cells were cultured for 5 passages, and FACS analysis was used to measure the transduction efficiency, which was over 90%. The TERT expression was confirmed by immunostaining using a TERT-specific antibody. Control cells expressed an undetectable level of TERT, whereas it was readily visible in the nucleus of TERT-

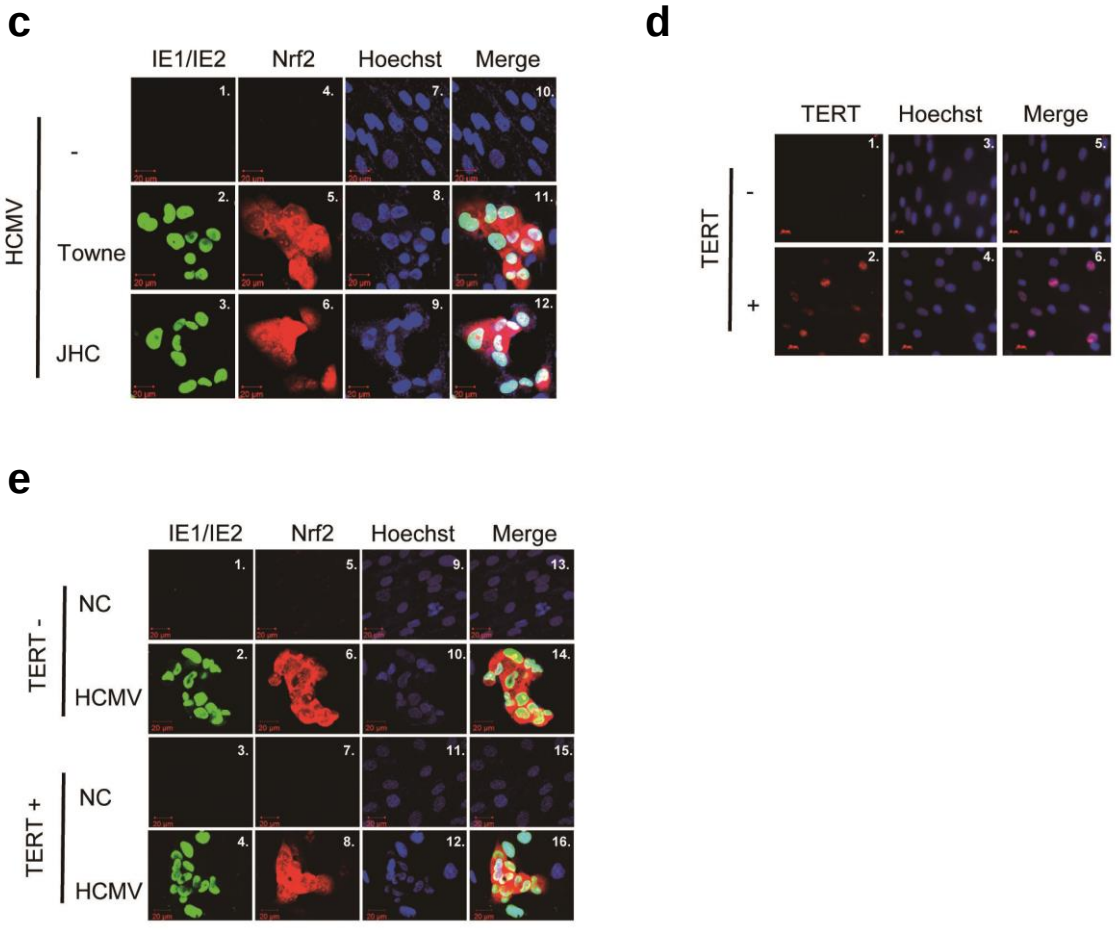
Figure III-1. Nrf2 Activation during HCMV infection. (a) HFFs were treated with 100uM tBHQ for 24h, and immunofluorescence (IF) was performed using an Nrf2-specific antibody (red). Nuclei were stained with Hoechst stain (blue). (b) HFFs were infected with HCMV and examined by IF at the time points indicated using IE1/IE2- (green) and Nrf2- (red) specific antibodies. (c) HFFs were infected with a laboratory-adapted strain (Towne) or a clinical isolate (JHC) of HCMV, and the localization of Nrf2 was analyzed by IF at 48 h p.i. (d) HFFs stably expressing the catalytic subunit of telomerase (TERT) were immunostained using TERT-specific antibodies (red). (e) HFFs expressing TERT and normal HFFs were infected with HCMV and examined by IF using IE1/IE2- (green) and Nrf2- (red) specific antibodies at 48 h p.i. Scale bar: 20 μm.

a


Nrf2
Hoechst
tBHQ
-
+
1.
2.
3.
4.
20 µm

b


IE1/IE2
Nrf2
Hoechst
Merge
HCMV
0h
24h
48h
72h
1.
2.
3.
4.
5.
6.
7.
8.
9.
10.
11.
12.
13.
14.
15.
16.


c
IE1/IE2
Nrf2
Hoechst
Merge
HCMV
-
Towne
JHC
1.
2.
3.
4.
5.
6.
7.
8.
9.
10.
11.
12.
d
TERT
Hoechst
Merge
TERT
-
+
1.
2.
3.
4.
5.
6.
e
IE1/IE2
Nrf2
Hoechst
Merge
TERT -
NC
HCMV
TERT +
NC
HCMV
1.
2.
3.
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11.
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13.
14.
15.
16.

expressing cells (Fig. III-1d, compare panels 5 and 6). Both cell types were then infected with HCMV and analyzed by immunofluorescence to observe IE1/IE2 and Nrf2 protein levels. In both the control and TERT-expressing cells, HCMV infection could upregulate the level of nuclear Nrf2 (Fig. III-1e, compare panels 6 and 8). Our data indicate that the expression of TERT does not affect HCMV-mediated Nrf2 activation. Additional studies are needed to determine what caused the differences between these two studies.

2.2 HCMV infection upregulates HO-1 expression

HO-1 is one of the major antioxidative stress genes, and its gene expression is regulated primarily by Nrf2. To test the effect of HCMV infection on HO-1 expression, HFFs were infected with HCMV, and total proteins were prepared at appropriate time points and subjected to immunoblotting analysis. HCMV infection induced the expression of the HO-1 protein in a transient manner (Fig. III-2a). The level of the HO-1 protein peaked at 48 h p.i., then decreased to a basal level. To investigate whether HCMV infection regulated the induction of HO-1 expression at the transcriptional level, the RNA level of HO-1 was determined by Northern blot hybridization. The steady state mRNA level of HO-1 was lower at 6 h p.i. than at basal level, but increased thereafter, peaking at 24 h p.i. followed by the decrease (Fig. III-2b). These data suggested that HCMV infection could alter HO-1 expression at the RNA level.

To exclude the possibility that HO-1 induction might have occurred in uninfected cells rather than in the infected cells (for example, by the action of cytokines or other materials secreted from neighboring HCMV-infected cells), HFFs were infected with HCMV, and HO-1 expression was analyzed by immunofluorescence using IE1/IE2- and HO-1-specific antibodies. Uninfected cells expressed undetectable levels of HO-1 (Fig.

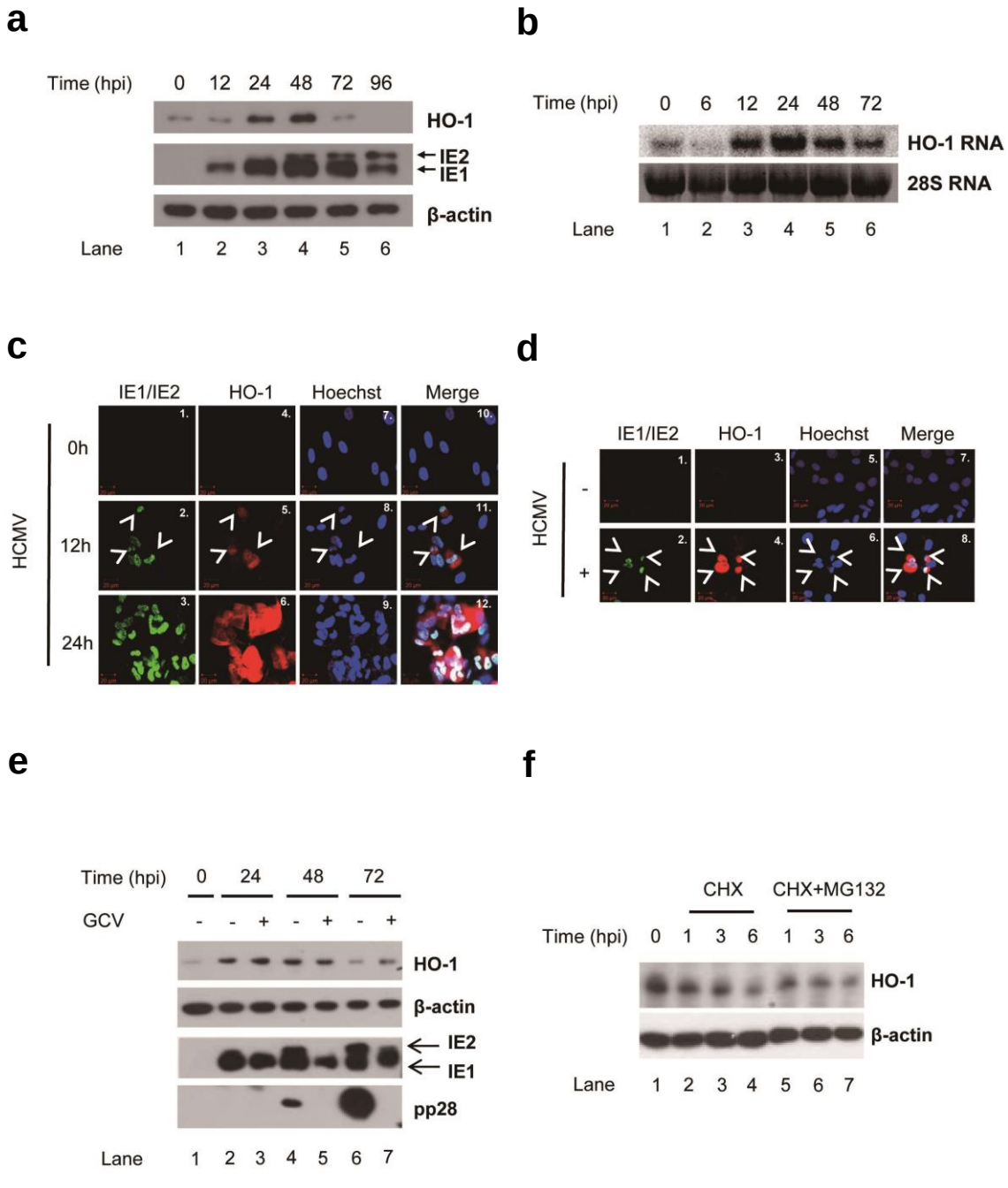
III-2c, panel 4). At 12 h p.i., some cells expressed IE1/IE2. Increased HO-1 protein expression was only observed in IE1/IE2-positive cells and undetectable in neighboring IE1/IE2-negative cells (Fig. III-2c, panel 11). At 24 h p.i., nearly all the cells expressed both IE1/IE2 and HO-1 (Fig. III-2c, panel 12). These data indicate that HO-1 expression is induced only in infected (IE1/IE2-positive) cells.

To confirm these results, the effect of HCMV infection was also tested on another cell line, SK-N-SH, known to be susceptible to this herpesvirus (Wang & Shenk, 2005). Cells were infected with HCMV and analyzed by immunofluorescence at 24 h p.i. HO-1 was not detected in uninfected cells (Fig. III-2d, panel 3). In infected cultures, nearly all IE1/IE2-positive cells showed increased HO-1 expression (Fig. III-2d, panel 8). These data indicate that our observations are not restricted to one particular cell type.

The possible involvement of viral late gene products in the HCMV-mediated regulation of HO-1 expression was also tested. HFFs were infected with HCMV in the absence or presence of 30 μM ganciclovir (GCV). Cells were harvested at the indicated time points, and Western blot analysis was performed on whole cell extracts to monitor HO-1 expression levels. As expected, GCV treatment inhibited the expression of a late gene, pp28 (UL99), as well as the expression of IE2, whose expression during the late stage of infection has been shown to be dependent on viral DNA synthesis (Fig. III-2e, compare lanes 4 and 5, and lanes 6 and 7) (Fehr & Yu, 2011). The kinetics of HO-1 protein expression were transient as observed above, and this expression pattern was not altered by the addition of GCV (Fig. III-2e, compare lanes 2,4,6 with 3,5,7). Our data suggest that the expression of viral late genes might be not involved in regulating HO-1 expression.

HO-1 protein levels were almost undetectable at 72 h p.i., whereas HO-1 mRNA

Figure III-2. HO-1 induction in HCMV-infected cells. (a) HFFs were infected with HCMV and harvested at the time points indicated. HO-1 protein levels were measured by immunoblotting using IE1/IE2-, HO-1, and β-actin specific antibodies. (b) HO-1 mRNA levels were determined by Northern blot hybridization. 28S ribosomal RNA levels served as a loading control. (c) HFFs were infected with HCMV, and cell monolayers were analyzed by IF using IE1/IE2- (green) and HO-1- proteins (red) specific antibodies. Scale bar: 20 μm. (d) SK-N-SH, a neuroblastoma cell line, was infected with HCMV and examined by IF at 24 h p.i. (e) HFFs were infected with HCMV, and at 72 h p.i., they were treated with cycloheximide (CHX; 100 μM) for 1 h, then incubated with MG132 (2 μM) for the times indicated. Protein levels were measured by immunoblotting using HO-1- and β-actin-specific antibodies. (f) HFFs were infected with HCMV in the absence or presence of 30 μM GCV. Protein levels were measured by immunoblotting using HO-1-, β-actin-, IE1/IE2-, and pp28-specific antibodies.


a
Time (hpi) 0 12 24 48 72 96
HO-1
IE2
IE1
β-actin
Lane 1 2 3 4 5 6
b
Time (hpi) 0 6 12 24 48 72
HO-1 RNA
28S RNA
Lane 1 2 3 4 5 6
c
IE1/IE2 HO-1 Hoechst Merge
HCMV
0h
12h
24h
d
IE1/IE2 HO-1 Hoechst Merge
HCMV
e
Time (hpi) 0 24 48 72
GCV - - + - + - +
HO-1
β-actin
IE2
IE1
pp28
Lane 1 2 3 4 5 6 7
f
CHX CHX+MG132
Time (hpi) 0 1 3 6 1 3 6
HO-1
β-actin
Lane 1 2 3 4 5 6 7

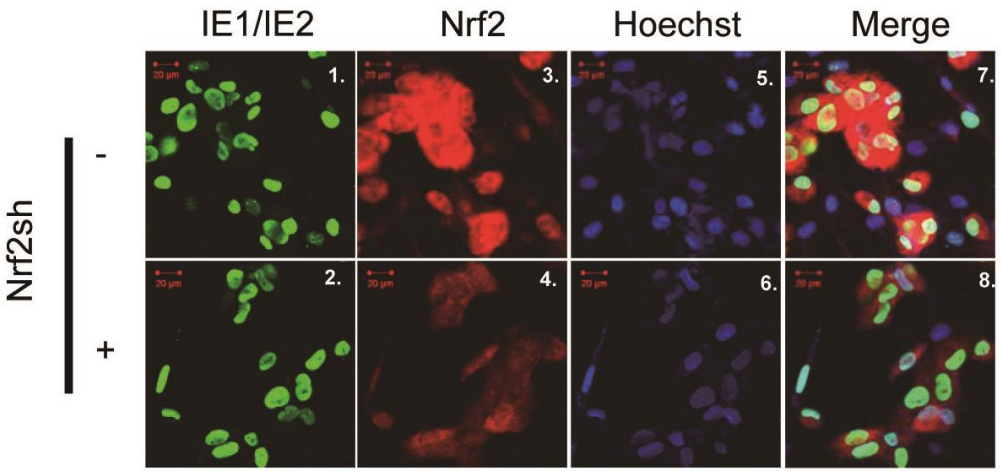
levels were still significantly upregulated (Fig. III-2a, compare lane 5 and lane 6 of Fig.2b), suggesting the possibility that HO-1 may be controlled post-translationally, including enhanced proteasome degradation, during the late stages of infection. The stability of HO-1 was investigated in the absence or presence of the proteasome inhibitor MG132 following cycloheximide treatment to inhibit further HO-1 protein synthesis. HFFs were infected with HCMV, treated with 100 μM cycloheximide at 72 h p.i. for 1 h, and then treated with 2μM MG132 for times indicated. After cycloheximide treatment, HO-1 protein levels gradually decreased over time (Fig. III-2f, lanes 1 to 4), and the addition of MG132 did not affect the stability of HO-1 protein levels (Fig. III-2f, compare lanes 2,3,4 with 5,6,7). These data suggest that proteasome activity may be not involved in controlling HO-1 protein levels during this stage of infection. Additional studies will be needed to determine how HCMV infection decreases HO-1 protein levels at this time.

2.3 Nrf2 is required for HCMV-mediated induction of HO-1 expression

To test the involvement of Nrf2 in HCMV-mediated regulation of HO-1, Nrf2-specific shRNAs were used. HFFs were transduced with retroviral vectors expressing an shRNA specific for Nrf2 (Nrf2sh) or luciferase (LUCsh) in a bicistronic message with GFP. Overall, the transduction efficiency was approximately 90% as determined by FACS. HFFs expressing either type of shRNA were infected with HCMV and immunostained using IE1/IE2- and Nrf2-specific antibodies. Nrf2 protein levels were reduced in Nrf2sh cells (Fig. III-3a, compare panels 3 and 4). In LUCsh cells, HO-1 protein levels were increased in response to HCMV infection (Fig. III-3b, lanes 1 and 2). The basal levels of the HO-1 protein expression were lower in Nrf2sh cells than in LUCsh cells (Fig. III-3b, lanes 1 and 3), and the HCMV-mediated increase in HO-1 protein

Figure III-3. Effects of Nrf2 knockdown on the HCMV-mediated induction of HO-1. (a) HFFs expressing either type of shRNA were infected with HCMV and examined by IF using IE1/IE2- (green) and Nrf2- (red) specific antibodies. Scale bar: 20 μm. (b) HFFs expressing shRNAs were infected with HCMV, and HO-1, GCLC, and β-actin proteins levels were measured at 48 h p.i. by immunoblotting.

a


IE1/IE2
Nrf2
Hoechst
Merge
Nrf2sh
-
+
1.
2.
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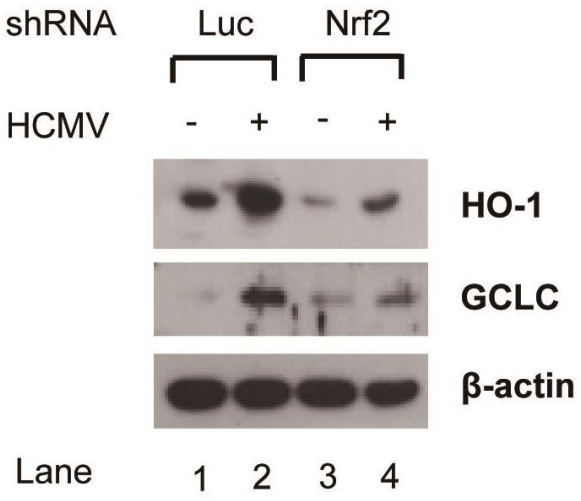
b


shRNA
Luc
Nrf2
HCMV
- + - +
HO-1
GCLC
β-actin
Lane
1 2 3 4

levels was suppressed in Nrf2sh cells (Fig. III-3b, compare lanes 3 and 4). These data indicate that the HCMV-mediated induction of HO-1 expression is controlled, at least in part, by Nrf2. The expression of glutamate cysteine ligase catalytic subunit (GCLC), an Nrf2 target gene, was also found to be regulated in a manner similar to HO-1 (Fig. III-3b). The level of Nrf2 expression could not be measured by Western blot due to the high background (data not shown), however, Nrf2 immunofluorescence clearly showed knockdown of Nrf2 expression (Fig. III-3a). These data indicate that HCMV infection increases the expression of various antioxidative genes through the activation of Nrf2.

2.4 The expression of viral gene products is required for HCMV-mediated activation of Nrf2

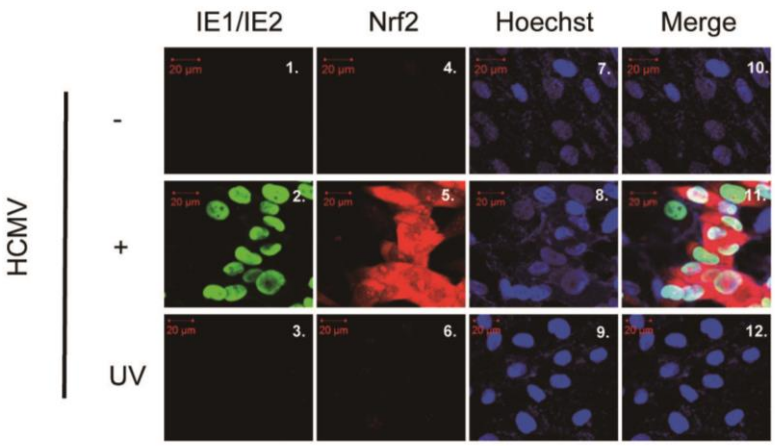
UV-irradiated virus is known to be taken up by cells, but does not support the expression of viral proteins due to the damage of viral DNA. To test whether viral gene expression was necessary for HCMV-mediated Nrf2 activation, the effect of UV-irradiated virus was used. HFFs were treated with normal or UV-irradiated virus, and cells were analyzed for the IE1/IE2 and Nrf2 proteins by immunofluorescence at 48 h p.i. UV-irradiation completely abolished the ability of the virus to induce the expression of Nrf2 and viral proteins, indicating that newly synthesized viral protein(s) might be needed for the upregulation of Nrf2 expression (Fig. III-4a, panels 2 and 3, and panels 5 and 6).

Reactive oxygen species (ROS) has been shown to activate Nrf2 by inducing the dissociation of Keap1 and Nrf2 (Niture *et al.*, 2010). Because HCMV infection was previously reported to increase the level of ROS, it was tested whether Nrf2 activation was the direct result of HCMV infection or the indirect consequence of the increased ROS levels (Speir *et al.*, 1996). HFFs were treated with normal or UV-irradiated virus, and the

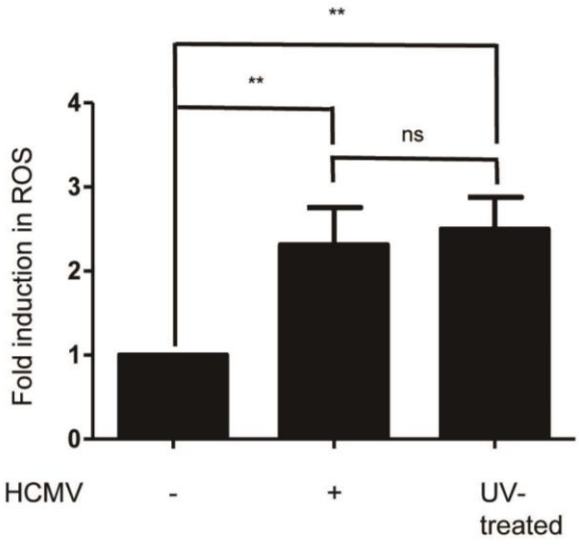
Figure III-4. Effects of UV-irradiated HCMV on Nrf2 and intracellular ROS.

(a) HFFs were treated with normal or UV-irradiated virus, and examined by IF using IE1/IE2 (green) or Nrf2 (red) specific antibodies at 48 h p.i. Scale bar: 20 μm. (b) The level of intracellular ROS was determined using CM-H2DCFDA. The geometric mean fluorescence intensity of cells was expressed as a fold induction relative to mock-infected cells. (c) HFFs were treated with UV-irradiated virus at an m.o.i. of 0.1, and the level of ISG54 mRNA was measured by quantitative RT-PCR at 5 h p.i. The level of GAPDH mRNA served as an internal control. Data are presented as mean±SD of triplicate samples; ns, non-significant, **, $P < 0.01$ (one-way ANOVA and Tukey's Multiple Comparison Test).

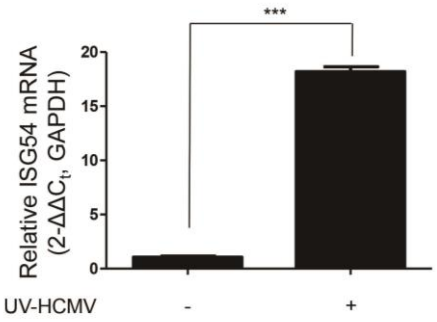
a


IE1/IE2
Nrf2
Hoechst
Merge
HCMV
-
+
UV
1.
2.
3.
4.
5.
6.
7.
8.
9.
10.
11.
12.
20 µm

b


**
**
ns
Fold induction in ROS
0
1
2
3
4
HCMV
-
+
UV-
treated

c


***
Relative ISG54 mRNA
(2-ΔΔC$_t$, GAPDH)
0
5
10
15
20
UV-HCMV
-
+

amount of intracellular ROS was measured, using DCFDA, a fluorescence-based ROS indicator. The level of ROS was increased in both cells treated with normal or UV-irradiated virus (Fig. III-4b). Because Nrf2 activation was not observed in cells treated with UV-irradiated virus, these data suggest that Nrf2 was activated by HCMV infection, not by the increased level of intracellular ROS that might have occurred in response to viral infection.

To exclude the possibility that UV-irradiation, prior to infection, might inhibit the infectivity of the virus, the induction of interferon stimulated gene 54 (ISG54) expression by UV-irradiated virus was tested. Recognition of viral DNA by a cytosolic DNA sensor, Z-DNA-binding protein 1 (ZBP1), was previously reported to activate interferon regulatory factor 3 (IRF3) in HCMV-infected cells (DeFilippis *et al.*, 2010), while the activation of IRF3 is well-known to induce ISG54 expression (Andersen *et al.*, 2008). HFFs were treated with UV-irradiated virus at an m.o.i. of 0.1. Total RNAs were isolated at 5 h p.i., and the level of ISG54 mRNA was analyzed by quantitative RT-PCR. The amount of ISG54 mRNA was significantly induced by the treatment (Fig. III-4c), indirectly indicating that UV-irradiated virus successfully penetrated into the target cells.

2.5 UL38 increases HO-1 expression in an Nrf2-independent manner.

It has been reported that the HCMV UL38 protein has multiple effects on the stress response system of the host cell. For examples, UL38 was reported to inhibit the TSC1/2-mediated response which suppresses mTOR activation under stressed conditions (Moorman *et al.*, 2008). Also, UL38 protected cells from ER-stress induced apoptosis (Xuan *et al.*, 2009). Because these two effects were mediated by different domains of UL38 (Qian *et al.*, 2011), this protein may have several mechanisms to modulate the host

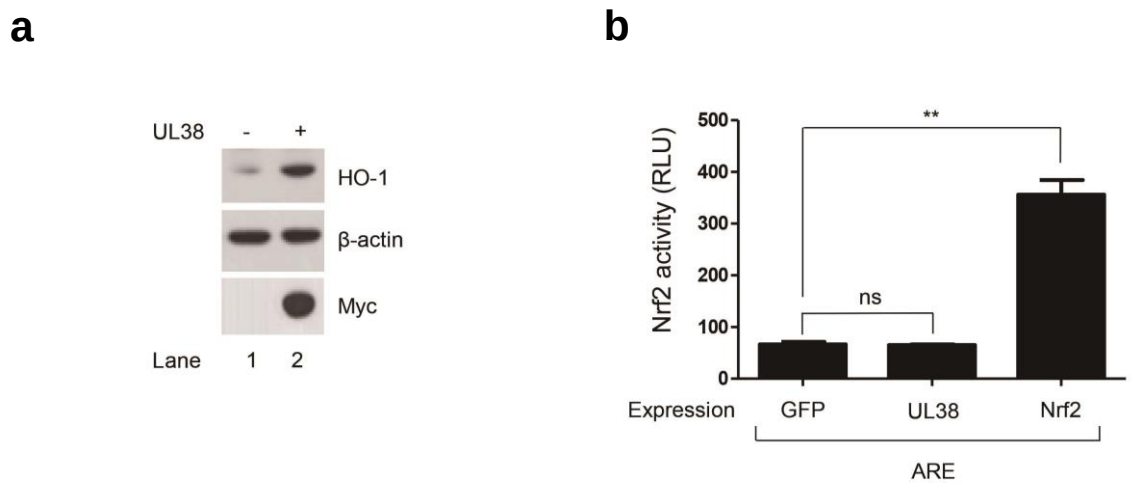


Figure III-5. Effects of UL38 expression on HO-1 expression and Nrf2 activity.

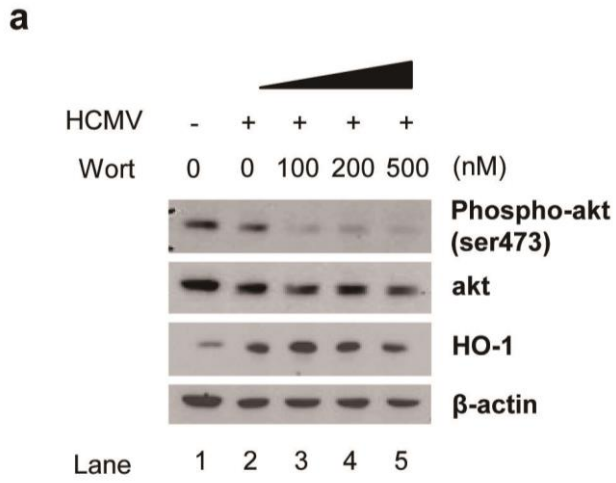
(a) The level of HO-1 was measured in HFFs overexpressing the HCMV UL38 protein by immunblotting analysis using a HO-1-specific antibody. The expression of UL38 was confirmed by anti-myc immunobloting. (b) HFFs were co-transfected with an ARE-driven luciferase reporter plasmid and the plasmid expressing GFP, UL38 or Nrf2. Total protein lysates were harvested at 2 days post-transfection, followed by the activity assay. Data are presented as mean±SD of duplicate samples; ns, non-significant, **, $P < 0.01$ (one-way ANOVA and Tukey's Multiple Comparison Test).

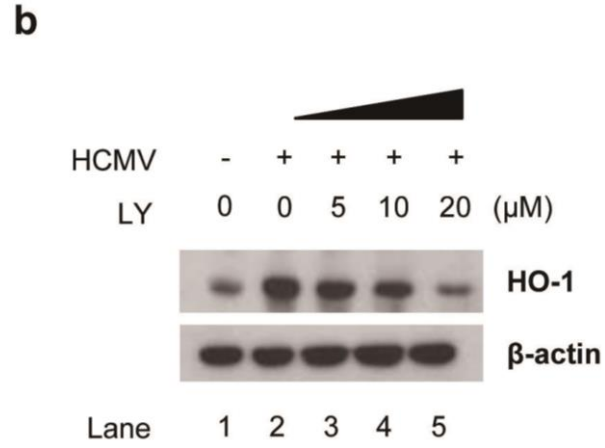
system. To test the possible involvement of UL38 in HCMV-mediated activation of Nrf2, HFFs stably expressing UL38, tagged with the myc epitope, were generated in the same manner as TERT-expressing cells. The expression of UL38 was confirmed by the Western blotting using a myc-specific antibody. UL38-expressing cells showed increased level of HO-1 expression (Fig. III-5a), suggesting that UL38 might be involved in the activation of the Nrf2 signaling pathway.

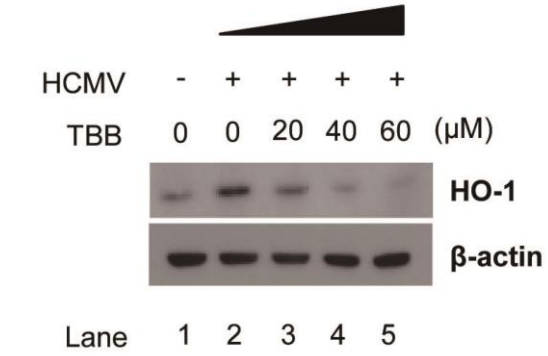
To test this possibility, HFFs were co-transfected with an ARE-driven luciferase reporter plasmid and expression plasmids for GFP, UL38, or Nrf2. Luciferase activity was determined in the lysates 2 days after transfection. UL38 expression did not influence luciferase activity, while it was induced by the expression of Nrf2 (Fig. III-5b). These data suggested that UL38 did not induce Nrf2 activation. Additional studies will be needed to find a viral factor(s) involved in the HCMV-mediated activation of Nrf2.

2.6 CK2 kinase is involved in HCMV-mediated activation of Nrf2

The PI3K pathway has been shown to mediate Nrf2 activation in various experimental settings (Nakaso *et al.*, 2003; Wang *et al.*, 2008). Because HCMV infection has been reported to activate the PI3K pathway, we tested whether this pathway is involved in HCMV-mediated activation of Nrf2 (Johnson *et al.*, 2001). HFFs were infected with HCMV, and at 24 h p.i., the cells were incubated with increasing doses of two different PI3K inhibitors, Wortmannin and LY294002, for an additional 24 h. Total protein extracts were prepared, and immunoblot analysis of Akt phosphorylation (Ser473), a downstream substrate of PI3K, was performed. Uninfected cells showed high levels of Akt phosphorylation, and HCMV infection did not significantly affect these levels (Fig. III-6a, lanes 1 and 2). Treatment with Wortmannin decreased Akt phosphorylation,


a
HCMV - + + + +
Wort 0 0 100 200 500 (nM)
Phospho-akt (ser473)
akt
HO-1
β-actin
Lane 1 2 3 4 5


b
HCMV - + + + +
LY 0 0 5 10 20 (μM)
HO-1
β-actin
Lane 1 2 3 4 5


c
HCMV - + + + +
TBB 0 0 20 40 60 (μM)
HO-1
β-actin
Lane 1 2 3 4 5

d

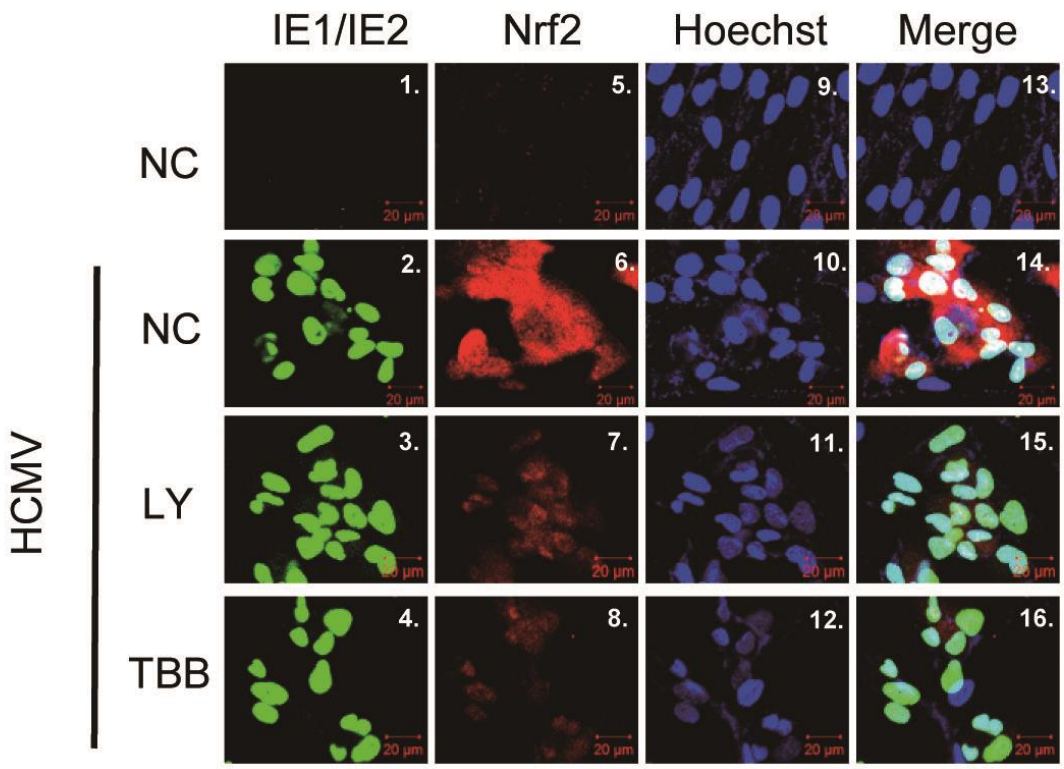


Figure III-6. Involvement of CK2 in HCMV-mediated Nrf2 activation. (a,b,c) HFFs were infected with HCMV, and at 24 h p.i., the cells were incubated with increasing doses of Wortmannin (a), LY294002 (b), or TBB (c) for an additional 24 h. Total protein extracts were prepared and analyzed by immunoblotting using phospho-akt- (Ser473), total akt-, HO-1-, and β-actin-specific antibodies. (d) HFFs were infected with HCMV and treated with the respective inhibitors. Cells monolayers were examined by IF using IE1/IE2- (green) and Nrf2- (red) specific antibodies. Scale bar: 20 µm.

indicating that the PI3K pathway was indeed inhibited by this chemical, which did not affect the HCMV-mediated induction of HO-1 (Fig. III-6a, lanes 2 to 5). These results suggest that the PI3K pathway is likely not involved in HCMV-mediated Nrf2 activation.

To be certain, the effect of LY294002 on HO-1 expression was also tested. Unlike Wortmannin, LY294002 treatment (20μM) suppressed the HCMV-mediated induction of HO-1 expression (Fig. III-6b). Because LY294002 is known to inhibit not only PI3K but also CK2, we tested whether inhibiting CK2 would affect HCMV-mediated HO-1 induction (Gharbi *et al.*, 2007). HCMV-infected HFFs were treated with a CK2 inhibitor, 4,5,6,7-tetrabromobenzotriazole (TBB) and examined using immunoblot analysis. TBB treatment inhibited the HCMV-mediated increase of HO-1 expression in a dose-dependent manner, suggesting that CK2 may be involved in Nrf2 activation by HCMV (Fig. III-6c).

To confirm these results, Nrf2 expression in LY294002- and TBB-treated cells was also analyzed by immunofluorescence detection of IE1/IE2 and Nrf2. In untreated control cells, HCMV infection increased Nrf2 protein levels (Fig. III-6d, compare panels 5 and 6), but LY294002 (20μM) and TBB (60μM) treatments abolished this increase without affecting IE1/IE2 expression (Fig. III-6d, compare panel 6 with panels 7 and 8, and panel 2 with panels 3 and 4). Together, these data suggest that CK2 activity is required for HCMV-mediated Nrf2 activation.

Previous study showed the role of CK2 in HCMV-mediated activation of the canonical NF-κB pathway, especially in the immediate-early stage of infection (Nogalski *et al.*, 2007). However, the regulation of CK2 activity in the entire HCMV life cycle is currently not known. Our data suggest that HCMV infection could activate CK2 at later time of infection. Although this finding should be further consolidated, it suggests a novel effect of HCMV infection.

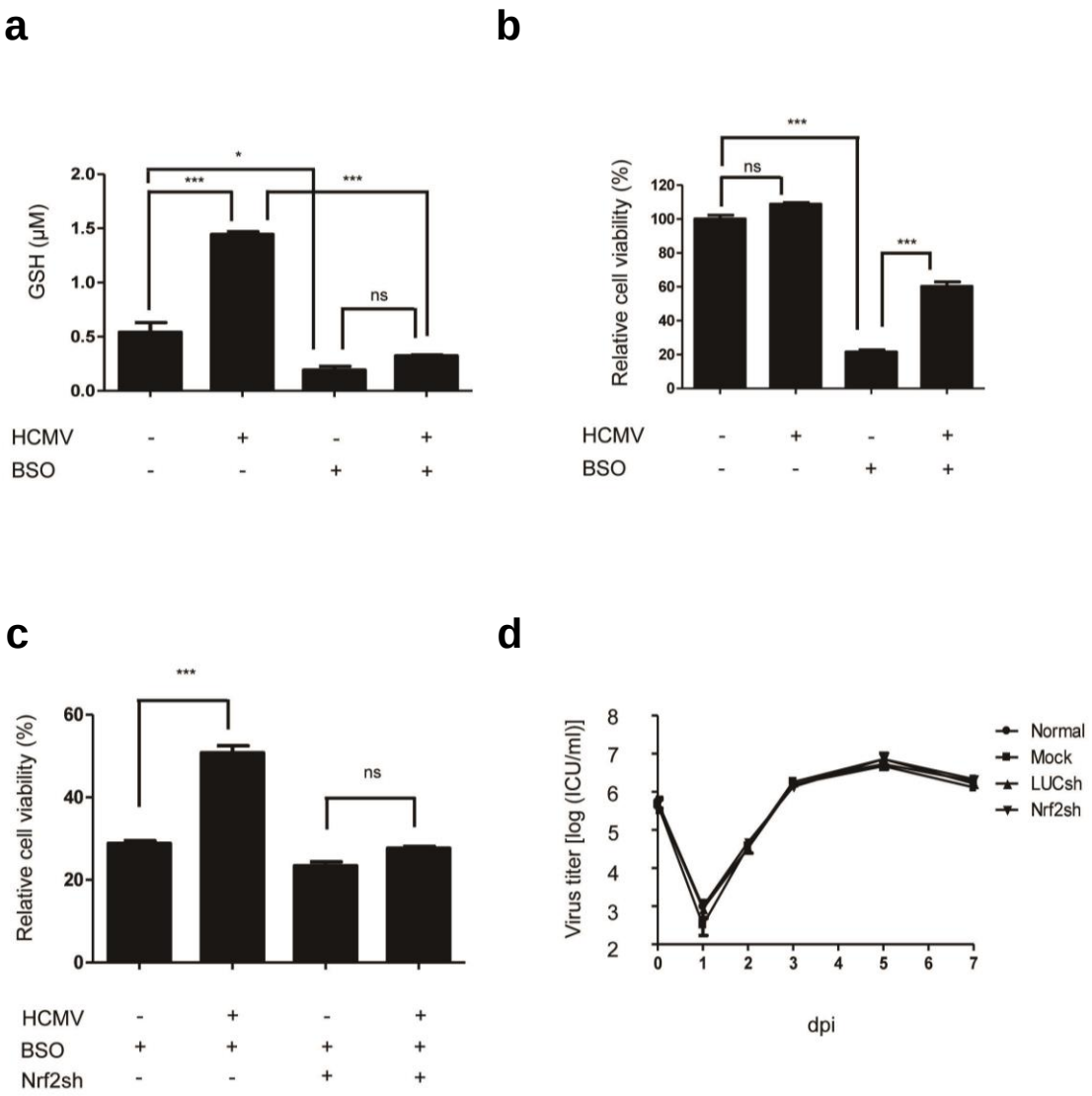
2.7 Nrf2 is involved in HCMV-mediated protection of host cells against oxidative stress

Nrf2 and its downstream genes are known to play a central role in cellular defense against oxidative stress. Based on these data, the possible role of the HCMV-mediated Nrf2 activation in the context of oxidative stress was investigated. HFFs were infected with HCMV for 2 h and then cultured in new medium containing 2mM BSO, a potent inducer of oxidative stress that functions by inhibiting the synthesis of the antioxidant glutathione. One day later, cellular glutathione levels were measured. HCMV infection increased the level of glutathione by approximately 3 fold compared to the uninfected control (Fig. III-7a, compare bars 1 and 2). BSO treatment reduced the glutathione levels in both control and HCMV-infected cells to similar low levels, indicating the synthesis of glutathione was effectively inhibited (Fig. III-7a, compare bars 1 and 3, and bars 2 and 4).

The effect of BSO-induced oxidative stress on cell viability was measured using a MTT assay. BSO treatment resulted in massive cell death and reduced cell viability to 20% compared to the untreated control cells (Fig. III-7b, compare bars 1 and 3). In the absence of BSO, HCMV infection did not affect cell survival under the experimental condition used (Fig. III-7b, compare bars 1 and 2). In the presence of BSO, however, HCMV infection significantly increased the percentage of viable cells (Fig. III-7b, compare bars 3 and 4). The difference was almost 3 fold, indicating that HCMV may protect host cells from BSO-induced cell death.

The possible involvement of Nrf2 in this process was also examined using shRNAs. HFFs expressing a control luciferase shRNA (LUCsh) or Nrf2 shRNA (Nrf2sh) were infected with HCMV and treated with BSO as described above. In HFFs expressing the control shRNA, the HCMV-infected cells were more resistant to BSO-induced cell

Figure III-7. The possible role of Nrf2 in HCMV-mediated cell protection against oxidative stress. (a) HFFs infected or mock-infected with HCMV were treated or left untreated with BSO (2mM) at 2 h p.i. The intracellular concentration of GSH was measured at 24 h following BSO treatment. (b) The viability of mock-infected and HCMV-infected cells was analyzed at 4 days following BSO treatment. (c) HFFs expressing either type of shRNA were treated as mentioned above, and the cell viability was measured. (d) HFFs expressing shRNAs, normal HFFs and mock-transduced HFFs were infected with HCMV. The titers of progeny virus in culture supernatant were measured at the times indicated using infectious center assays. Data are presented as mean ±SD of triplicate samples; ns, non-significant; *, $P< 0.05$; ***, $P < 0.001$ (one-way ANOVA and Tukey's Multiple Comparison Test).


a
b
c
d
GSH (μM)
2.0
1.5
1.0
0.5
0.0
*
***
***
ns
HCMV - + - +
BSO - - + +
Relative cell viability (%)
120
100
80
60
40
20
0
***
ns
***
HCMV - + - +
BSO - - + +
Relative cell viability (%)
60
40
20
0
***
ns
HCMV - + - +
BSO + + + +
Nrf2sh - - + +
Virus titer [log (ICU/ml)]
8
7
6
5
4
3
2
0 1 2 3 4 5 6 7
dpi
Normal
Mock
LUCsh
Nrf2sh

death (Fig. III-7c, compare bars 1 and 2). When Nrf2 expression was inhibited, however, HCMV no longer had a protective effect on the cells (Fig. III-7c, compare bars 3 and 4). In the absence of BSO, HCMV infection did not affect the viability of HFFs expressing either type of shRNA (data not shown).

The role of Nrf2 in viral replication under normal experimental conditions was also investigated. Nrf2sh cells were infected with HCMV, together with three control cell types including normal, mock-transduced, and LUCsh cells. The culture supernatants were collected at various time points, and viral titers were determined using an infectious center assay. The amount of progeny virus was similar between all four samples (Fig. III-7d). Similar data were obtained using a primary JHC strain (data not shown). These data suggest that HCMV-mediated Nrf2 activation may protect cells from oxidative stress but does not seem to play a noticeable role under normal culture conditions.

3. Discussion

Nrf2 is one of the key transcription factors involved in regulating the expression of antioxidative stress genes. In this study, we demonstrated that HCMV infection of HFFs could activate Nrf2, thus resulting in increased HO-1 expression by the CK2 pathway. When host cells were stressed by BSO, HCMV-infected cells survived more efficiently than uninfected control cells through the activation of Nrf2. Our data suggest that HCMV may control cellular Nrf2 for its own benefit, e.g., to overcome the oxidative stress imposed by itself or the host's disease status.

It has recently been reported that hsp90 expression is increased following

antioxidant or heat shock treatment and subsequently interacts with Keap1, which is phosphorylated by CK2, leading to the dissociation of the Keap1-Nrf2 complex and the stabilization of Nrf2 (Niture & Jaiswal, 2010). HCMV has been shown to increase hsp90 expression, and our data indicate that HCMV-mediated Nrf2 activation was abolished following treatment with CK2 inhibitors (Stanton *et al.*, 2007). Altogether, these data would suggest that HCMV activates Nrf2 by manipulating the hsp90 and Keap1 interaction. These data may also explain how HCMV infection activates Nrf2 independent of ROS production.

Our data showed that viral UL38 protein induced HO-1 expression probably through Nrf2-independent mechanism. UL38 is known to activate mTOR and previous reports suggest a possible link between mTOR and oxidative stress. For instance, hydrogen peroxide inhibits mTOR by activating AMPKα (Chen *et al.*, 2010). These data suggest that mTOR may be involved in UL38-mediated induction of HO-1 expression. Because direct involvement of mTOR in the regulation of antioxidative stress responses is not yet clear, this potential should be studied further.

It has recently been reported that Nrf2 is not activated by HCMV infection in HFFs expressing the catalytic subunit of human telomerase. It is not yet clear what contributed to this discrepancy (Tilton *et al.*, 2011). One possibility is the difference in the manner in which the telomerase-expressing cells were constructed. In our case, HFFs were used after only 5 to 6 passages following the initial retroviral transduction, while Tilton et al. established cell line through drug selection and subcloning.

Gene expression profile of HCMV-infected HFFs confirms that HO-1 and GCLC expression is induced by HCMV infection. It also suggests that expressions of other Nrf2 target genes are up-regulated (Browne *et al.*, 2001). These include glutamate-cysteine

ligase regulatory subunit (GCLM), glutathione S-transferase M3 (GSTM3), and glutathione reductase. However, the expression of NAD(P)H:quinone oxidoreductase 1 (NQO1), a prototypical Nrf2 target gene (Venugopal & Jaiswal, 1996), was highly decreased by HCMV infection, which is also confirmed in recent studies (Tilton *et al.*, 2011). These data suggest that HCMV infection may have differential effects on the expression of Nrf2 target genes. Consolidating this finding and investigating its molecular mechanism would provide a novel insight into the regulation of the antioxidative stress response. It would also be interesting to examine the role of NQO1 during HCMV infection and its downregulation may be beneficial to the virus or to the host cell.

Viral infections in general have been known to generate oxidative stress in host cells, which would impose negative effects on the virus because the host cell would be damaged as has been shown in the case of HIV, HSV, HBV, and HCV infections (Valyi-Nagy & Dermody, 2005) (Liu *et al.*, 2008) (Joyce *et al.*, 2009). Oxidative stress can be caused directly by the actual viral infection and/or an indirect consequence of the host inflammatory response. HCMV infection has been reported to induce oxidative stress in cell culture as well as a systemic inflammatory response in patients who present with a primary HCMV infection, suggesting that HCMV infection generates oxidative stress (Speir *et al.*, 1996; van de Berg *et al.*, 2010). Pre-existing inflammation could be another source of oxidative stress because HCMV has been shown to be reactivated from latency in several inflammatory diseases (Varani & Landini, 2011). To cope with these stresses, HCMV may have developed the mean(s) to survive or reduce the detrimental effects of oxidative stress, as in the case of HBV, which has also been shown to activate Nrf2 (Schaedler *et al.*, 2010).

Neither the viral growth rate nor the cell viability were significantly affected in

host cells engineered to express Nrf2 at low levels by knockdown. Therefore, it appears that Nrf2 does not play a significant role under unstressed conditions, even in the context of HCMV infection. The magnitude of the oxidative stress imposed by *in vitro* HCMV infection might not be severe enough for Nrf2 to show any visible effects. Indeed, it has been shown that Nrf2 does not have any clear phenotypic effects under unstressed conditions, as evidenced in experiments involving mice that were genetically modified to lack or overexpress Nrf2 (Calkins *et al.*, 2010; Chan *et al.*, 1996). When the cells were treated with BSO, however, the expression level of Nrf2 was important for the survival of host cells under stressful conditions. Taken together, it is possible that HCMV infection may render cells more resistant to oxidative stress, such as that induced by inflammatory responses, for its own benefit.

Our findings have implications for understanding the chronic inflammation characteristic of HCMV-related diseases. The cell-protective effect of Nrf2 might lead to increased survival of HCMV during oxidative stress and thus HCMV persistence under these conditions. Persistence would result in further oxidative stress, contributing to the establishment of chronic inflammation.

CHAPTER IV

Role of C/EBPα during HCMV infection as a downstream mediator of mTOR pathway

1. Backgrounds

HCMV DNA replication is initiated at the lytic origin of replication (oriLyt), a cis-acting sequence in the viral genome (Anders *et al.*, 1992; Anders & Punturieri, 1991; Hamzeh *et al.*, 1990; Masse *et al.*, 1992). Two sequence elements within oriLyt, called the essential regions 1 and 2, are necessary for viral DNA replication (Pari, 2008). The essential region 1 contains promoter activity, suggesting the involvement of transcription in the replication process (Xu *et al.*, 2004), and essential region 2 forms a RNA-DNA hybrid structure (Colletti *et al.*, 2007). Viral-encoded trans-acting factors are also required for viral DNA replication (Pari *et al.*, 1993). These include the 6 core replication proteins that are components of the viral DNA replication machinery and other proteins such as UL84, UL112/113, and IE2, which are known to be involved in the initiation and regulation of this process (Pari, 2008). The involvement of cellular factors such as nucleolin has also been reported (Strang *et al.*, 2010; Strang *et al.*, 2012). Nucleolin binds the viral protein UL44 and maintains the architecture of the viral DNA replication compartment (Strang *et al.*, 2012).

The CCAAT/enhancer-binding proteins (C/EBPs) are a family of basic leucine/zipper (bZIP) transcription factors (Tsukada *et al.*, 2011). The C/EBP family proteins form homodimers or heterodimers with other family members or with members of other transcription factor families (Agre *et al.*, 1989; Landschulz *et al.*, 1988; Vinson *et al.*, 1993). The intra-family dimers bind to the consensus sequence RTTGCGYAAY (R = A or G and Y = C or T) and regulate the expression of target genes (Osada *et al.*, 1996). C/EBPα, the founding member of this family, plays important roles in adipocyte differentiation, hematopoiesis, and cell cycle regulation (Freytag *et al.*, 1994; Lefterova *et*

al., 2008; Tontonoz *et al.*, 1994) (Radomska *et al.*, 1998; Scott *et al.*, 1992; Zhang *et al.*, 1997) (Johnson, 2005). C/EBPα exists as two isoforms, the full-length p42, and the N-terminal truncated p30 which is generated by translation initiation at a downstream AUG codon within the same transcript (Lin *et al.*, 1993). C/EBPα-p30 is a dominant-negative inhibitor of p42 and might play additional role(s) that are not yet clearly understood (Kirstetter *et al.*, 2008; Pabst *et al.*, 2001). Therefore, the transcriptional activity of C/EBPα at the consensus C/EBP sequence depends on the relative expression levels of the two C/EBPα isoforms. The p42/p30 ratio is regulated by various mechanisms involving transcriptional and translational controls (Calkhoven *et al.*, 2000; Cao *et al.*, 1991; Perrotti *et al.*, 2002; Yeh *et al.*, 1995).

C/EBPα is involved in the lytic cycles of two gamma-herpesviruses: KSHV and EBV. C/EBPα acts cooperatively with viral transactivators to activate viral lytic promoters and mediates the cell cycle arrest induced by viral proteins (Huang *et al.*, 2006; Wang *et al.*, 2003a; Wang *et al.*, 2003b; Wu *et al.*, 2003a; Wu *et al.*, 2002; Wu *et al.*, 2004a; Wu *et al.*, 2003b). Notably, the oriLyt regions of KSHV and EBV have C/EBPα binding sites, and the binding of C/EBPα to these sites enhances the replication of the viral genome (Huang *et al.*, 2006; Wang *et al.*, 2004). The HCMV oriLyt region also contains multiple C/EBPα binding sites (Kagele *et al.*, 2009). In this study, we investigated the effect of HCMV infection on C/EBPα using permissive fibroblasts. We found that HCMV infection increased the C/EBPα p42/p30 ratio and resulted in the translocation of C/EBPα-p42 to the viral DNA replication compartment. Our data suggested that the binding of C/EBPα to the oriLyt region might facilitate viral DNA replication by regulating the binding of UL44 to oriLyt.

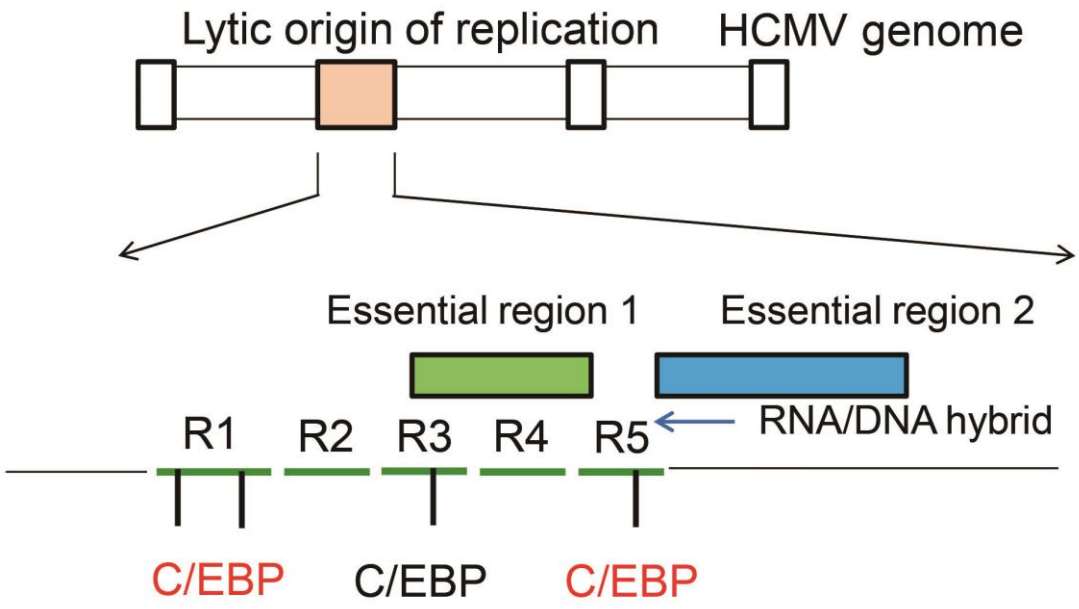


Figure IV-1. Schematic diagram of HCMV oriLyt. The genome replication of HCMV is initiated at the lytic origin of replication (oriLyt), the cis-acting region in the viral genome. The presence of two essential regions and C/EBP binding sites within oriLyt are shown. A previous study demonstrated that C/EBP binding sites in the R1 and R5 regions of oriLyt (shown in red), but not one in the R3 region, interacted with the C/EBPα protein in HCMV-infected cells, by using ChIP analysis. See text for the details (from Pari *et al.*, 2008).

2. Results

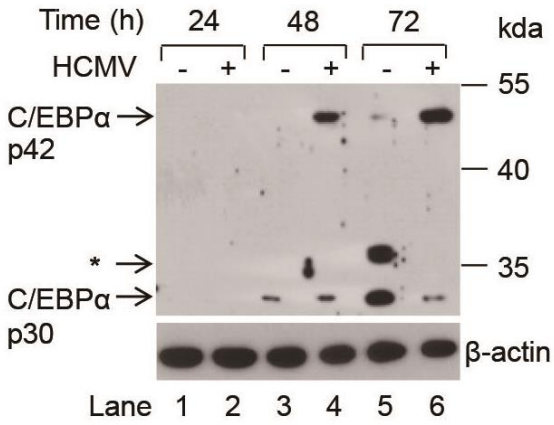
2.1 Effect of HCMV infection on the expression, localization, and DNA binding activity of C/EBPα

To test the effect of HCMV infection on C/EBPα expression, HFFs were infected with HCMV (Towne strain). The total cellular proteins were extracted at the indicated time points and subjected to immunoblotting analysis using an antibody to C/EBPα. C/EBPα exists in two different isoforms, p42 and p30, with distinct expression patterns during the culture period. In uninfected HFFs, the level of p42 was undetectable or very low over the 72 h culture period. The expression of p30 was initially undetectable but greatly increased at 72 h of culture (Fig. IV-2a, compare lanes 1, 3, and 5). It is well-known that 72 h culture of confluent HFFs lead to the induction of cell quiescence, which might have resulted in the up-regulation of C/EBPα-p30 expression. In HCMV-infected cells, the expression of p42 was greatly increased at 48 h p.i. and this induction was maintained thereafter, while the level of p30 did not significantly change during the entire culture period unlike in uninfected cells (Fig. IV-2a, compare lanes 2, 4, and 6). These data indicated that the viral infection led to an increased p42/p30 ratio, which is an important determinant of the transcriptional activity of C/EBPα.

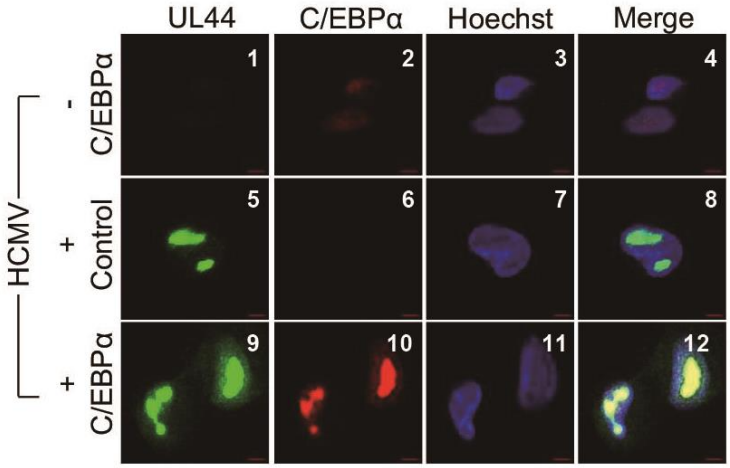
The expression level and intracellular localization of the C/EBPα protein at 48 h p.i. were also investigated using immunofluorescence. The HCMV infection increased the fluorescence intensity of C/EBPα (red), whereas no change was observed when the purified IgG from normal serum was used instead of a C/EBPα-specific antibody (Fig. IV-2b, panels 2, 6, and 10). C/EBPα was present within the nuclei of the infected cells in areas with a globular shape that resembled the viral DNA replication compartment (Fig.

Figure IV-2. Effects of HCMV infection on C/EBPα. (a) HFFs were infected with HCMV at an m.o.i. of 3 and harvested at the indicated time points. The expression level of C/EBPα was measured by immunoblotting analysis using a C/EBPα-specific antibody. β-actin protein levels were detected as a loading control. Asterisk (*) indicates an unidentified protein of C/EBPα origin, probably a SUMOylated C/EBPα-p30 (Muller *et al.*, 2010). (b) HFFs were infected with HCMV, and examined by immunofluorescence at 48 h p.i. using UL44- (green) and C/EBPα- (red) specific antibodies. IgG purified from preimmune serum served as a negative control for the C/EBPα-specific antibody. Nuclei were stained with Hoechst stain (blue). Scale bar: 5 μm. (c) HFFs were infected with HCMV, and the DNA binding activity of C/EBPα was analyzed by chromatin immunoprecipitation using the C/EBPα-specific antibody and primers to amplify the R1 and R5 regions of HCMV oriLyt. IgG from normal rabbit serum was included as a control.

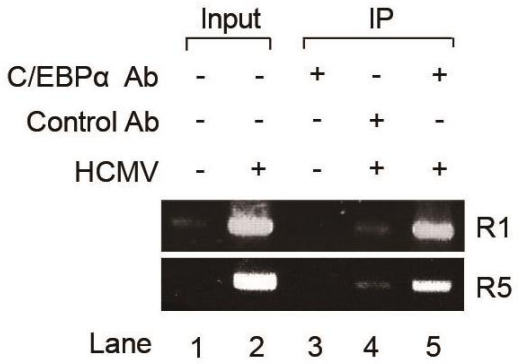
a


Time (h)
24
48
72
kda
HCMV
- + - + - +
55
C/EBPα →
p42
40
* →
35
C/EBPα →
p30
β-actin
Lane 1 2 3 4 5 6

b


UL44
C/EBPα
Hoechst
Merge
HCMV
-
C/EBPα
1
2
3
4
+
Control
5
6
7
8
+
C/EBPα
9
10
11
12

c


Input
IP
C/EBPα Ab - - + - +
Control Ab - - - + -
HCMV - + - + +
R1
R5
Lane 1 2 3 4 5

IV-2b, panels 10 and 11). The fluorescence corresponding to C/EBPα also co-localized with that of the viral protein UL44 (green), which is known to be localized to this sub-nuclear structure (Fig. IV-2b, panel 12) (Penfold & Mocarski, 1997). These results indicated that C/EBPα accumulated in the viral DNA replication compartment in infected cells during the late stage of infection. According to Figure 1A, this C/EBPα consists mostly of the p42 isoform.

It was previously reported that C/EBPα binds to the oriLyt region of the HCMV genome (Kagele *et al.*, 2009). To confirm this binding, HFFs were infected with HCMV and fixed at 48 h p.i. The DNA was immunoprecipitated using C/EBPα-specific or nonspecific control antibodies, followed by PCR amplification using primers specific to the R1 and R5 regions of oriLyt; these regions were previously observed to interact directly with C/EBPα. The uninfected control samples and the samples that were immunoprecipitated using the nonspecific antibody showed little enrichment of the R1 and R5 regions (Fig. IV-2c, lanes 3 and 4), but these regions were amplified in the DNA samples immunoprecipitated using the C/EBPα-specific antibody (Fig. IV-2c, lane 5). These data indicated that C/EBPα bound to the HCMV oriLyt region, suggesting that this transcription factor might play a role in viral DNA synthesis.

2.2 mTOR-mediated activation of host cell translation is required for the induction of C/EBPα-p42 expression by HCMV infection

To identify the step at which the HCMV infection regulated C/EBPα expression, the level of C/EBPα mRNA was measured. HFFs were infected with HCMV and the total RNA was extracted and analyzed by Northern blotting using a ^{32}P-labeled cDNA probe for C/EBPα. The level of C/EBPα mRNA was high in uninfected control cells (Fig. IV-3a,

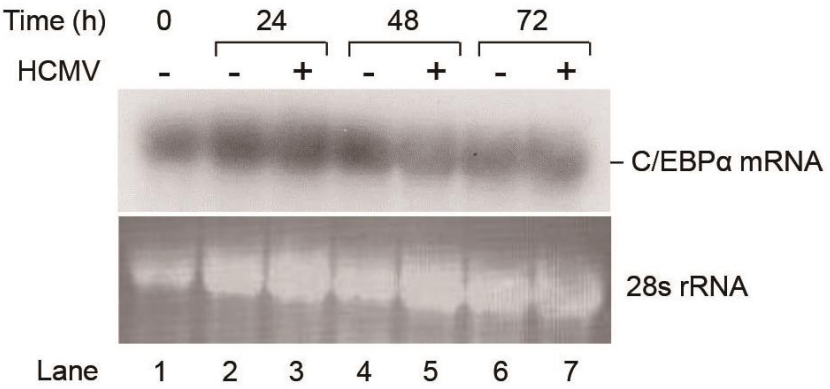
lanes 1, 2, 4, and 6) and changed negligibly upon HCMV infection (Fig. IV-3a, compare lanes 2, 4, 6 with 3, 5, 7). These results suggested that the HCMV-mediated regulation of C/EBPα expression most likely occurred at the post-transcriptional level.

Next, the stability of C/EBPα-p42 protein was tested. HFFs were infected with HCMV and treated with 100μM of cycloheximide (CHX) to inhibit protein synthesis at 2 d p.i. Total proteins were harvested at the indicated time points after CHX treatment, followed by immuoblotting analysis. HCMV infection up-regulated the level of C/EBPα-p42 (Fig. IV-3b, compare lanes 1 and 2). When protein synthesis was inhibited, however, the level of C/EBPα-p42 in HCMV-infected cells was rapidly decreased, reaching that of uninfected cells at 12 h post-treatment (Fig. IV-3b, compare lanes 2, 3, and 4). The amounts of the UL44 and β-actin proteins were maintained at high levels during CHX treatment. Therefore, the C/EBPα-p42 protein seems to have a relatively short half-life, approximately 6 h, suggesting that continuing translation might be needed for high level expression of C/EBPα-p42 in HCMV-infected cells.

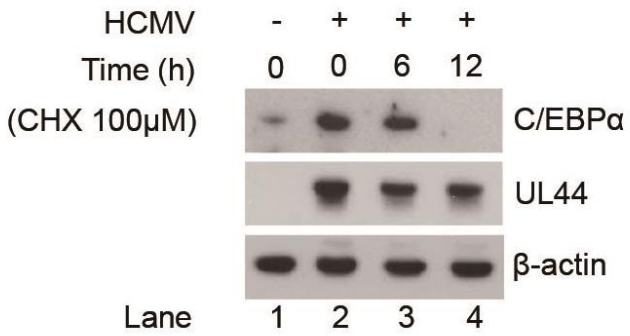
Because HCMV infection stimulates translation of host cell by activating mTOR (Moorman & Shenk, 2010), the effect of PP242, which is known to inhibit mTOR, on the HCMV-mediated induction of C/EBPα expression was tested; PP242 is an inhibitor targeting the ATP-binding site of mTOR (Feldman *et al.*, 2009). HFFs were infected with HCMV and treated with 0.5 and 1 μM of PP242 at 1 d p.i. The C/EBPα expression level in the treated cells at 2 d p.i. was analyzed by Western blotting. HCMV infection increased the expression of C/EBPα-p42 (Fig. IV-3c, compare lanes 1 and 2). Treatment with 1 μM PP242 abolished the HCMV-mediated induction of p42 expression, slightly decreased the level of UL44, and did not affect that of β-actin (Fig. IV-3c, compare 2, 3, and 4). These data indicated that the mTOR-mediated activation of host cell translation

Figure IV-3. Involvement of mTOR-mediated translational control in the induction of C/EBPα expression in HCMV-infected cells. (a) C/EBPα mRNA levels were determined by Northern blot hybridization using a ^{32}P-labeled cDNA probe specific for C/EBPα. 28S ribosomal RNA levels served as a loading control. (b) HFFs infected with HCMV were treated with cycloheximide at 2 d p.i. and harvested at the indicated time points after the treatment. The expression levels of C/EBPα, UL44, and β-actin were determined by immunoblotting analysis, using specific antibodies to respective proteins. (c) HFFs were infected with HCMV and treated with PP242, an mTOR inhibitor, at 1 d p.i. Total protein were extracted at 2 d p.i., followed by Western blot analysis using C/EBPα-, UL44-, and β-actin-specific antibodies.

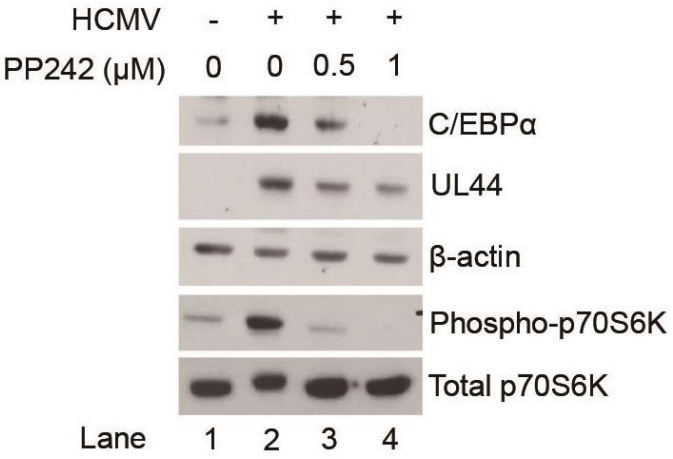
a


Time (h) 0 24 48 72
HCMV - - + - + - +
C/EBPα mRNA
28s rRNA
Lane 1 2 3 4 5 6 7

b


HCMV - + + +
Time (h) 0 0 6 12
(CHX 100μM)
C/EBPα
UL44
β-actin
Lane 1 2 3 4

c


HCMV - + + +
PP242 (μM) 0 0 0.5 1
C/EBPα
UL44
β-actin
Phospho-p70S6K
Total p70S6K
Lane 1 2 3 4

might be required for the induction of C/EBPα-p42 expression by HCMV infection.

2.3 Viral DNA replication is required for the induction of C/EBPα-p42 expression by HCMV infection

The possible involvement of the viral DNA replication process in the regulation of C/EBPα expression was investigated. HFFs were infected with HCMV in the absence or presence of 30 μM ganciclovir (GCV), an inhibitor of viral DNA synthesis. Cells were harvested at 72 h p.i., followed by Western blot analysis to monitor the C/EBPα expression level. In control cells, HCMV infection increased the level of C/EBPα-p42 expression (Fig. IV-4, compare lanes 1 and 3), which was highly suppressed by treatment with GCV (Fig. IV-4, compare lanes 3 and 4), indicating the requirement for the initiation of viral DNA replication. Treatment with GCV did not change the phosphorylation status of the p70S6 kinase (Fig. IV-4, compare lanes 3 and 4), suggesting that viral DNA replication might be involved in the regulation of p42 expression downstream of the mTOR pathway. Together, these data suggested that the initiation of viral DNA replication was necessary for the HCMV-mediated control of C/EBPα-p42 expression.

2.4 CHOP overexpression reduces the interaction between C/EBPα and oriLyt

The data described above suggested that C/EBPα might play a role in the replication of the HCMV DNA. To understand the underlying mechanism, HFFs were engineered to stably express the CHOP protein that inhibits the DNA binding activity of C/EBPα in a dominant-negative fashion by its direct association with C/EBPα (Ron & Habener, 1992). HFFs were transduced with retroviral vectors expressing CHOP and GFP from a bicistronic construct. The transduced cells were enriched for GFP-expressing cells,

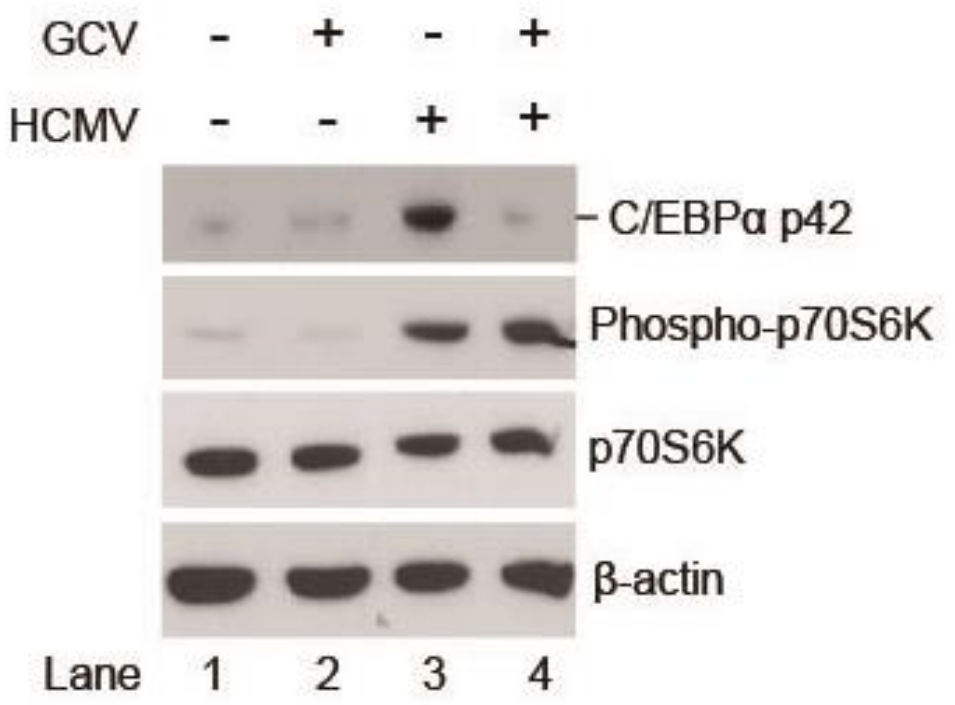


Figure IV-4. Effects of GCV on HCMV-mediated regulation of C/EBPα expression and p70S6K phosphorylation. HFFs were infected with HCMV in the absence or presence of 30 μM GCV. Protein lysates were prepared at 3 d p.i., followed by immunoblotting analysis using specific antibodies to C/EBPα, phospho-p70S6K (Thr389), total p70S6K, and β-actin.

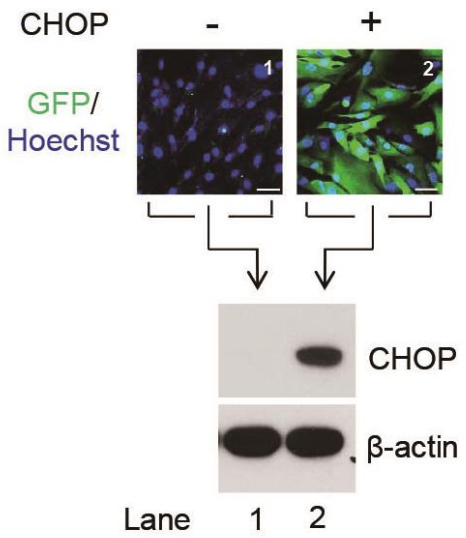
and the remaining GFP-negative cells served as a negative control. The GFP expression was confirmed by immunofluorescence (Fig. IV-5a, upper panel). The CHOP expression was also confirmed by immunoblotting analysis using a specific antibody. The CHOP protein was virtually undetectable in the GFP-negative cells, whereas it was present at high levels in the GFP-positive cells (Fig. IV-5a, lower panel).

To study the effect of CHOP overexpression, HFFs expressing CHOP or control GFP-negative cells were infected with HCMV. Chromatin immunoprecipitation was performed at 48 h p.i. using an antibody specific to C/EBPα and primers to amplify the R1 and R5 regions of oriLyt. In the control cells, HCMV infection induced the binding of C/EBPα to the R1 and R5 regions (Fig. IV-5b, compare lanes 3 and 4). The association of C/EBPα with the R5 region but not the R1 region was suppressed in the cells overexpressing the CHOP protein (Fig. IV-5b, compare lanes 5 and 6). These results suggested that C/EBPα directly bound to the R5 region of oriLyt and that elevated CHOP expression could inhibit this binding of C/EBPα. It remains unclear how CHOP overexpression selectively inhibited the binding of C/EBPα to the R5 region. The R5 region has unique structures not found in the R1 region such as the RNA-DNA hybrid, which would account for the differential effect of CHOP overexpression on C/EBPα.

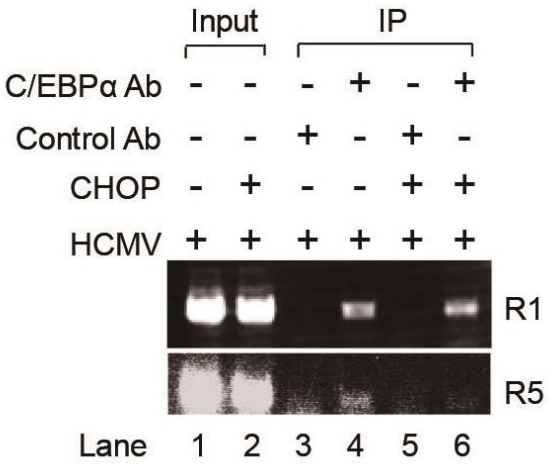
CHOP expression is induced by the unfolded protein response (UPR) through the action of ATF4 (Wang *et al.*, 1996), and HCMV infection increases the expression of ATF4 (Isler *et al.*, 2005). Therefore, the kinetics of endogenous CHOP expression in HCMV-infected cells was investigated by Western blot analysis using a CHOP-specific antibody. The CHOP protein was undetectable in uninfected control cells (Fig. IV-5c, lanes 1). When HFFs were infected with HCMV, the CHOP protein was not detected at 2 d p.i., but its level highly increased at 3 d p.i. (Fig. IV-5c, lanes 2, 3, and 4). Because the

Figure IV-5. Effects of CHOP overexpression on the interaction between C/EBPα and oriLyt. (a) GFP-positive cells acquired by FACS were immunostained with an anti-GFP antibody along with the control GFP-negative cells (upper panel). Nuclei were stained with Hoechst stain (blue). Scale bar: 50 μm. Protein lysates were prepared from each type of cells and the expression level of CHOP was determined by immunoblotting analysis using CHOP- and β-actin- specific antibodies (lower panel). (b) HFFs overexpressing CHOP and the control cells were infected with HCMV, and the interaction between C/EBPα and oriLyt was analyzed by chromatin immunoprecipitation as described in the legend of Fig. IV-1C. (c) HFFs were infected with HCMV and protein lysates were obtained at the indicated time points. The level of endogenous CHOP protein was determined by immunoblotting analysis using antibodies specific to CHOP and β-actin.

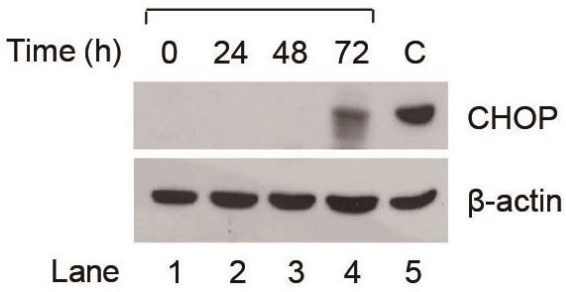
a


CHOP
-
+
GFP/
Hoechst
1
2
CHOP
β-actin
Lane 1 2

b


Input
IP
C/EBPα Ab - - - + - +
Control Ab - - + - + -
CHOP - + - - + +
HCMV + + + + + +
R1
R5
Lane 1 2 3 4 5 6

c


Time (h) 0 24 48 72 C
CHOP
β-actin
Lane 1 2 3 4 5

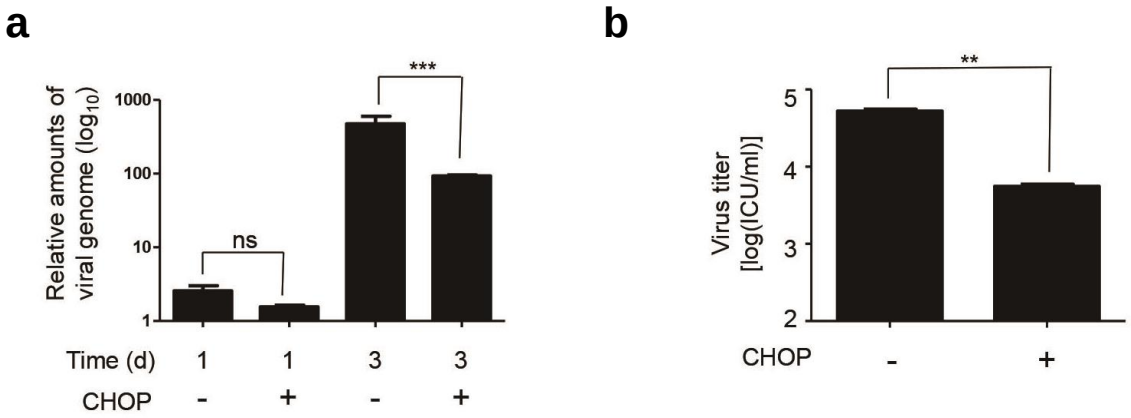
binding of C/EBPα to the oriLyt region occurs before the upregulation of CHOP expression, the HCMV-mediated induction of endogenous CHOP expression could not have suppressed the binding of C/EBPα to oriLyt at least during the first 48 h p.i.

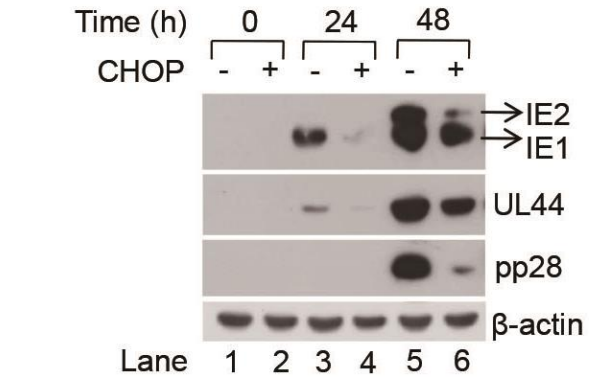
2.5 The binding of C/EBPα to the oriLyt region is required for efficient viral DNA replication

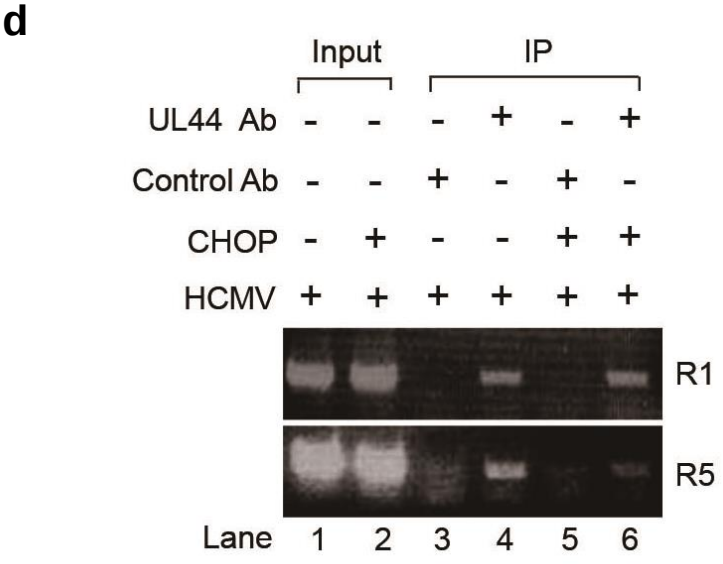
The effect of elevated CHOP protein level on viral DNA synthesis was investigated. Control and CHOP-overexpressing cells were infected with HCMV. The genomic DNAs were extracted at the indicated time points and subjected to real-time quantitative PCR analysis. At 1 d p.i., the amount of the viral genome was low in both the CHOP-overexpressing and control cells (Fig. IV-6a, compare lanes 1 and 2), but at 3 d p.i., it was 5-fold lower in the CHOP-overexpressing cells than in the controls (Fig. IV-6a, compare lanes 3 and 4). Consistent with this result, the CHOP-overexpressing cells produced approximately 10-fold fewer viral progeny than did the control cells at 3 d p.i., as measured by an infectious center assay (Fig. IV-6b). These results suggested that the interaction between C/EBPα and the oriLyt region was required for the efficient replication of the viral genome and, ultimately, for the increased production of viral progeny.

To exclude the possibility that the elevated CHOP expression indirectly reduced viral DNA replication by inhibiting the expression of the viral IE and E genes, the expression of selected viral genes in the control and CHOP-overexpressing cells was compared using immunoblotting analysis. The elevated CHOP expression reduced the levels of IE1 and UL44 at 1 d p.i. (Fig. IV-6c, compare lanes 3 and 4). These differences were less prominent at 2 d p.i., when the interaction between C/EBPα and the oriLyt

Figure IV-6. Involvement of C/EBPα in viral DNA replication. (a-d) HFFs overexpressing CHOP were infected with HCMV along with the control cells. (a) Genomic DNA was extracted at 1 and 3 d p.i., and subjected to real-time quantitative PCR using primers specific to the viral and cellular DNA. The amount of cellular DNA served as an internal control. (b) Culture supernatants were harvested at 72 h p.i., and the titers of progeny virus in culture supernatant were measured using infectious center assays. (c) Total proteins were harvested at the indicated time points, and the levels of viral IE1/IE2, UL44, and pp28 proteins were analyzed by immunoblotting using antibodies specific to each protein. (d) Control and CHOP-overexpressing cells were infected with HCMV, and fixed at 48 h p.i. The binding of UL44 to the oriLyt region was measured by chromatin immunoprecipitation using an UL44-specific antibody and primers which amplifies the R1 and R5 regions of oriLyt. IgG purified from normal mouse serum was used as a negative control. Statistical analysis was done as described in the Materials & Methods.


a
Relative amounts of viral genome (log_{10})
1000
100
10
1
***
ns
Time (d) 1 1 3 3
CHOP - + - +
b
Virus titer [log(ICU/ml)]
5
4
3
2
**
CHOP - +


c
Time (h) 0 24 48
CHOP - + - + - +
IE2
IE1
UL44
pp28
β-actin
Lane 1 2 3 4 5 6


d
Input
IP
UL44 Ab - - - + - +
Control Ab - - + - + -
CHOP - + - - + +
HCMV + + + + + +
R1
R5
Lane 1 2 3 4 5 6

region had already occurred (Fig. IV-6c, compare lanes 5 and 6). The expression levels of IE2 and pp28, which are both known to be dependent on viral DNA synthesis, were highly decreased in the CHOP-overexpressing cells (Fig. IV-6c, compare lanes 5 and 6). These data suggested that the elevated CHOP expression suppressed viral DNA replication by inhibiting the binding of C/EBPα to the oriLyt region, but not by affecting the expression of the viral IE and E genes.

The R1 and R5 region of oriLyt that directly interacts with C/EBPα is also bound by other viral proteins such as UL44 (Kagele *et al.*, 2009). The effect of elevated CHOP expression on the association between oriLyt and UL44 was tested. The CHOP-overexpressing and control cells were infected with HCMV, followed by chromatin immunoprecipitation at 48 h p.i. using a UL44-specific antibody and primers complementary to the R1 and R5 regions of oriLyt. As expected, UL44 was found to associate with these regions in the HCMV-infected cells (Fig. IV-6d, compare lanes 3 and 4). The binding of UL44 to the R5 region was reduced by the overexpression of CHOP (Fig. IV-6d, compare lanes 5 and 6), indicating that the interaction of C/EBPα with the R5 region might be required for the optimal association of UL44 with this region. The interaction between UL44 and the R1 region was not inhibited by CHOP overexpression (Fig. IV-6d, compare lanes 5 and 6). These results suggested that the binding of C/EBPα to the oriLyt region was required for optimal viral DNA synthesis, probably through the regulation of the interactions between replication proteins and the oriLyt region.

3. Discussion

Our data showed that HCMV infection increased the C/EBPα p42/p30 ratio in

infected cells by the mTOR-mediated activation of host cell translation. C/EBPα accumulated in the viral DNA replication compartment, where it directly interacted with the R5 region of oriLyt. This DNA-protein interaction facilitated viral DNA replication probably by regulating the interaction between UL44 and oriLyt.

The activation of mTOR has been reported to increase the expression of C/EBPα-p30 but not C/EBPα-p42 by inducing translation initiation at a downstream AUG codon in the C/EBPα mRNA (Calkhoven *et al.*, 2000). However, our data showed that the HCMV-mediated activation of mTOR led to the induction of p42 expression. Also, GCV treatment abolished the HCMV-mediated induction of p42 expression without decreasing the activity of mTOR, indicating the possible involvement of viral late gene products. These data suggested that HCMV might utilize a distinct virus-specific mechanism(s) in the regulation of mTOR-mediated translation initiation to increase the expression of C/EBPα-p42.

One can argue that CHOP overexpression could indirectly suppress viral DNA replication, for examples, by the inhibition of the expression of viral IE and E genes or the induction of apoptosis (Marciniak *et al.*, 2004). However, the suppressive effect of CHOP on the level of early viral genes was not sustained to the late stage of infection. Also, CHOP overexpression selectively abrogated the association of UL44 with the specific region (R5) of oriLyt without affecting the UL44-R1 interaction. These data suggested that the CHOP-mediated delay during the early stage of infection might not have a significant effect on late events such as viral DNA replication. In addition, when cell rounding and DNA fragmentation were analyzed, CHOP-overexpression did not seem to induce apoptosis in our experimental setting (data not shown). In summary, our data suggest that CHOP overexpression may suppress viral DNA replication by the inhibition

of the interaction between C/EBPα and oriLyt, but not by its indirect effect.

HCMV infection triggers multiple mechanisms to maintain the activity of mTOR in response to various stress conditions that normally inhibit this pathway, suggesting the importance of this kinase in viral pathogenesis (Moorman *et al.*, 2008; Tilton *et al.*, 2011). The activation of mTOR is required for viral DNA replication (Moorman & Shenk, 2010), and our data showed that it was necessary for the induction of C/EBPα-p42 expression and, consequently, for efficient viral DNA replication. Together, these data suggested that mTOR might regulate the synthesis of viral DNA, at least in part, by increasing the expression of C/EBPα-p42.

The C/EBPα binding sites within the HCMV oriLyt region also interact with UL84, a viral protein necessary for the initiation of viral DNA replication; this interaction is not dependent on the C/EBPα protein (Kagele *et al.*, 2009). The interaction between UL84 and the C/EBPα binding sites has been detected in the purified virion and in HCMV-infected cells, indicating that UL84 binds to these sites from the early stage of infection (Kagele *et al.*, 2009). Our data indicated that C/EBPα interacts with the oriLyt region after the initiation of viral DNA replication. These data suggest that these two proteins might bind to the C/EBPα-binding sites in the HCMV oriLyt region in a sequential manner: UL84 first interacts with oriLyt and subsequently initiates viral DNA replication, and C/EBPα is then recruited and further facilitates viral genome replication.

Our data showed that the interaction between C/EBPα and the R5 region of oriLyt was required for the efficient binding of UL44 to the oriLyt region. The binding of C/EBPα to the oriLyt region might increase the accessibility of the local chromatin through the action of chromatin remodeling complexes such as SWI/SNF and CBP/p300, which bind to C/EBPα (Erickson *et al.*, 2001; Kovacs *et al.*, 2003; Pedersen *et al.*, 2001).

The change in the local chromatin structure might indirectly aid the recruitment of replication proteins such as UL44 to the oriLyt region (Mechali, 2010). It is also possible that C/EBPα directly regulates the binding of UL44 to oriLyt through an interaction with a novel viral and/or cellular factor(s), independent of its transactivation ability. The interaction between viral proteins and the R5 region of oriLyt seems to play an important role in viral DNA replication based on a mutational analysis of oriLyt (Kagele *et al.*, 2009). These data suggest that the binding of C/EBPα to oriLyt contributes to efficient viral DNA replication by indirectly or directly regulating the interaction between replication proteins such as UL44 and the oriLyt region.

In conclusion, C/EBPα might be a downstream target of mTOR in control of HCMV DNA replication by recruiting the viral protein UL44 to the oriLyt region. Therefore, the transcription factor C/EBPα appears to act as an important positive regulator of HCMV lytic infection.

CHAPTER V

Activation of GSK3β by HCMV infection and its involvement in the regulation of NF-κB

1. Backgrounds

The nuclear factor κB (NF-κB) family of transcription factors comprises five proteins: RelA (p65), RelB, c-Rel, NF-κB1 p50, and NF-κB2 p52 (Shih *et al.*, 2011). NF-κB proteins can form heterodimers with any other member of the family. Under unstimulated conditions, NF-κB dimers are maintained in an inactive form in the cytoplasm by the inhibitor of κB (IκB) family of proteins, which includes IκBα, IκBβ, IkBε, NF-κB1 p105 (the precursor of p50), and NF-κB2 p100 (the precursor of p52). In the canonical NF-κB pathway, proinflammatory cytokines such as tumor necrosis factor α (TNF-α) induce the degradation of IκBα and the subsequent release of NF-κB dimers composed of p65 and p50. However, the activation of some membrane receptors, such as CD40, B-cell activating factor (BAFF) receptor and lymphotoxin-β receptor, induces a different pathway called the non-canonical NF-κB pathway, in which the p52-RelB dimer is a transcriptional effector (Razani *et al.*, 2011). A key step in the activation of the non-canonical pathway is the proteasomal processing of p100 to p52 by NF-κB-inducing kinase (NIK) and IκB kinase α.

Glycogen synthase kinase 3β (GSK3β) is a serine/threonine kinase that regulates physiological processes such as development, energy homeostasis, and immune and inflammatory responses (Sutherland, 2011). Several studies have indicated the involvement of GSK3β in the regulation of NF-κB activity. For example, embryonic fibroblasts from GSK3β-knockout mice are susceptible to TNF-α-induced apoptosis because TNF-α is unable to induce anti-apoptotic NF-κB target genes in these cells (Hoeflich *et al.*, 2000). GSK3β seems to act downstream of IκBα degradation and regulates the expression of target genes in a gene-specific manner (Steinbrecher *et al.*,

2005). Mechanistically, GSK3β regulates NF-κB activity directly by phosphorylating p65 (Gong *et al.*, 2008), and indirectly by repressing the activity of cAMP response element-binding protein, which competes with p65 for the limited amount of the CBP/p300 transcriptional co-activator (Martin *et al.*, 2005). GSK3β also phosphorylates the IκB protein NF-κB2 p100, leading to degradation of this protein and subsequent activation of NF-κB (Arabi *et al.*, 2012) (Fukushima *et al.*, 2012) (Busino *et al.*, 2012).

The NF-κB pathway mediates various aspects of virus-host interaction, and viral infection can modulate its activity (Hiscott *et al.*, 2001). HCMV encodes gene products that activate or inhibit this pathway; during HCMV infection, the canonical NF-κB pathway is activated through the induction of p65 and p50 expression by the viral IE protein (Yurochko *et al.*, 1995), activation of the ataxia telangiectasia mutated-dependent pathway by the viral UL76 protein (Costa *et al.*, 2013), and promotion of interactions between TNF receptor-associated factor 6 and the viral UL144 protein (Poole *et al.*, 2006). By contrast, the IE86 protein of HCMV inhibits virus- and TNF-α-induced activation of NF-κB, and hence the induction of cytokines such as IL-6 and IL-8 by these stimuli (Taylor & Bresnahan, 2006). Through the combined effects of these regulatory proteins, HCMV infection persistently induces some level of canonical NF-κB activation in the host cell (Caposio *et al.*, 2007). However, the possible involvement of cellular factors such as GSK3β in HCMV-mediated regulation of NF-κB is still unknown, and the effect of HCMV infection on the non-canonical NF-κB pathway has not been addressed.

Here, permissive human fibroblasts were used to investigate the effect of HCMV infection on GSK3β activity and the possible role of GSK3β in HCMV-mediated regulation of NF-κB. The results demonstrate that HCMV infection activates GSK3β during the late stage of infection. Knockdown of GSK3β did not affect the activation of

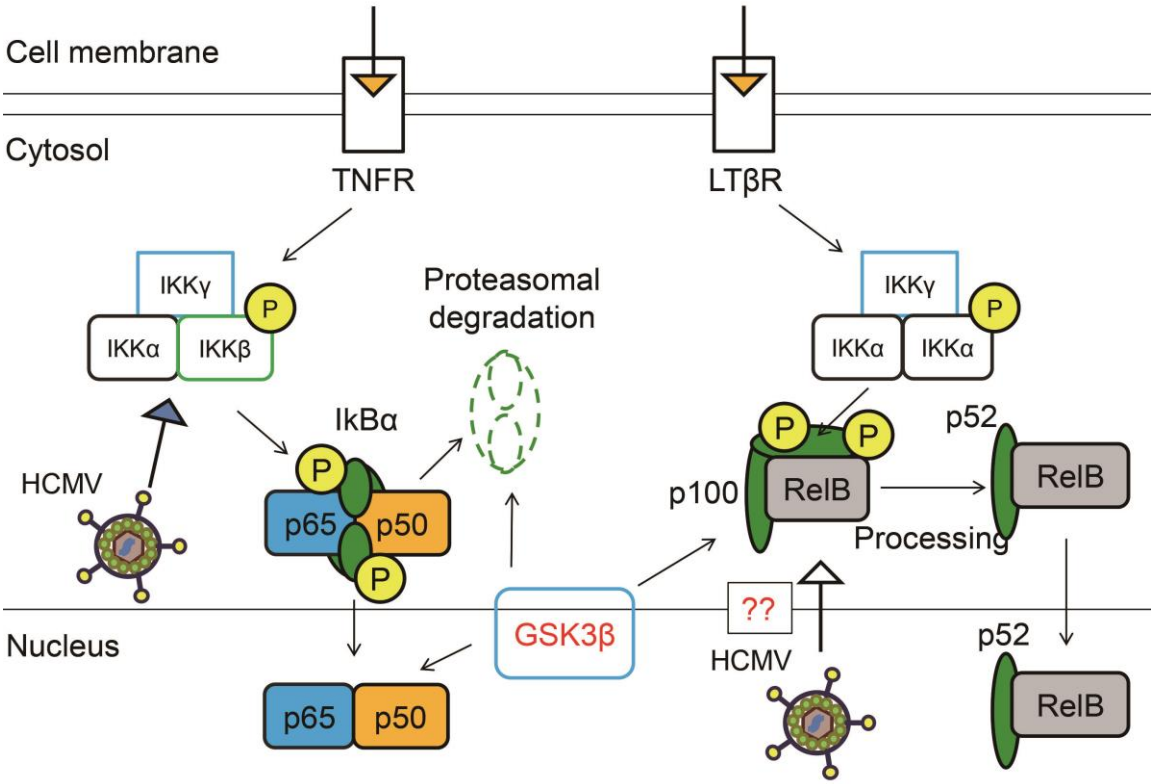


Figure V-1. Canonical and non-canonical NF-κB pathways. Schematic diagram of both canonical and non-canonical NF-κB pathways are shown. The signaling pathway downstream of TNFR (TNF-receptor) is the canonical pathway (left panel). Ligation of LTβR (lymphotoxic-β-receptor) activates a different pathway called the non-canonical pathway (right panel) (from Oeckinghaus and Ghosh *et al.*, 2009). The known involvements of GSK3β in both pathways are also depicted. HCMV infection is reported to activate canonical NF-κB, however, its effect on non-canonical NF-κB is not yet revealed.

canonical NF-κB by HCMV infection; however, non-canonical NF-κB activation was induced by viral infection in a GSK3β-dependent manner, as evidenced by the up-regulation of p52 and BAFF expression.

2. Results

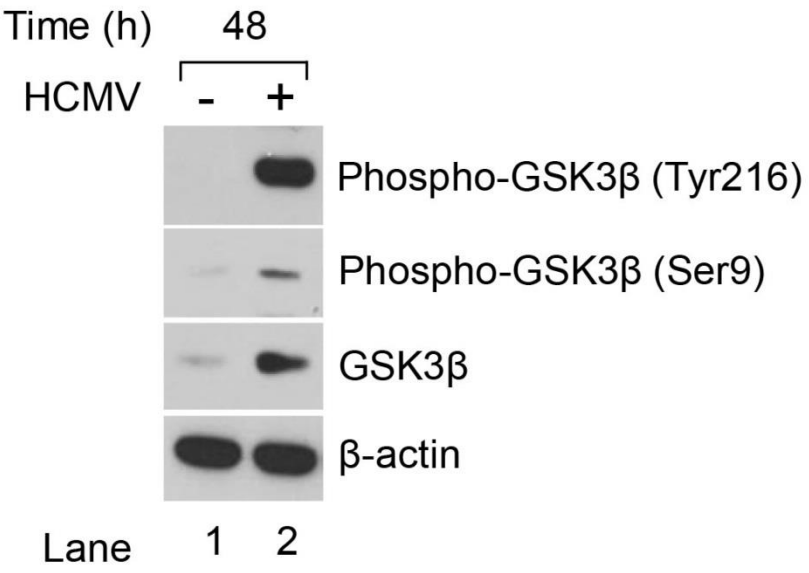
2.1 HCMV infection activates GSK3β during the late stage of infection

GSK3β activity is regulated by autophosphorylation at Tyr216 and Ser9; phosphorylation at these sites increases and decreases the activity of GSK3β, respectively (Dajani *et al.*, 2001) (Cross *et al.*, 1995). To determine the effect of HCMV infection on GSK3β activity, the phosphorylation state of the protein in uninfected and HCMV-infected primary human foreskin fibroblasts (HFFs) was examined by immunoblotting with antibodies specific to total or phosphorylated GSK3β. In uninfected cells, the expression level of GSK3β phosphorylated at Tyr216 was low (Fig. V-2a, lane 1), but viral infection induced the phosphorylation of GSK3β at this residue significantly (Fig. V-2a, lanes 1 and 2). The level of serine-phosphorylated GSK3β was also increased by HCMV infection, albeit to a lesser extent (Fig. V-2a), suggesting that the inhibitory phosphorylation of GSK3β occurred rarely in infected cells. HCMV infection also up-regulated the total GSK3β expression level (Fig. V-2a). Overall, these data suggest that HCMV infection activates GSK3β in HFFs, especially during the later stage of infection.

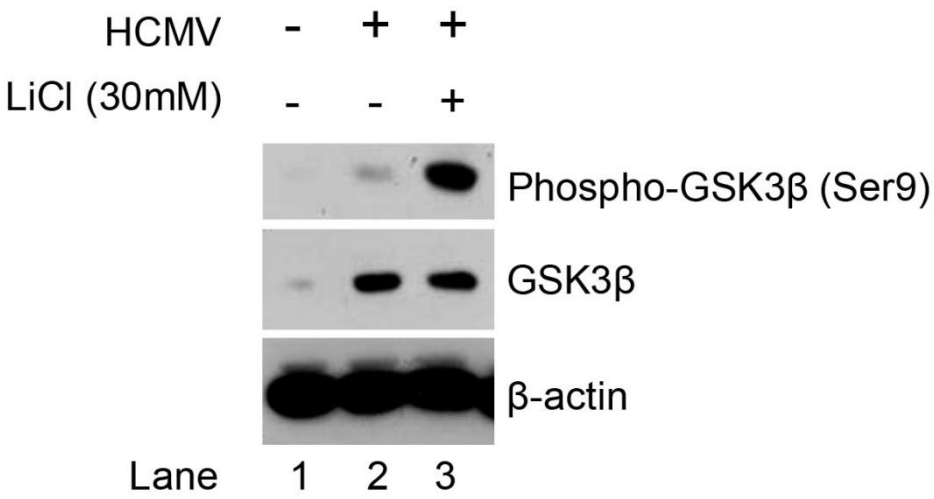
To determine whether serine phosphorylation actually occurred in a small fraction of the total GSK3β proteins in HCMV-infected cells, the cells were treated with LiCl, a known inducer of the level of serine-phosphorylated GSK3β (Chalecka-Franaszek & Chuang, 1999; De Sarno *et al.*, 2002). HFFs infected with HCMV were treated with 30

Figure V-2. Effect of HCMV infection on GSK3β activity. (a) Immunoblot analyses of phosphorylated GSK3β and total GSK3β levels in HFFs infected with HCMV (multiplicity of infection = 3). Total cellular proteins were extracted at the indicated time points and immunoblotting was performed using antibodies specific to phospho-GSK3β (serine 9 or tyrosine 216) and total GSK3β. The expression level of β-actin was detected as a loading control. (b) Immunoblot analyses of total GSK3β and serine-phosphorylated GSK3β levels in HFFs infected with HCMV, treated with 30 mM LiCl at 2 days post-infection, and then incubated for an additional 6 h after the treatment. (c) Immunoblot analyses of HFFs infected with HCMV and then subjected to cell fractionation at 2 days post-infection. The level of nuclear β-catenin was detected by immunoblotting using a specific antibody. The levels of TFIIB and α-tubulin served as loading controls for nuclear and cytoplasmic extracts, respectively. (d) Immunoblot analyses of HFFs infected with either intact or UV-irradiated HCMV. Analyses were performed at 2 days post-infection using antibodies specific to IE1/IE2, total GSK3β, and β-actin.

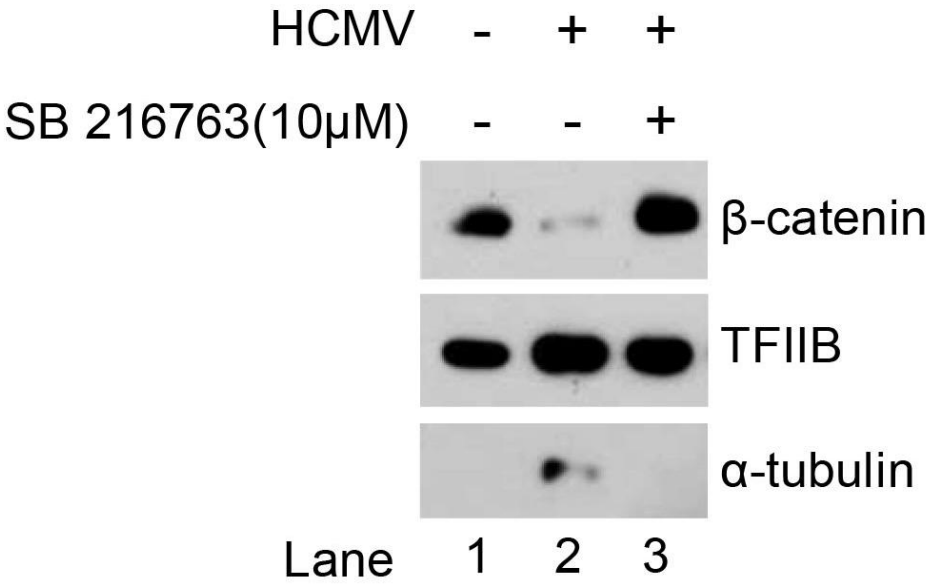
a


Time (h)
48
HCMV
-
+
Phospho-GSK3β (Tyr216)
Phospho-GSK3β (Ser9)
GSK3β
β-actin
Lane
1
2

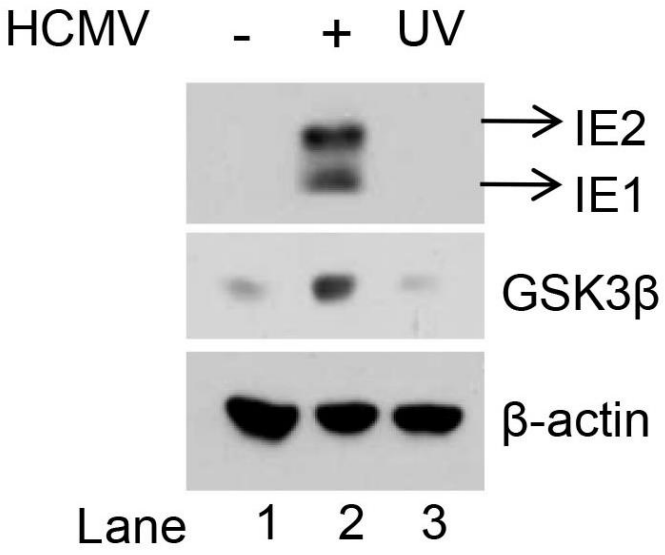
b


HCMV
-
+
+
LiCl (30mM)
-
-
+
Phospho-GSK3β (Ser9)
GSK3β
β-actin
Lane
1
2
3

c


HCMV - + +
SB 216763(10μM) - - +
β-catenin
TFIIB
α-tubulin
Lane 1 2 3

d


HCMV - + UV
IE2
IE1
GSK3β
β-actin
Lane 1 2 3

mM LiCl and incubated for 6 h, after which total proteins were extracted and examined by immunoblotting. Serine-phosphorylated GSK3β was not detected in uninfected cells and its level was increased only marginally by HCMV infection (Fig. V-2b, compare lanes 1 and 2). However, when HCMV-infected cells were treated with LiCl, serine phosphorylation of GSK3β was highly induced (Fig. V-2b, compare lanes 2 and 3). LiCl treatment did not affect the total GSK3β expression level (Fig. V-2b). These data suggest that the majority of GSK3β proteins in HCMV-infected HFFs are not phosphorylated at the serine residue and are therefore active.

GSK3β-mediated phosphorylation of β-catenin leads to the ubiquitination and degradation of this protein (Wu & Pan, 2010); therefore, the effect of HCMV infection on the level of β-catenin was examined. Two hours after HCMV infection, the cells were treated with 10 μM SB216763, an ATP-competitive inhibitor of GSK3, and the nuclear fraction was isolated at 2 days post-infection. Immunoblot analyses revealed that uninfected cells expressed a high level of nuclear β-catenin (Fig. V-2c, lane 1). This level was decreased significantly by HCMV infection (Fig. V-2c, compare lanes 1 and 2), and SB216763 treatment reversed the HCMV-mediated suppression of nuclear β-catenin expression (Fig. V-2c, compare lanes 2 and 3). β-catenin expression in SB216763-treated cells was higher than uninfected control cells, which might have resulted from the inhibition of basal GSK3 activity by SB216763 (Fig. V-2c, compare lanes 1 and 3). The expression levels of TFIIB and α-tubulin were measured as loading controls for the nuclear and cytoplasmic fractions, respectively (Fig. V-2c). Consistent with its phosphorylation state, these data suggest that HCMV infection activates GSK3β in HFFs.

To examine the possible involvement of viral gene products in HCMV-mediated induction of GSK3β expression, the effect of UV-irradiated HCMV on the total GSK3β

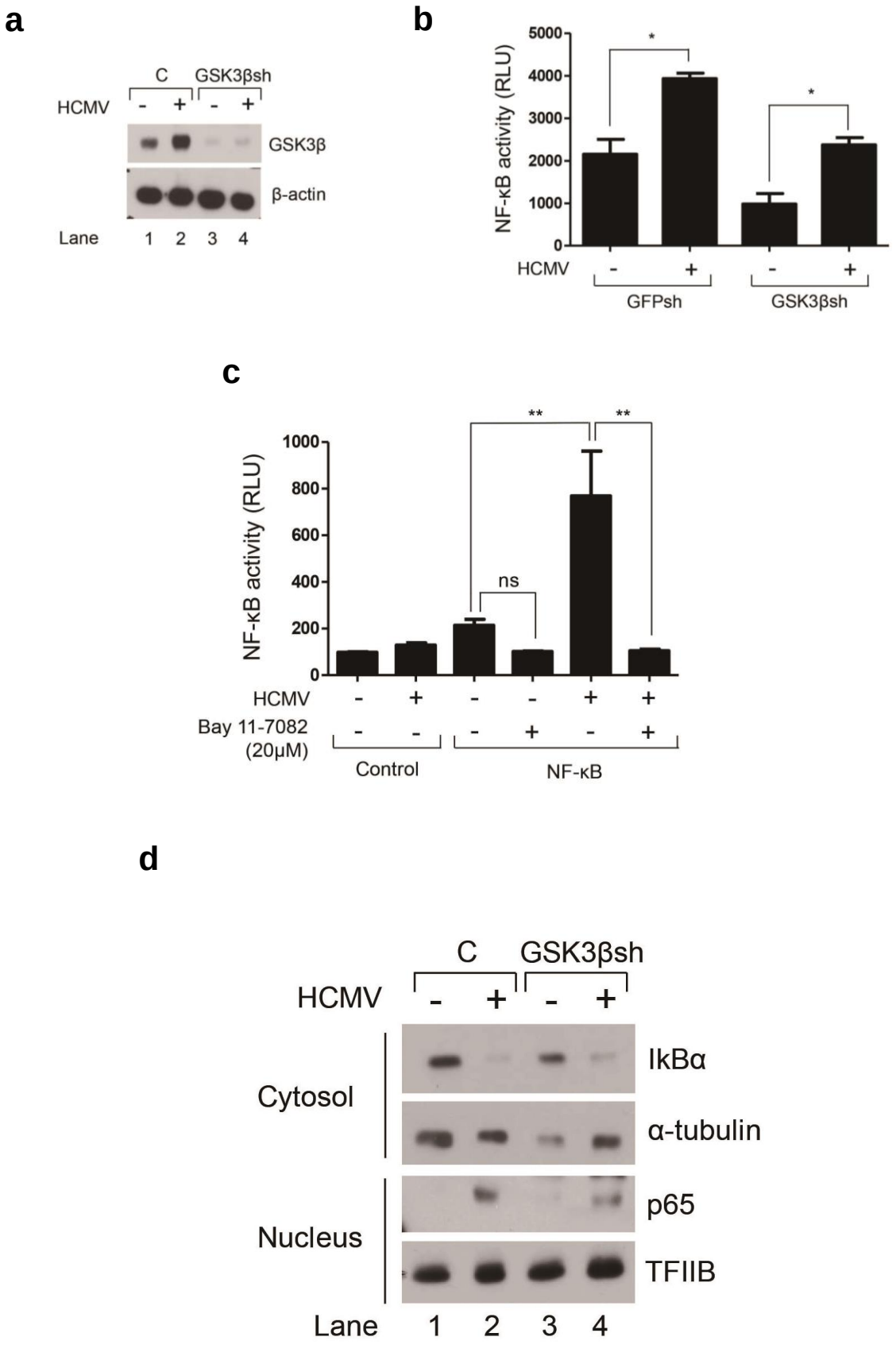
level was examined. Cells were infected with either HCMV or UV-irradiated HCMV, and total proteins were harvested at 48 h post-infection; immunoblot analyses were then performed using antibodies specific to IE1/IE2 and GSK3β. UV-irradiation of the virus stock prior to infection abolished the expression of IE1 and IE2, as expected (Fig. V-2d, compare lanes 2 and 3). Unlike non-irradiated virus infection, UV-irradiated HCMV infection had no effect on GSK3β expression (Fig. V-2d, lanes 1–3). These data suggest that viral gene expression is required for the induction of GSK3β expression by HCMV infection

2.2 GSK3β is not required for HCMV-mediated activation of canonical NF-κB

GSK3β activity is required for full activation of canonical NF-κB signaling (Gong *et al.*, 2008; Hoeflich *et al.*, 2000; Martin *et al.*, 2005; Steinbrecher *et al.*, 2005), which is induced by HCMV infection. Therefore, the possible involvement of GSK3β in HCMV-mediated activation of canonical NF-κB was determined. HFFs were transduced with a retroviral vector expressing a short hairpin RNA (shRNA) specific for GSK3β (GSK3βsh) or GFP (GFPsh) as a bicistronic message together with puromycin, and the transduced population was enriched by puromycin selection. HFFs expressing each shRNA were infected with HCMV, and the level of GSK3β expression was analyzed by immunoblotting at 2 days post-infection. In GFPsh-transduced cells, HCMV infection increased the level of GSK3β expression (Fig. V-3a, compare lanes 1 and 2). However, in GSK3βsh-transduced cells, the basal and HCMV-induced levels of GSK3β were lower than those in the GFPsh-transduced cells (Fig. V-3a, compare lanes 1 and 2 with 3 and 4), confirming that GSK3β expression was reduced by the GSK3β-specific shRNA.

Next, the effect of GSK3β knockdown on the HCMV-mediated activation of

Figure V-3. Effects of GSK3β knockdown on canonical NF-κB activation by HCMV infection. (a) Immunoblot analyses of total GSK3β levels in HFFs expressing GSK3βsh or GFPsh as a control. Cells expressing each shRNA were infected with HCMV, and protein lysates were prepared at 2 days post-infection. The expression levels of β-actin were detected as a loading control. (b) Luciferase activity assays of HFFs expressing each shRNA that were transfected with an NF-κB-driven luciferase reporter plasmid and then infected with HCMV 2 days after transfection. Total protein lysates were harvested at 2 days post-infection. (c) Luciferase activity assays of HFFs transfected with the NF-κB reporter plasmid or a control plasmid lacking NF-κB binding sequences. HCMV infection and the activity assay were performed as described for (b). Where indicated, the cells were treated with 20 μM BAY 11-7082, a NF-κB inhibitor, at 2 h post-infection. (d) Immunoblot analyses of HCMV-infected HFFs expressing each shRNA. The cells were fractionated at 2 days post-infection and immunoblotting was performed using antibodies specific to p65, IκBα, TFIIB, and α-tubulin. The statistical analysis was performed as described in the Methods.


a
C
GSK3βsh
HCMV
- + - +
GSK3β
β-actin
Lane 1 2 3 4
b
NF-κB activity (RLU)
5000
4000
3000
2000
1000
0
*
*
HCMV - + - +
GFPsh
GSK3βsh
c
NF-κB activity (RLU)
1000
800
600
400
200
0
**
**
ns
HCMV - + - - + +
Bay 11-7082 (20μM) - - - + - +
Control
NF-κB
d
C
GSK3βsh
HCMV - + - +
Cytosol
IkBα
α-tubulin
Nucleus
p65
TFIIB
Lane 1 2 3 4

canonical NF-κB was tested. HFFs expressing each shRNA were transfected with an NF-κB-driven luciferase reporter plasmid and then infected with HCMV 2 days after transfection. Luciferase activity was measured in the lysates at 2 days post-infection. HCMV infection up-regulated luciferase activity in both the control GFPsh-expressing cells and the GSK3β knockdown cells (Fig. V-3b), suggesting that GSK3β is not required for HCMV-driven activation of canonical NF-κB. HCMV infection did not affect transcription of the control plasmid lacking NF-κB binding sites, and treatment of the cells with 20 μM BAY-11-7082, a NF-κB inhibitor, completely suppressed virus-induced activation of the NF-κB reporter (Fig. V-3c). To confirm these findings, the effects of HCMV infection and GSK3β knockdown on cytoplasmic IκBα degradation and nuclear p65 translocation, which are the hallmarks of canonical NF-κB activation, were determined. HFFs expressing each shRNA were infected with HCMV and then subjected to subcellular fractionation and immunoblot analyses at 2 days post-infection. HCMV infection induced IκBα degradation and p65 translocation in control GFPsh-expressing cells (Fig. V-3d, compare lanes 1 and 2), and knockdown of GSK3β did not affect these processes (Fig. V-3d). Taken together, data suggest that GSK3β is not involved in the regulation of canonical NF-κB activity by HCMV.

2.3 HCMV infection induces non-canonical NF-κB activation in a GSK3β-dependent manner

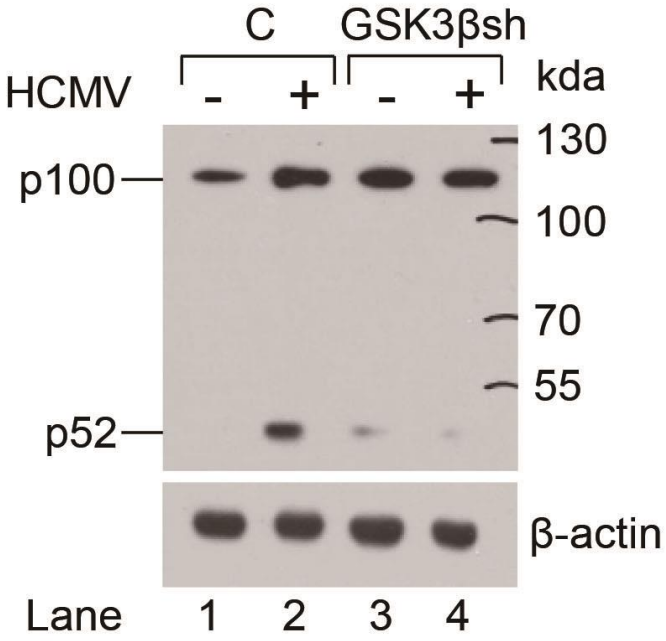
Viral infections such as HIV reportedly activate the non-canonical NF-κB pathway (Manches *et al.*, 2012) (de Jong *et al.*, 2010). Proteasomal processing of p100 to p52 is a hallmark of the activation of this pathway (Razani *et al.*, 2011); therefore, to determine the effect of HCMV infection on non-canonical NF-κB signaling and the

possible involvement of GSK3β in this process, HFFs expressing GSK3βsh or GFPsh were infected with HCMV and the expression levels of p100 and p52 were analyzed by immunoblotting at 2 days post-infection. In uninfected GFPsh-transduced cells, p100 was expressed at basal levels and p52 was undetectable (Fig. V-4a, lane 1). However, HCMV infection increased the expression levels of both of these proteins, although the effect on p52 was much more pronounced (Fig. V-4a, compare lanes 1 and 2). This result suggests that processing of p100 to p52 might be induced by HCMV infection. Uninfected GSK3β knockdown cells showed a high basal expression level of p100 and a lower expression level of p52, which might have resulted from the loss of GSK3β-mediated phosphorylation and degradation of the p100 protein, as reported recently (Fig. V-4a, compare lanes 1 and 3) (Busino *et al.*, 2012; Fukushima *et al.*, 2012). In contrast to the control GFPsh-expressing cells, HCMV infection did not induce p50 expression in the GSK3β knockdown cells (Fig. V-4a, compare lanes 3 and 4). These data suggest that, in addition to its known role in the regulation of p100 stability, GSK3β might also be involved in the activation of non-canonical NF-κB by HCMV infection by facilitating the expression of p52.

The effects of HCMV infection and GSK3β knockdown on the expression levels of p105 and p50 were also determined; p105 is constitutively processed to p50, a major player in the canonical NF-κB pathway (Lin *et al.*, 1998). Uninfected GFPsh-expressing cells displayed basal levels of p105 and p50 expression (Fig. V-4b, lane 1), and HCMV infection increased the levels of both proteins (Fig. V-4b, compare lanes 1 and 2). Knockdown of GSK3β did not affect the basal and HCMV-induced levels of p105 and p50 (Fig. V-4b, compare lanes 1 and 2 with 3 and 4), suggesting that GSK3β might specifically regulate the expression levels of p100 and p52 without affecting those of

Figure V-4. Involvement of GSK3β in the induction of non-canonical NF-κB signaling by HCMV infection. (a, b) Immunoblot analyses of HFFs expressing GSK3βsh or GFPsh as a control. Cells expressing each shRNA were infected with HCMV, and protein lysates were obtained at 2 days post-infection. Immunoblotting was performed using antibodies specific to p100/p52 (a) and p105/p50 (b). (c) Immunoblot analyses of HFFs infected with HCMV and treated with 10 μM SB216763, a GSK3 inhibitor. Total proteins were extracted at the indicated time points after SB216763 treatment, and the levels of p100 and p52 were detected using a p100/p52-specific antibody. (d–f) Quantitative RT-PCR analyses of HFFs expressing GSK3βsh or GFPsh as a control. The cells were infected with HCMV, and the level of the mRNA encoding BAFF was measured at 2 days post-infection (d) or 3 days post-infection (e). As a control, the expression level of the mRNA encoding GCLC was determined at 2 days post-infection (f). The statistical analysis was performed as described in the Methods.

a


C
GSK3βsh
HCMV
-
+
-
+
kda
130
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70
55
p100
p52
β-actin
Lane
1
2
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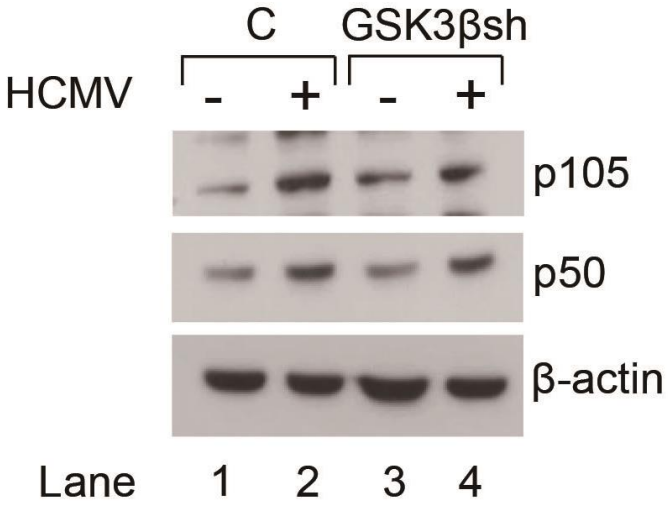
b


C
GSK3βsh
HCMV
-
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p105
p50
β-actin
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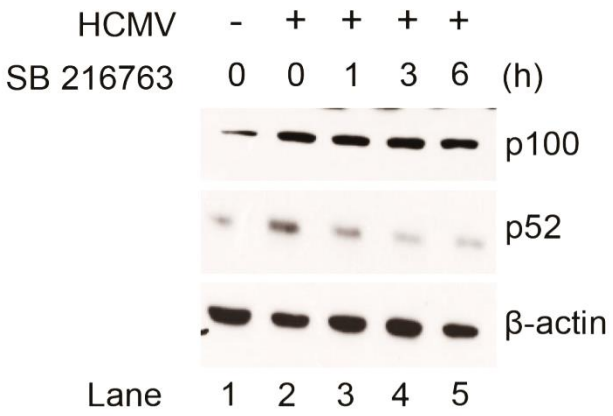
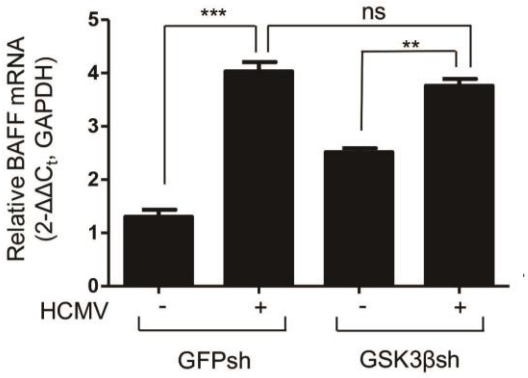
c


HCMV - + + + +
SB 216763 0 0 1 3 6 (h)
p100
p52
β-actin
Lane 1 2 3 4 5

d


Relative BAFF mRNA (2-ΔΔCt, GAPDH)
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ns
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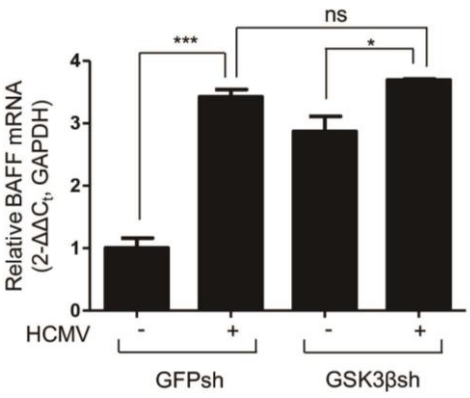
HCMV - + - +
GFPsh
GSK3βsh

e


Relative BAFF mRNA (2-ΔΔCt, GAPDH)
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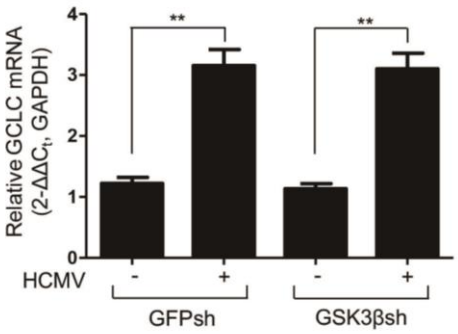
GFPsh
GSK3βsh

f


Relative GCLC mRNA (2-ΔΔCt, GAPDH)
**
**
HCMV - + - +
GFPsh
GSK3βsh

other NF-κB proteins in HCMV-infected cells.

The effect of transient inhibition of GSK3β on the expression levels of p100 and p52 was also determined by treating HCMV-infected HFFs with 10 μM SB216763 at 2 days post-infection. Immunoblot analyses revealed that HCMV infection up-regulated the levels of p100 and p52, as expected (Fig. V-4c, compare lanes 1 and 2). Although treatment of the cells with SB216763 did not affect p100 expression, HCMV-mediated induction of p52 expression was suppressed completely (Fig. V-4c, compare lanes 2, 3, 4, and 5). These data suggest that GSK3β may induce the processing of p100 to p52 in HMCV-infected cells.

The non-canonical p52-RelB NF-κB dimer up-regulates the expression of target genes such as those encoding BAFF and the CXCL12, CXCL13, CCL19, and CCL21 chemokines (Wharry *et al.*, 2009). Therefore, the effect of HCMV infection and GSK3β knockdown on the expression of the mRNA encoding BAFF was examined by quantitative reverse transcription polymerase chain reaction (RT-PCR) analyses at 48 h post-infection. In GFPsh-expressing cells, HCMV infection increased the amount of BAFF transcripts (Fig. V-4d, compare lanes 1 and 2), indicating activation of the non-canonical NF-κB pathway by HCMV infection. GSK3β knockdown also up-regulated the basal level of the mRNA encoding BAFF (Fig. V-4d, compare lanes 1 and 3), which might have resulted from increased p52 expression in the GSK3β knockdown cells (Fig. V-4a). However, the HCMV-mediated induction of this mRNA was suppressed in the absence of GSK3β (Fig. V-4d, compare lanes 3 and 4). To confirm these results, the effect of GSK3β knockdown on HCMV-mediated induction of BAFF expression was also tested at a later stage of infection. At 3 days post-infection, the expression level of the mRNA encoding BAFF was not affected when GSK3β expression was suppressed (Fig. V-4e). As

a negative control, the effect of GSK3β knockdown on HCMV-mediated induction of the mRNA encoding GCLC, an antioxidant enzyme, was determined. HCMV infection increased the level of this mRNA in both control and GSK3β knockdown cells, excluding the possibility that GSK3β knockdown non-specifically suppressed the induction of BAFF by HCMV infection (Fig. V-4f). These data suggest that HCMV infection induces the activation of the non-canonical NF-κB pathway and that HCMV-mediated activation of GSK3β is involved in this process.

2.4 HCMV infection does not affect NIK expression in HCMV-infected cells

Because the up-regulation of NIK expression is a key step during the activation of non-canonical NF-κB signaling (Sun, 2011), the effect of HCMV infection and GSK3β knockdown on NIK expression was tested. HFFs expressing GSK3βsh or GFPsh were infected with HCMV, and the expression level of NIK was detected by immunoblotting at 2 days post-infection. Uninfected cells expressing GFPsh displayed a basal level of NIK expression that was decreased slightly by viral infection (Fig. V-5, compare lanes 1 and 2), indicating that HCMV infection might utilize a NIK-independent mechanism to induce the processing of p100 to p52. Knockdown of GSK3β had no effect on NIK expression in either uninfected or infected cells (Fig. V-5, compare lanes 1 and 2 with 3 and 4), indicating that GSK3β does not regulate NIK expression. These data suggest that HCMV infection might activate the non-canonical NF-κB pathway by a novel and NIK-independent mechanism. Additional studies such as the effect of HCMV infection on IKKα are needed to elucidate the exact mechanism by which HCMV induces the processing of p100 to p52.

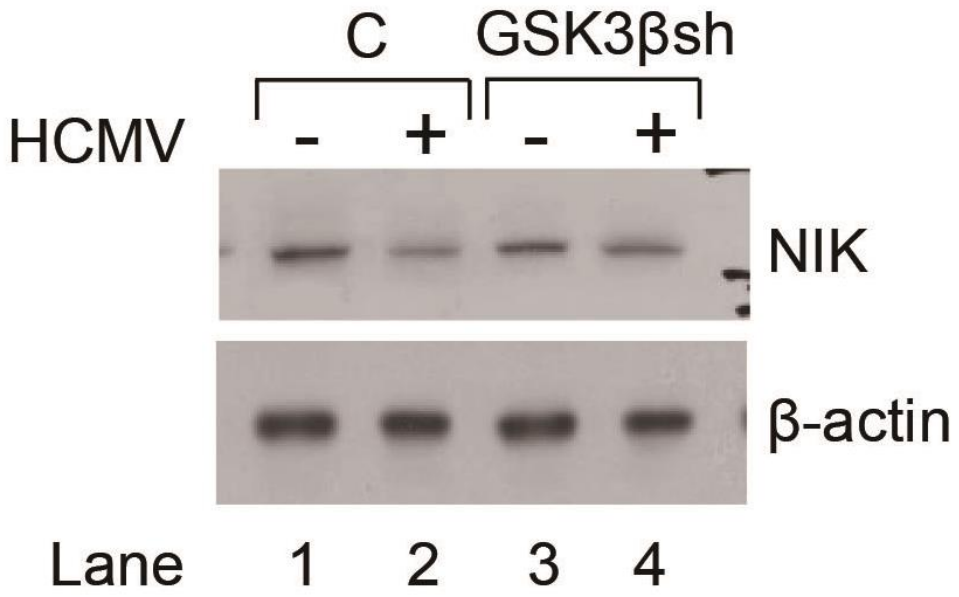


Figure V-5. Effect of HCMV infection on NIK expression. Immunoblot analyses of HFFs expressing GSK3βsh or GFPsh. The cells were infected with HCMV and the levels of NIK and β-actin were detected using specific antibodies.

3. Discussion

For the first time our data showed that GSK3β could be activated when permissive cells like HFFs were infected with HCMV and that this virally activated GSK3β could upregulate the expression of the non-canonical NF-κB subunit (p52) and BAFF, a downstream target gene of p52-RelB. These results strongly suggest that GSK3β may be involved in the activation of the non-canonical NF-κB pathway in the context of HCMV infection.

There are two types of the phosphorylated GSK3β protein; tyrosine-phosphorylated GSK3β as an active form and serine-phosphoryated one as an inactive form (Doble & Woodgett, 2003). HCMV infection appears to have differential effects on these two forms. When cells were infected with HCMV, the level of total GSK3β was greatly increased. However, the magnitude of induction of the tyrosine-phosphorylated form (10-fold) was much greater than that of the serine-phosphorylated one (2.5-fold). LiCl is a well-known compound that increases the serine phosphorylation of GSK3β (Chalecka-Franaszek & Chuang, 1999; De Sarno *et al.*, 2002). When HCMV-infected cells were treated with LiCl, the level of serine-phosphorylated GSK3β was further increased by 10-fold. These results show that when cells are infected with HCMV, viral infection seems to generate the condition favorable for the production of active tyrosine-phosphorylated GSK3β, while the level of the serine phosphorylated form still remains low or limited.

HCMV infection appears to activate non-canonical NF-κB in a way(s) different from other known inducers. For example, the binding of a well-known inducer of non-canonical NF-κB, such as LTβ, to its cellular receptor always leads to the stabilization of NIK, resulting in the activation of IKKα and the processing of p100 to p52 (Sun, 2011).

This was not the case for HCMV infection. It was reported that a GSK3β inhibitor (BIO) did not affect the processing of p100 to p52 induced by LTβ, indicating no involvement of GSK3β in this step (Busino *et al.*, 2012). However, data from our experiments involving shRNA or a specific chemical inhibitor (SB216763) showed that in the context of HCMV infection, GSK3β was required for the processing of p100 to p52. Although the exact mechanism remains to be elucidated, HCMV-mediated activation of the non-canonical NF-κB pathway seems to be using a signaling pathway different from those adopted by other known inducers.

Our results may provide an insight on the understanding of HCMV's role(s) in the modulation of cell signaling pathways involved in cell proliferation and oncogenesis. For example, the interaction of BAFF with its receptor has been shown to lead to proliferation and activation of B-cells through the induction of non-canonical NF-κB (Razani *et al.*, 2011). In addition, activation of non-canonical NF-κB was reported to enhance the progression of glioma (Tchoghandjian *et al.*, 2013), while HCMV has been suspected to act as a significant cofactor in this tumor (Dziurzynski *et al.*, 2012). Together, it is interesting to postulate that the activation of non-canonical NF-κB by HCMV infection may explain, at least in part, how this virus is involved in B-cell hyperactivation or oncogenesis of glioma.

β-catenin appears to be involved in tumorigenesis, but its role(s) is not yet clear and there are even contradictory data (Anastas & Moon, 2013). It was previously reported that HCMV infection facilitated the degradation of β-catenin (Angelova *et al.*, 2012). We showed that the virus does so through GSK3β as the effect of HCMV infection was inhibited when cells were treated with a GSK3β inhibitor (SB216763). β-catenin is a positive effector of the canonical wnt signaling pathway which has been shown to be

involved in the tumorigenesis of breast and colon cancers among others. On the other hand, high levels of β-catenin were reported to be correlated with improved prognosis in some cancer cases (Anastas & Moon, 2013). Clearly, the roles of the wnt pathways in oncogenesis, as well as that of b-catenin, are complex and the exact mechanisms remain to be elucidated

It is clear that the effects of HCMV infection on the host cell are very complex, even only in the context of GSK3β. Given such important roles of GSK3β and the non-canonical NF-κB pathway in cell signaling, proliferation, and tumorigenesis, further investigations are warranted to better understand the mechanisms underlying the effects of HCMV infection on signaling pathways and eventually to unravel the pathophysiology of this ubiquitous herpes virus.

CHAPTER VI

Conclusion

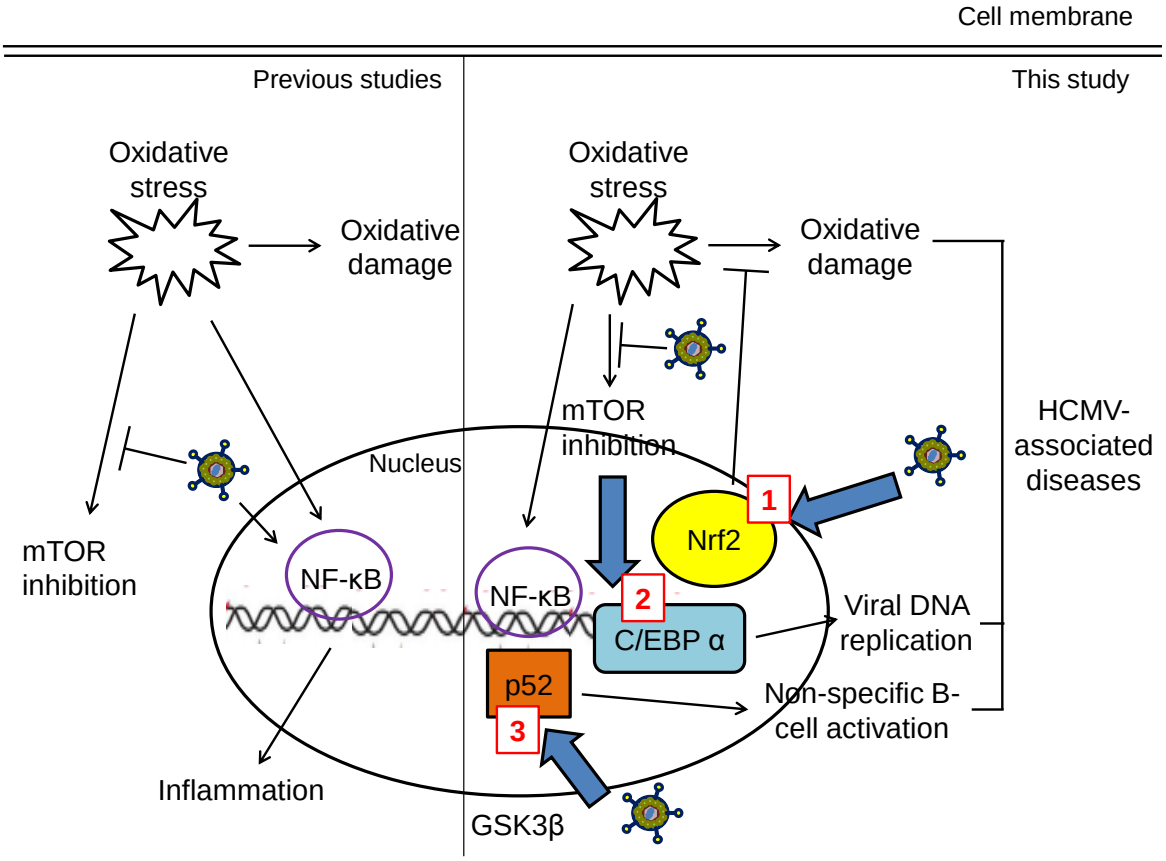
The activation of cellular stress responses plays a crucial role in maintaining the integrity of macromolecules against various stresses. The dysfunction of these mechanisms, therefore, is often associated with a variety of human diseases such as neurodegenerative diseases, cancer, and metabolic diseases. It is well established that viral infection imposes stresses to host cell and host system responds to them in many different ways, depending on the type of virus.

HCMV infection adopts several different ways of regulating the stress response system of the host cell. The pathways targeted by HCMV include canonical NF-κB, mTOR, the UPR, and the DDR. In this thesis research, I focused on the effect of HCMV infection on cellular factors involved in the oxidative stress responses such as Nrf2, mTOR, and NF-κB. Our data discovered novel effects of HCMV infection such as activation of the Nrf2 pathway and the non-canonical NF-κB pathway. Also, I revealed that the molecular mechanism of mTOR activation leading to enhanced viral DNA replication, in which C/EBPα played an important role. These data highlight complex interactions between HCMV infection and the stress response system, and together with the previous studies, I propose that my observations can help elucidate the characteristics of HCMV infection under inflammatory condition, which is closely related to the generation of oxidative stress.

HCMV infection induces oxidative stress directly and/or indirectly by immune and inflammatory response, which, if severe, can have anti-viral effects on HCMV infection such as the induction of oxidative damage and the inhibition of mTOR (Fig. VI-1, left panel). Therefore, HCMV infection may have mechanisms to combat negative consequences of oxidative stress to establish infection, which is exemplified by previous studies showing that HCMV infection maintained mTOR activation even under stressed

conditions (Fig. VI-1, left panel). According to my data, the activation of mTOR may lead to the up-regulation of C/EBPα expression, resulting in enhanced viral DNA replication, as demonstrated in Chapter IV (Fig. VI-1, right panel, box 2). In addition to ensuring replication of the viral genome, HCMV may also activate Nrf2 to protect host cells from premature cell death caused by oxidative stress, as shown in Chapter III (Fig. VI-1, right panel, box 1). Low level of ROS caused by HCMV infection can lead to the activation of canonical NF-κB pathway, which mediates the pro-inflammatory action of this virus (Fig. VI-1, left panel). My data described in Chapter V showed that non-canonical NF-κB may also play a crucial role in immuno-modulatory effects of HCMV through the induction of BAFF expression, which regulates activation, proliferation and survival of B-cells (Fig. VI-1, right panel, box 3) (Razani *et al.*, 2011). In conclusion, the results of this thesis research explain how HCMV infection persists in the organs or tissues of patients with chronic inflammation (Britt, 2008), and how it induces the production of autoantibodies (Varani & Landini, 2011), which all contribute to the development of HCMV-related diseases.

Figure VI-1. Findings made in this thesis and their possible roles in HCMV infection under oxidative stress. Oxidative stress can have various effects on HCMV infection such as the induction of oxidative damage, the inhibition of mTOR, and the activation of canonical NF-κB (left panel). HCMV infection protects mTOR from stress-mediated inhibition and has multiple mechanisms to regulate canonical NF-κB (left panel). In this thesis, I revealed that HCMV activated Nrf2 which promotes host cell survival under oxidative stress (right panel, arrow 1), HCMV-mediated mTOR activation led to the up-regulation of C/EBPα expression and to the increased genome replication of the virus (right panel, arrow 2), and that HCMV infection induced non-canonical NF-κB signaling, resulting in the induction of BAFF expression (right panel, arrow 3). These findings may explain the known capability of HCMV to persist under oxidative stress and its pathogenic effects such as non-specific B-cell activation, which all contribute to the pathology of HCMV-related diseases.


Cell membrane
Previous studies
This study
Oxidative stress
Oxidative damage
mTOR inhibition
NF-κB
Inflammation
Oxidative stress
Oxidative damage
mTOR inhibition
Nucleus
Nrf2
1
NF-κB
2
C/EBP α
Viral DNA replication
p52
3
Non-specific B-cell activation
GSK3β
HCMV-associated diseases

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국문초록

세포의 스트레스 반응은 다양한 종류의 스트레스가 가해졌을 때 DNA나 단백질과 같은 세포의 거대분자들이 손상되는 것을 막는 역할을 한다. 바이러스 감염 또한 숙주세포에 스트레스를 일으키며 숙주의 시스템이 바이러스의 종류에 따라 이런 자극에 서로 다르게 반응하는 것이 잘 알려져 있다. 이 연구에서는 인간 거대세포 바이러스 (human cytomegalovirus, HCMV) 감염과 숙주 세포의 스트레스 반응 시스템간의 상호작용에 대해 연구하였다.

Nrf2 (NF-E2-related factor 2)는 산화스트레스 시에 여러 유전자들의 발현을 조절하는데 핵심적인 역할을 하는 인자 중의 하나이다. 활성화 되면, Nrf2의 발현이 증가하고 증가한 Nrf2는 핵으로 이동하여 heme oxygenase-1 (HO-1)나 glutamate-cysteine ligase catalytic subunit (GCLC)과 같은 여러 하위단계 유전자들의 발현을 조절한다. 이 연구를 통해 나는 HCMV 감염이 Nrf2, HO-1, 그리고 GCLC의 발현을 증가시키며 이 과정에 바이러스의 유전자 발현과 casein kinase 2 (CK2)의 활성이 필요하다는 것을 밝혔다. HCMV에 감염된 숙주세포는 항산화물질인 glutathione를 생산하는 효소인 GCLC의 저해제인 buthionine sulfoximine (BSO)를 처리해 산화스트레스를 유도하였을 때 감염되지 않은 세포보다 더 잘 살아남았다. 그러나 HCMV의 이러한 세포보호효과는 Nrf2 특이적인 shRNA를 발현시키면 사라졌다. 이 결과는 HCMV 감염에 의해 활성화 되는 Nrf2가 감염 혹은 염증 반응 등에 의해 유도되는 산화스트레스로부터 숙주세포를 보호하는데 필수적인 역할을 한다는 것을 암시한다.

HCMV 감염은 스트레스 상황에서도 mTOR (mammalian target of rapamycin)를 활성화 하며 활성화 된 mTOR는 바이러스의 DNA 복제를 촉진하는 것으로 알려져 있다. 그러나 이 과정의 하부 메커니즘은 잘 알려져 있지 않았다. 나는 mTOR의 하위에 C/EBPα (CCAAT/enhancer binding protein α)라는 전사인자가 있다는 것을 보였다. mTOR는 아마 C/EBPα의 translation을 조절하여 위와 같은 효과를 내는 것으로 보인다. CHOP (C/EBP homologous protein) 단백질을 과발현하여 C/EBPα의 활성을 억제하면, 바이러스의 DNA 복제와 late 유전자의 발현이 저해되었고 그 결과 progeny 바이러스의 생산이 감소하였다.

바이러스 감염은 숙주의 면역 및 염증 반응을 활성화 하며 그 결과 숙주세포에 심한 스트레스를 주는 것으로 알려져 있다. NF-κB (Nuclear Factor-κB)는 이 과정에 주요한 역할을 하는 인자 중의 하나이다. NF-κB의 활성화는 각각 canonical 혹은 non-canonical pathway라 불리는 두 가지 경로를 통해 일어난다. Glycogen synthase kinase 3β (GSK3β)가 두 pathway를 모두 조절하는 것이 알려진 바 있다. 나는 HCMV 감염이 GSK3β를 활성화 한다는 것을 보였다. GSK3β 특이적인 shRNA를 발현시켜 HCMV에 의한 GSK3β의 활성화를 억제하였을 때 canonical NF-κB는 여전히 활성화 되는 것을 알 수 있었다. 그러나 GSK3β 특이적인 shRNA는 HCMV 감염에 의해 non-canonical NF-κB subunit인 p52와 p52-RelB의 하위단계 유전자인 B-cell activating factor (BAFF)의 발현이 증가되는 것을 억제하였다. 이 결과는 바이러스 감염에 의해 활성화 된 GSK3β가 HCMV에 감염된 세포에서 non-canonical NF-κB signaling을 활성화 시킨다는 것을 암시한다. HCMV 감염에 의한 non-canonical NF-κB signaling의 활성화는 B-cell hyperactivation과 같은 HCMV 감염과 연관된 여러 pathologic effect를 설명할 수 있다.

본 연구를 통해, 나는 HCMV와 숙주 세포가 주고 받는 새로운 상호작용을 발견하였다. 바이러스는 Nrf2나 C/EBPα와 같은 숙주세포의 인자를 발현시켜 숙주세포의 생존을 돕거나 바이러스의 DNA 복제가 효과적으로 일어나게 만들 수 있다. 또한 HCMV 감염은 잠재적으로 감염과 연관된 여러 immunopathy를 매개할 수 있는 non-canonical NF-κB pathway를 활성화 하는 것으로 관찰되었다. 이 결과들은 특히 만성염증과 같은 스트레스 상황에서 HCMV 감염이 어떻게 질병을 심화시킬 수 있는지 이해하는데 도움을 줄 것이다.

핵심어

스트레스반응시스템 / 인간 거대세포 바이러스 (human cytomegalovirus) / Nrf2 / 세포의 생존 / mTOR / C/EBPα / 바이러스의 DNA 복제 / GSK3β / non-canonical NF-κB / 만성염증