Cytomegalovirus-Encoded Chemokine Receptors and Mechanisms of Cellular Signal Transduction

By

Ryan Michael Melnychuk

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CERTIFICATE OF APPROVAL

This is certify that the Ph.D. thesis of

Ryan M. Melnychuk

has been approved

Jay Nelson, Ph.D, *thesis advisor*

Scott Wong, Ph.D, *committee member*

David Johnson, Ph.D, *committee member*

Klaus Frueh, Ph.D, *committee member*

Mihail Iordanov, Ph.D, *committee member*

"Freedom is the right to be wrong, not the right to do wrong."

John George Diefenbaker

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List of Abbreviations:

α: alpha

a.a.: aminoacid

A(nucleotide context): adenine

Ad: adenovirus

AIDS: acquired immunodeficiency syndrome

AP: activating protein

ApoE: apolipoprotein E

β: beta

BAC: bacterial artificial chromosome

BMT: bone marrow transplantation

bp: base pair

C: cytosine

C-: carboxyl

Ca^{2+}: calcium

cAMP: 5' cyclic adenosine monophosphate

CASMC: carotid artery smooth muscle cells

CCR: CC-chemokine receptor

cDNA: complementary DNA

CID: cytomegalic inclusion disease

CIDV: cytomegalic inclusion disease virus

CMV: cytomegalovirus

CR: chronic rejection

DAG: diacylglycerol

DARC: Duffy antigen/receptor for chemokine

DMEM: Dulbecco's modified Eagle's Medium

DN: dominant negative

DNA: deoxyribonucleic acid

E: refers to the β or early class of CMV expressed genes

ERK: extracellular regulated kinase

FAK: focal adhesion kinase

FCS: fetal calf serum

FITC: fluorescein-isocyanate-tetramethyl

γ: gamma

g: glycoprotein

G: guanine

gCI-gCIII: refers to glycoprotein complexes I through III of HCMV

GDP: guanosine 5' diphosphate

GEF: guanine nucleotide exchange factor

GPCR: G-protein coupled receptor

GRK: G-protein coupled receptor kinase

GTP: guanosine 5' triphosphate

HAART: highly active anti-retroviral therapy

HCMV: human cytomegalovirus

HHV: human herpesvirus

HIV: human immunodeficiency virus

hpi: hours post-infection

HSGV: human salivary gland virus

HSV: Herpes Simplex Virus Type

IE: refers to the α or immediate early class of CMV expressed genes

IE1-72: major gene product of UL123, refers to the 72-kilodalton protein expressed from this gene locus

IE2-86: major gene product of UL122, refers to the 86-kilodalton protein expressed from this gene locus

IKK: IκB kinase

IL: Interleukin

IP3: inositol (1,4,5)-triphosphate

IPTG: isopropyl-β-D-thiogalactopyranoside

IR_L: internal repeat of the unique long (UL) segment of the viral genome

IR_S: internal repeat of the unique short (US) segment of the viral genome

JNK: c-Jun NH_2-kinase

kb: kilobase

kbp: kilobase pairs

kd: kilodalton

L: refers to the γ or late class of CMV expressed genes

LDL: low-density lipoprotein

MCMV: mouse cytomegalovirus

MCP: viral capsid context, major capsid protein

mCP: minor capsid protein

MCP: chemokine context, monocyte chemoattractant protein

mC-AP: minor capsid associated protein

MDV: Marek's disease virus

MIP: macrophage inflammatory polypeptide

MIEP: major immediate early promoter and enhancer region of cytomegaloviruses

MLC: myosin light chain

moi: multiplicity of infection

mRANTES: mouse RANTES

ND10: nuclear domain 10

NH_2: amino-terminal

NHDF: normal human dermal fibroblasts

nm: nanometer

nM: nanomolar

ORF: open reading frame

oriLyt: HCMV origin of replication

PAR: protease activated receptor

PBS: phosphate buffered saline

PDGF: platelet derived growth factor

pi: post-infection

pp: phospho-protein

PKC: protein kinase C

PSG: penicillin-streptomycin-L-glutamine

PTK: protein tyrosine kinase

PTX: pertussis toxin

RANTES: regulated on activation, normal T cell expressed and secreted

Rat AoSMC: primary rat aortic smooth muscle cells

RCMV: rat cytomegalovirus

RhCMV: rhesus cytomegalovirus

RNA: ribonucleic acid

SCP: smallest capsid protein

SGV: salivary gland virus

SMC: smooth muscle cell

SOT: solid organ transplantation

TBS: tris-buffered saline

TBST: tri-buffered saline supplemented with 1% triton-X

TGN: trans-golgi network

TNF: tumor necrosis factor

TVS: transplant vascular sclerosis

TR_L: terminal repeat sequence of the unique long (UL) genome segment

TR_S: terminal repeat sequence of the unique short (US) genome segment

UL: unique long

US: unique short

vRNA: viral RNA packaged in CMV capsids

VLDL: very low-density lipoprotein

WT: Wild-type

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Abstract

Human cytomegalovirus (HCMV) is ubiquitous in the human population, causes significant disease in immunocompromised individuals, and has been implicated in vascular diseases such as atherosclerosis. HCMV encodes over 200 proteins including multiple chemokine receptors. The function of these chemokine receptors during the virus life cycle is unknown, although one of the HCMV G-protein coupled receptors (GPCR), US28, has been shown to induce smooth muscle cell (SMC) migration, suggesting a role in vascular disease. US28 is the most characterized HCMV GPCR and has been demonstrated to bind multiple chemokines. The characterization of US28 signaling has primarily been based on experiments performed in human fibroblasts including calcium flux and ERK-1/2 activation through $G\alpha_i1$ and $G\alpha16$. However, the mechanisms of US28 induction of SMC migration and the signaling involved in these migratory events are unknown. Therefore, in the first part of my thesis I have characterized the G-proteins that couple to US28 to promote SMC migration. I have observed that members of the $G\alpha12$ family are critical for SMC migration and that these G-proteins mediate migration through the RhoA signaling pathway. In the second part of my thesis, I examined the ligand specificity of US28 for induction of SMC migration. I observed that the CC-chemokines RANTES and MCP-1 but not the CX_3C-chemokine Fractalkine induced RhoA resulting in SMC migration. In addition, DNA microarray analysis indicated that RANTES induced cellular genes involved in cellular motility, while Fractalkine down-modulated these same genes. These data represent the first characterization of a chemokine receptor that binds multiple ligands to elicit alternative signaling responses. In the last part of my thesis, I characterized a functional US28

homologue (M33) in mouse CMV (MCMV). I observed that M33 induced mouse SMC migration either in the context of viral infection or independently in the presence of ligand. The signaling involved in migration was mediated through the Rac family of small G proteins in contrast to US28 induction of RhoA. These findings provide an animal model to examine the role of viral chemokine receptors in vascular disease.

Preface

The work presented in this dissertation was performed by the author under the supervision of Dr. Jay A. Nelson in the Program of Molecular and Cellular Biosciences, the department of Molecular Microbiology and Immunology at Oregon Health Sciences University. The data are presented in three chapters that have been published or submitted for publication in peer reviewed scientific journals. Chapter two, entitled "The Human Cytomegalovirus-Encoded G-protein Coupled Receptor US28 Mediates Smooth Muscle Cell Migration Through Gα12" was published in the Journal of Virology 78: 8382-8391. This publication describes US28-G-protein coupling and the activation of associated signaling molecules required for US28-mediated SMC migration. Chapter three, entitled "Ligand-Specific Smooth Muscle Cell Migration and Signaling Induced by The Human Cytomegalovirus Chemokine Receptor US28" describes the ligand specific nature of US28 signaling. Chapter four, entitled "Mouse Cytomegalovirus M33 is both Necessary and Sufficient in Virus-induced Vascular Smooth Muscle Cell Migration" establishes the MCMV-encoded chemokine receptor M33 as a functional homologue of US28 that promotes the ligand-dependent migration of SMC. Finally, Chapter five contains the overall conclusions derived from these studies, as well as future directions of CMV-encoded chemokine receptor studies.

Chapter 1

Introduction

1.0 The Herpesviridae

As established by the Herpesvirus Study Group of the International Committee on the Taxonomy of viruses, inclusion in the family Herpesviridae is based on virion architecture and four biological properties. In general, Herpesviruses encode enzymes involved in nucleotide metabolism, synthesize their DNA and assemble icosadeltahedral capsids of 100-110 nanometers (nm) in the nucleus, destroy infected host cells upon the release of progeny virus, and have the capacity to establish latency in the infected host. The family Herpesviridae is further divided into three subfamilies: the Alphaherpesvirinae, the Betaherpesvirinae and the Gammaherpesvirinae. The Alphaherpesviruses have a variable host range, display rapid growth kinetics and destructive spread in culture, and are able to establish latency in sensory neurons. In contrast, Betaherpesviruses display a restricted host range, slow growth kinetics often resulting in the production of enlarged cells, and establish latent/persistent infections in salivary glands, lymphoid cells and numerous tissues and organs. Gammaherpesviruses have a restricted host range, productively infect lymphoblastoid cells, display specificity for T or B cells and establish latency in lymphoid tissues.

1.1 Human Cytomegalovirus: A brief history

Human Cytomegalovirus (HCMV) is a ubiquitous β-herpesvirus that causes severe disease in neonates and immunocompromised individuals. The earliest descriptions of HCMV infection date back to the turn of the 20th century, specifically 1904, when large, abnormal cells with prominent intranuclear inclusions from the lungs, kidneys and liver of a syphilitic child were described (Jesionek, 1904). Within months, another report was

published describing cells of similar appearance in the kidneys of a stillborn infant (Ribbert, 1904). These abnormal cells possessed features characteristic of CMV infection, including prominent intranuclear inclusions surrounded by a zone of clearing. Some suggested that the appearance of abnormal cells in various organs and tissues was indicative of protozoal infection (Tietze, 1905). However, in 1921 Goodpasture and Talbot concluded that protozoa were not responsible for the intranuclear inclusions and suggested that the cellular abnormalities resembled cytological changes described in a previous study of skin lesions caused by varicella (Goodpasture and Talbot, 1921). Goodpasture and Talbot also coined the term 'cytomegalia' to describe these enlarged cells. Cells of similar appearance had also been observed in the salivary glands of guinea pigs (Jackson, 1920), and using the guinea pig model, the viral etiology of 'cytomegalia' was confirmed (Cole and Kuttner, 1926). Salivary gland suspensions from infected guinea pigs retained infectivity after passage through Berkefeld N filters, resulting in the production of cytomegalic cells in the salivary glands of injected animals (Cole and Kuttner, 1926). During these early years of research the agent responsible for 'cytomegalia' was simply referred to as salivary gland virus (SGV). The 1950's witnessed the adoption of the term generalized cytomegalic inclusion disease (CID) to describe infants with an often fatal disease characterized by the presence of enlarged cells with prominent intranuclear inclusions in multiple organs, and the virus associated with this condition became known as CID virus (CIDV).

Three groups independently isolated CIDV or human SGV (HSGV) during the mid-1950s. Margaret Smith first isolated mouse SGV in tissue culture in 1954 (Smith, 1954).

Using similar techniques she was able to isolate serially propagated HSGV as well as an isolate from the kidney of an infant who had succumbed to CID (Smith, 1956). Concurrently, the Weller group, who were attempting to isolate Toxoplasma from a liver biopsy of an infant suffering from hepatosplenomegaly, cerebral calcification and chorioretinitis; and the Rowe group, who were identifying new adenovirus isolates from adenoids of children undergoing tonsillectomy-adenoidectomy, isolated the Davis and AD169 strains of HSGV/CIDV respectively (Rowe et al., 1956; Weller, 1970; Weller et al., 1957). Finally, in 1960, the names SGV and CIDV were re-examined and dismissed since an unrelated SGV had previously been described in bats, and the tropism of the human virus was not restricted to the salivary glands, in favor of the name "cytomegalovirus" (Weller et al., 1960).

1.2 Epidemiology of Human Cytomegalovirus

HCMV is a ubiquitous pathogen that is found in virtually every human population, from developed industrialized nations to isolated aboriginal groups (Alford and Pass, 1981; Pass, 1985). HCMV infection is common, with an incidence of infection ranging from 40-100% depending on age (the frequency of infection increases with age) and socioeconomic status (Britt and Alford, 1996). In North America, approximately 90% of the urban population and 50% of individuals residing outside of cities display evidence of HCMV infection. Generally, HCMV is acquired earlier in life and at a greater frequency in developing countries and in the lower socioeconomic groups in developed nations. Although readily prevalent in most human populations, HCMV is not considered to be

highly infectious, as transmission requires direct contact with infected individuals or materials.

HCMV is the most common congenital viral infection in humans, infecting 0.2-2% of American infants *in utero,* and is considered to be the leading infectious cause of central nervous system maldevelopment and sensorineural deafness in neonates (Fowler et al., 1997; Larke et al., 1980; Peckham et al., 1983). HCMV may also be acquired during the childhood years through the consumption of infected breast milk or transmission through close contact with infected individuals, especially in the day care setting where approximately 8-60% of infants are infected during the first six months of life (Britt and Alford, 1996). In addition to infected breast milk, HCMV may be transmitted intrapartum or via the transplacental route. Epidemiological studies indicate that approximately 10% of all infants acquire HCMV from a maternal source (Pass, 1985).

While HCMV infection of normal immunocompetent individuals generally results in a life-long asymptomatic infection, this pathogen is responsible for approximately 8% of all cases of mononucleosis and 20-50% of the cases of heterophile-negative mononucleosis (Klemola et al., 1970). Infection of normal immunocompetent hosts has also been linked to a number of other clinical pathologies including: Guillain-Barr syndrome, peripheral thrombocytopenia, retinitis, gastrointestinal ulceration, hepatitis and pneumonitis (Ho, 1995; Nesmith and Pass, 1995).

Immunosuppressed and immunocompromised patients (transplant or graft recipients on immunosuppressive drug therapy or individuals suffering from acquired immunodeficiency syndrome (AIDS)) are particularly susceptible to opportunistic pathogens and HCMV infection is a significant cause of morbidity and mortality in these individuals. Infection of immunocompromised patients is common and can occur by reactivation of latent/persistent virus, re-infection or by primary infection (Britt and Alford, 1996). HCMV is the most common life threatening opportunistic viral infection in AIDS patients, infecting numerous tissues and organs including the lungs and more frequently the retina. HCMV-induced retinitis occurs in 85% of AIDS patients with AIDS related HCMV disease (Nichols and Boeckh, 2000). Most adults and approximately 50% of children with human immunodeficiency virus (HIV) have evidence of CMV infection, and prior to the advent of highly active anti-retroviral therapies (HAART) the incidence of CMV-associated disease in adults and children with AIDS was 40% and 9% respectively (Britt and Alford, 1996). Since the inception of HAART therapies, these numbers have decreased significantly (Nichols and Boeckh, 2000; Selik et al., 2002). The risk of developing clinical pathologies relating to HCMV infection in individuals suffering from AIDS is linked with CD4 T cell levels in the infected host (Gallant et al., 1992). Common clinical manifestations of HCMV infection in AIDS patients include: retinitis, esophagitis, colitis and less commonly: encephalitis, peripheral neuropathy, polyradiculoneuritis, pneumonitis, gastritis and hepatitis (Cheung and Teich, 1999).

HCMV infection is also problematic in immunosuppressed patients undergoing solid organ transplantation (SOT) or bone marrow transplantation (BMT). Primary HCMV infection in SOT recipients has been linked to organ failure, specifically, renal dysfunction in kidney recipients, hepatitis in liver recipients, coronary stenosis or organ rejection in heart transplant patients and pneumonia after lung or heart transplantation (Britt and Alford, 1996). In the absence of prophylaxis, 8-39% of SOT recipients develop symptomatic HCMV disease, including infections of the gastrointestinal tract, which occur in 5% of all transplant recipients (Sepkowitz, 2002). The most common clinical manifestation of HCMV infection in BMT recipients is pneumonitis. The sources of CMV in SOT and BMT recipients are reactivation of latent virus in seropositive patients or the acquisition of virus from transplanted cells, organs or blood products.

Increased incidence and severity of vascular diseases including atherosclerosis, arterial restenosis following angioplasty and solid organ transplant vascular sclerosis (TVS) or chronic rejection have been associated with HCMV infection. Since HCMV is a ubiquitous pathogen that causes both lytic and life-long persistent infections, and HCMV pathogenesis and vascular disease development are complex multi-factoral processes, there have been difficulties in establishing HCMV as a contributing factor in the development of vascular pathologies. However, there is increasing epidemiological and molecular evidence that suggest a role for CMVs in vascular disease. The initiation and progression of vascular disease, including the roles of chemokines, chemokine receptors and CMVs is discussed elsewhere (see Chapter1.6.1-1.6.3).

1.3 Genome and Structure of Cytomegalovirus

HCMV is the prototypical member of the β-herpesviruses, which are slow growing salivary gland tropic and highly species specific (Britt and Alford, 1996). Like other herpesviruses, CMVs have a large double stranded deoxyribonucleic acid (DNA) genome, an icosahedral nucleocapsid, a protein tegument, and a lipid envelop derived from host cell membranes.

1.3.1 Genome

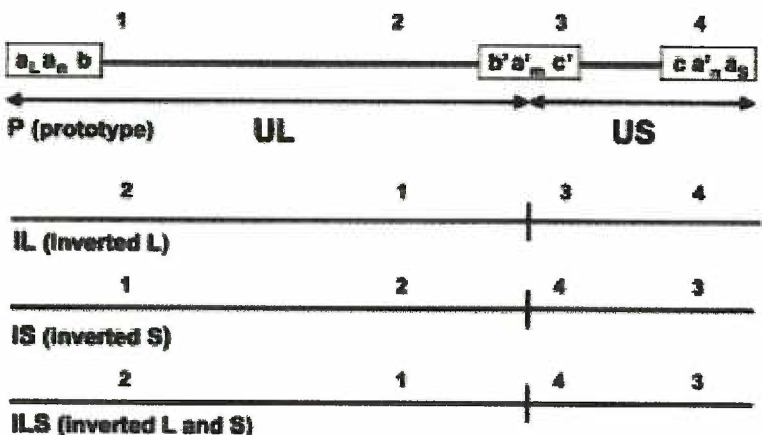
At 230 kilobase pairs (kbp), the genome of HCMV has the largest coding capacity of all known mammalian viruses. HCMV's genome is Guanine (G)+Cytosine (C) rich and is composed of two segments, the unique long (UL) and unique short (US). The UL segment is flanked by the terminal repeat TR_L and the corresponding internal repeat IR_L, while the US segment is bounded by and the internal repeat IR_S and the terminal repeat TR_S (Chee et al., 1990a). The UL and US regions may undergo genome inversion events mediated by *a* sequences found in the direct repeat sequences at the genome termini and in the inverted repeats at the UL-US junction. The *a* sequences contain cis-acting packaging elements that are required for DNA cleavage and packaging of the viral genome (Kemble and Mocarski, 1989; McVoy et al., 1998; Mocarski et al., 1987; Spaete and Mocarski, 1985). Ultimately, four genome isomers where the UL and US regions are oriented in either direction may be produced (Figure 1.1A). In contrast, the genomes of all other characterized β-herpesviruses, including all mammalian CMVs are linear and lack internal repeats.

Figure 1.1 Genome isomers and organization of HCMV genome.

(A) Laboratory strains of HCMV have the potential to produce four genome isomers in virus infected cells (adapted from Landolfo et al., 2003).

(B) Genome organization of laboratory and clinical isolates of HCMV (modified from Murphy et al., 2003)

A.



B.

Until recently, the only completely sequenced isolate of HCMV was strain AD169 (Chee et al., 1990a; Rowe et al., 1956). Based on this sequence, strain AD169 (230283 basepairs (bp)) was predicted to encode at least 208 non-overlapping open reading frames (ORFs) of greater than 80 amino acids (a.a.), 14 of which are duplicated within the TR_L/IR_L repeats (Chee et al., 1990a). Subsequent analysis revealed errors in the original sequence, including the absence of a 929bp sequence in the UL42-UL43 region (Dargan et al., 1997), as well as errors or deletions in the UL21.5, UL111a, UL48/49 and UL80.5 (Gibson et al., 1996b; Kotenko et al., 2000; Mullberg et al., 1999; Wood et al., 1997). Partial sequencing of the clinical isolate Toledo led to the identification of an additional 15 kpb region believed to encode 19 ORFs that were absent from AD169 (Cha et al., 1996). When chimpanzee CMV was sequenced and aligned with AD169, ten previously unrecognized ORFs were identified in both viruses (Davison et al., 2003). More recently, a number of clinical isolates including Toledo, Fix, Phoebe, and TR, as well as the laboratory strains AD169 and Towne were sequenced and compared (Murphy et al., 2003). Using an 80 a.a. cut-off these analyses revealed 252 ORFs with the potential to encode proteins, including 29 previously unrecognized ORFs that were conserved in all four clinical isolates (Murphy et al., 2003). Importantly, all of the clinical isolates possessed varying compliments of novel viral genes in place of the IR_L repeat (Figure 1.1B). The lack of an internal repeat suggests that clinical isolates only produce two genome isomers in infected cells. Therefore, there are significant differences in the DNA sequences of laboratory adapted strains and clinical isolates, and these differences are manifested in the ability of clinical isolates but not most laboratory strains to productively infect endothelial cells and monocyte/macrophages (see Chapter 1.5).

1.3.2 Capsid

The HCMV genome is surrounded by a 100nm icosahedral nucleocapsid (Figure 1.2A) that is composed of at least four viral proteins. The formation of icosahedral nucleocapsids requires the function of at least three additional viral proteins. The major structural proteins of the HCMV capsid include: the major capsid protein (MCP) encoded by UL86 (Chee et al., 1989), the minor capsid protein (mCP) encoded by UL85, the minor capsid associated protein (mC-AP) encoded by UL46, and the smallest capsid protein (SCP) encoded by UL48.5 (Baldick and Shenk, 1996; Gibson, 1983; Gibson, 1996). Three assembly related proteins encoded by UL80, UL80a and UL80.5, including a serine like protease have roles in capsid assembly and maturation (Baldick and Shenk, 1996; Chen et al., 1999; Gibson, 1996; Gibson et al., 1996a; Gibson et al., 1996b; Trus et al., 1999). The primary component of capsid pentamers and hexons is MCP (pUL85), while mCP (pUL85) and mC-AP (pUL46) associate in a 2:1 ratio to form triplexes that interdigitate the hexons and pentons (Butcher et al., 1998; Chen et al., 1999; Trus et al., 1999). The SCP is believed to associate with the tips of capsid hexons and have a role in tegumentation similar to VP-35 of Herpes Simplex Virus (HSV)-1 (Thomsen et al., 1994). Three different capsid forms are detectable in HCMV infected cells. Type A capsids are fully formed capsids that due to a failure to package viral DNA are devoid of genomes. Type B capsids are mature capsids that lack tegument proteins and type C capsids are fully mature B capsids with tegument and containing viral genomes (Figure 1.2B).

1.3.3 Tegument

Between the viral nucleocapsid and envelope lies an amorphous protein layer termed the tegument (Figure 1.2A). The HCMV tegument is acquired during viral egress and is composed of at least 25 proteins of cellular and viral origin (Gibson, 1983). Many of the tegument proteins detected in virion and dense body preparations are highly immunogenic phospho-proteins (pp) (Gibson, 1983; Landini and La Placa, 1991; Landini et al., 1989; Lazzarotto et al., 1991; Roby and Gibson, 1986). Two of the most abundant proteins made during viral replication, which are also major constituents of the virion, are the tegument proteins pp65 and pp150 (Gibson, 1983; Stinski, 1976), encoded by UL83 (lower matrix protein) and UL32 (basic phospho-protein) respectively (Jahn et al., 1987).

The tegument proteins are hypothesized to promote the creation of an intracellular environment that is optimal for viral gene expression and replication. In accordance with this hypothesis, the tegument proteins pp71 (UL82) and ppUL69 (UL69) have been demonstrated to have roles in the transactivation of viral and cellular gene expression (Liu and Stinski, 1992; Winkler and Stamminger, 1996). The latter tegument protein may also have a role in modulating cell cycle progression (Hayashi et al., 2000; Lu and Shenk, 1999). In addition to roles in cell cycle regulation and transcriptional transactivation, tegument proteins have also been reported to contribute to HCMV evasion of the host immune response (Jones et al., 1995). The cellular proteins beta-2-microglobulin, actin, actin-related protein 2/3 (Arp2/3), protein phosphatase I, annexin II and CD13 have also been detected in HCMV virion preparations (Baldick and Shenk, 1996; Gallina et al., 1999; Giugni et al., 1996; Grundy et al., 1987a; Grundy et al., 1987b;

Michelson et al., 1989; Michelson et al., 1996; Stannard, 1989; Wright et al., 1995); however, the importance of most of these cellular proteins during HCMV growth has not been established. Finally, five ribonucleic acid (RNA) transcripts corresponding to ORFs UL21.5, UL106-109, TR_L/IR_L 2-5, TR_L/IR_L 7 and TR_L/IR_L 13 and designated as virion RNAs (vRNAs) have been detected in preparations of highly purified, infectious HCMV particles (Bresnahan and Shenk, 2000). More recent studies indicate that at least the UL21.5 vRNA is packaged in proportion to the transcripts' intracellular concentration (Terhune et al., 2004). vRNAs are believed to be associated with the tegument and delivered to the host cell upon infection.

1.3.4 Envelope

A lipid envelope acquired from the infected cell surrounds the nucleocapsid and tegument (Figure 1.2A). Six major viral glycoproteins (g) have been detected in the HCMV envelope: gB (UL55), gH (UL75), gL (UL115), gO (UL74), gM (UL100), and gN (UL73), and at least three glycoprotein complexes gCI, gCII and gCIII are formed in HCMV infected cells (Gretch et al., 1988). The gCI and gCIII complexes are required for virus entry into the host cell (Theiler and Compton, 2001). The gCI complex is composed of homodimers of the major envelope glycoprotein gB. Recently, gB was demonstrated to interact with the epidermal growth factor (EGF) receptor to mediate the intitiaion of intracellular signaling cascades and virus entry (Wang et al., 2003). The gCI complex is also critical for cell to cell transmission of virus and HCMV-mediated cell fusion (Bold et al., 1996; Britt and Mach, 1996; Compton et al., 1993; Navarro et al., 1993; Tugizov et al., 1994). The glycoproteins gH, gL and gO associate to form the

heterotrimeric gCIII complex (Huber and Compton, 1998), whose transport to the cell surface is dependent upon the presence of gL (Kaye et al., 1992; Spaete et al., 1993). The gCIII complex is necessary for viral envelope fusion with the plasma membrane by means of a pH-independent mechanism (Huber and Compton, 1998). The gCII complex is a heterodimer composed of gM (UL100) and gN (UL73) (Mach et al., 2000), and this glycoprotein complex has been detected in the HCMV envelope (Kari et al., 1994; Lehner et al., 1989). With the exception of gO, which is dispensable for HCMV growth and replication in cultured fibroblasts (Hobom et al., 2000), gB, gH, gL and gM are all essential for *in vitro* infection. A number of other viral proteins have been detected in the HCMV envelope including gp48 (encoded by UL4) and the viral-encoded chemokine receptors UL33 and US27; however, the role of these proteins has not been determined.

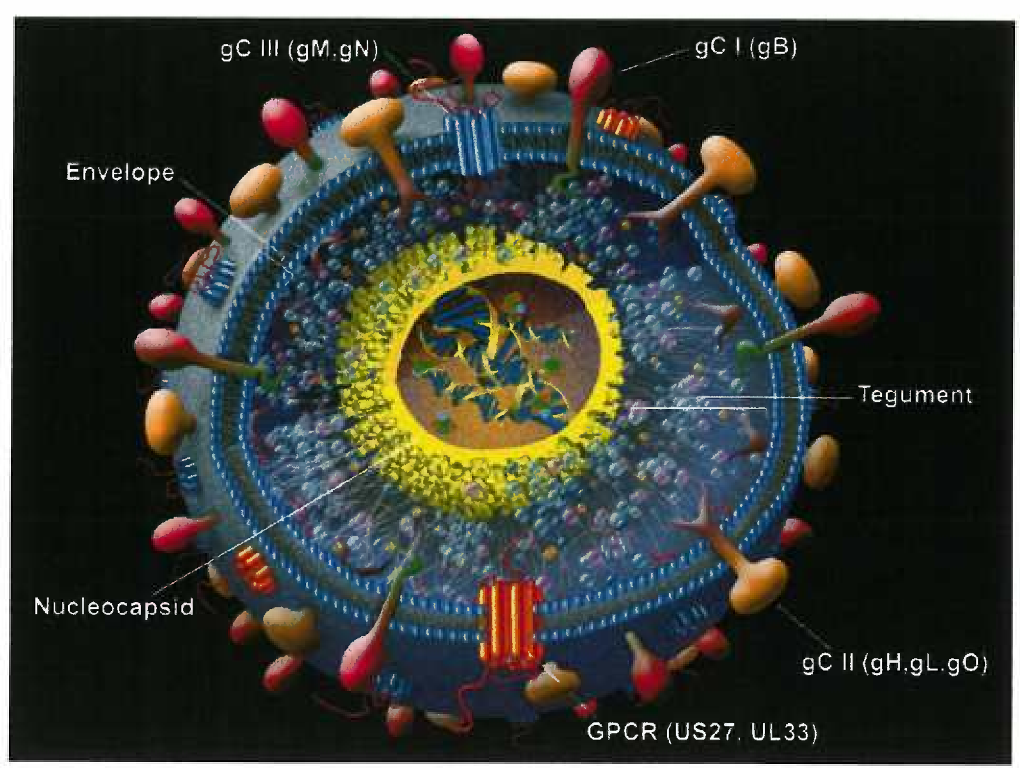
Figure 1.2 HCMV capsids and particles.

(A) Schematic of HCMV particle. The double stranded DNA genome is encased in a nucleocapsid. A tegument composed of viral and cellular proteins surrounds the nucleocapsid. A lipid envelope containing three viral-glycoprotein complexes (gCI, gCII and gCIII) as well as other proteins (including UL33 and US27) is acquired from the infected host cell (used with permission from J.A. Nelson and D.N. Streblow).

(B) Digital representation of three different capsids types that are produced in HCMV infected cells.

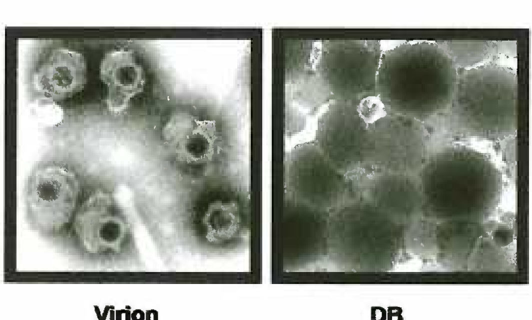
(C) Electron micrographs of infectious virions and dense bodies (Varnum et al., 2004).

A.



B.

C.



1.3.5 HCMV particles

Three different types of particles are produced in HCMV infected cells: mature infectious virions, noninfectious enveloped particles (NIEPs), and dense bodies (Mocarski, 2001). Mature infectious HCMV virions are composed of a linear 230kb double-stranded DNA genome encased in an icosahedral nucleocapsid that is surrounded by the protein tegument, and a viral-glycoprotein containing lipid envelope derived from host cell membranes (Figure 1.2C). Mature particles that lack viral genomes are designated as NIEPs. HCMV also produces unique particles termed dense bodies (Figure 1.2C). The tegument protein pp65 is the primary constituent of the nonreplicating enveloped dense bodies, accounting for approximately 60% of the protein abundance of these particles (Varnum et al., 2004).

Recently, our laboratory analyzed purified virions and dense bodies utilizing "gel-free" two-dimensional capillary liquid chromatography MS/MS and Fourier transform ion cyclotron resonance mass spectrometry. In addition to already identified proteins, a number of previously unknown proteins were identified in highly purified HCMV virions (Varnum et al., 2004). The known virion proteins identified included five capsid proteins (UL46, UL48-49, UL80, UL85, and UL86), fourteen tegument proteins (UL24, UL25, UL26, UL32, UL43, UL47, UL48, UL82, UL83, UL94, UL99, US22, US23, and US24), eleven glycoproteins (TRL10, UL22A, UL41A, UL55, UL73, UL74, UL75, UL77, UL100, UL115, and UL119), twelve proteins involved in DNA replication and transcription (IRS1, TRS1, UL44, UL45, UL54, UL57, UL69, UL72, UL84, UL89, UL97, and UL122), and two G-protein coupled proteins (UL33 and US27). In addition,

twelve HCMV-encoded polypeptides not previously associated with the virion were identified: UL5, UL38, UL50, UL71, UL79, UL93, UL96, UL103, UL132, US23, US24, and TRL14. This analysis also revealed the presence of far more cellular proteins than originally thought, including: ATP binding proteins, calcium (Ca^{2+}) binding proteins, chaperones, cytoskeletal proteins, enzymes, signaling molecules, transcription/translation factors and transport proteins. UL4, UL23, UL53, UL56, UL98a, and US28 had previously been reported to be associated with HCMV particles (Adair et al., 2002; Bogner et al., 1993; Chang et al., 1989; Dal Monte et al., 2002; Spaete et al., 1994), and although peptides corresponding to these proteins were detected, the peptides failed to meet the criteria for inclusion in the database of virion associated proteins.

1.4 Gene expression, regulation and replication

Once a viral pathogen has gained entry into a host cell, the virus must initiate a preordained transcriptional/replication program prior to the cell becoming aware of a foreign presence. Similar to other herpesviruses, HCMV lytic cycle gene expression is divided into one of three kinetic classes or temporal phases: immediate early (IE or α), early (E or β) and late (L or γ) (Stinski, 1978a; Stinski, 1978b; Wathen and Stinski, 1982; Wathen et al., 1981). Each temporal phase is characterized by the expression of a specific subset of viral genes. Treatment of infected cells with cycloheximide inhibits the expression of E and L genes but does not influence the trancription of IE genes. Therefore *de novo* protein synthesis is not required for IE expression (Wathen et al., 1981), rather, efficient transcription of the IE genes is dependent upon host cell and virion associated factors. The IE genes primarily encode transactivating proteins that

promote the expression of subsequent kinetic classes of viral genes, as well as the expression of selected cellular genes (Figure 1.3). E gene expression is dependent upon cellular factors, expression of IE gene products, and precedes viral DNA replication (Stinski, 1978b). The majority of the E genes encode factors that have direct roles in viral DNA replication, and other proteins that induce the expression of viral and/or cellular genes critical for the creation an intracellular environment that is optimal for viral replication (Figure 1.3). Expression of L genes requires IE and E gene products, as well as contributions from cellular factors and most L genes are not transcribed in the presence of inhibitors of viral DNA replication. L genes encode structural proteins including capsid, tegument and envelope glycoproteins, as well as proteins that have roles in virion assembly and morphogenesis (Figure 1.3).

1.4.1 Organization of the Major Immediate Early Promoter

Productive CMV infection requires efficient expression of the IE genes and expression of these genes is regulated by powerful promoter/enhancer regions. Driving expression of the HCMV major immediate early genes IE1-72 (72 kilodalton (kd) gene product of IE1) and IE2-86 (86kd gene product of IE2) is the major immediate early promoter (MIEP) and an adjacent enhancer region. The MIEP of HCMV is considered to be one of the strongest promoters and is commonly used in mammalian expression vectors to drive the expression of genes of interest. Within the MIEP are numerous repeat elements that contain binding sites for cellular transcription factors (Meier and Stinski, 1996; Stinski and Roehr, 1985). Although many of these repeat elements and transcription factor binding sites are conserved amongst CMVs, the number and organization of these sites

vary (Sandford and Burns, 1996). For example, within the MIEP of HCMV are four 18-bp repeats that contain consensus NF-κB or rel-binding sites, five 19-bp repeats that contain ATF/CREB binding sites, two activating protein (AP)-1 binding sites, as well as numerous SP-1 binding sites (Figure 1.4) (Meier and Stinski, 1996). The MIEP of MCMV on the other hand is almost exclusively composed of NF-κB (6 sites), and AP-1 (7 sites) binding sites, with a single ATF/CREB site located upstream of the NF-κB and AP-1 sites (Figure 1.4) (Dorsch-Hasler et al., 1985).

HCMV infection has been demonstrated to induce rapid nuclear translocation of NF-κB and promote NF-κB dependent transcription (Cherrington and Mocarski, 1989; Kowalik et al., 1993; Prosch et al., 1995; Sambucetti et al., 1989; Yurochko and Huang, 1999; Yurochko et al., 1997). Use of NF-κB inhibitors and expression of dominant negative proteins that inhibit NF-κB activity has revealed that efficient HCMV IE gene expression is dependent upon the activation of NF-κB family transcription factors (DeMeritt et al., 2004). The majority of these studies were performed in fibroblasts, and in addition to cell-type specific requirements for cellular transcription factors, HCMV may also require the activation of different factors for lytic and/or latent/persistent gene expression. Therefore, ATF/CREB, NF-κB and AP-1 transcription factors likely co-operate and contribute to MIEP transactivation (DeMeritt et al., 2004; Lee et al., 2004; Meier et al., 2002). MCMV has also been demonstrated to promote NF-κB (p50/p65) nuclear translocation (Gribaudo et al., 1995), and induce NF-κB p50/p105 transcription (Gribaudo et al., 1996); however, NF-κB activity is not necessary for MCMV transcription and replication *in vitro* (Benedict et al., 2004). The role of AP-1 family

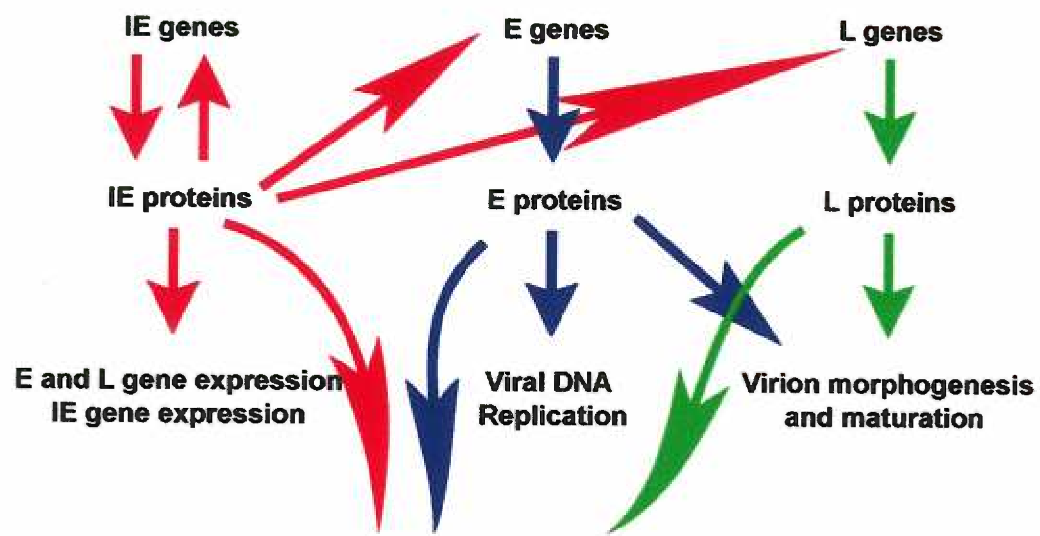
transcription factors in HCMV and MCMV gene expression and replication has not been determined.

1.4.2 Immediate Early Genes:

There are four regions within the HCMV genome that express IE transcripts: UL36-38, UL122/123, TRS1/IRS1 and US3 (Colberg-Poley, 1996; Spector, 1996; Stenberg, 1996), and a fifth region that may also fall into the IE kinetic class (Colberg-Poley, 1996; Stinski et al., 1983). IE transcripts are detectable within 1 hour post-infection (hpi), peaking between 4 and 8 hpi. IE proteins have been demonstrated to have key roles in the regulation of gene expression (Castillo and Kowalik, 2002; Castillo et al., 2000), immune modulation through the down-regulation of major immuno-histocompatibility complex I (MHC-1 by US3) (Ahn et al., 1996; Jones et al., 1996), and inhibition of apoptosis (UL36-38) (Goldmacher et al., 1999; Skaletskaya et al., 2001). The major IE gene products, IE1-72 and IE2-86, are expressed as alternatively spliced transcripts from the UL122/123 locus, and share a common 85 a.a. NH_2-terminal exon, differing in splicing to either UL123 for IE1-72 and UL122 for IE2-86 (Spector, 1996; Stenberg, 1996). Expression of these gene products is cell-type dependent and varies with respect to infection conditions. For example during HCMV infection of fibroblasts IE1-72 expression is sustained through out the course of infection, while expression of IE2-86 decreases during the early phase and increases at later time points (Stamminger et al., 1991; Stenberg et al., 1989).

Figure 1.3 HCMV gene expression and function.

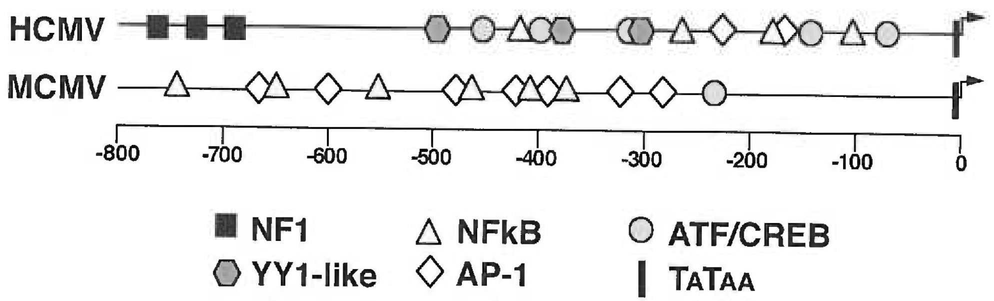
The IE proteins influence IE, E and L gene expression as well as cellular functions. E genes are critical for viral DNA replication, and influence host cell functions. L genes also regulate host cell function and have roles in virion morphogenesis and maturation (modified from Landolfo et al., 2003).



Regulation of host cell functions
-gene expression
-cell cycle progression
-apoptosis
-immune response

Figure 1.4 Organization and distribution of transcription factor binding sites in the MIEP of HCMV and MCMV.

The distribution of NF-κB, AP-1, NF-1, SP-1 and ATF/CREB binding sites in HCMV and MCMV (modified from Sandford and Burns, 1996).


HCMV
MCMV
-800
-700
-600
-500
-400
-300
-200
-100
0
NF1
NFkB
ATF/CREB
YY1-like
AP-1
TATAA

IE1-72 and IE2-86 regulate both viral and host gene expression; however, IE2-86 appears to be a dominant viral regulatory protein. IE2-86 functions as an autoregulator by binding several sites between the TATA box and transcription initiation sites of UL122/UL123 and is believed to control the transition from E (β) to L (γ) gene expression (Cherrington et al., 1991; Liu et al., 1991; Pizzorno and Hayward, 1990). The transactivator functions of IE2-86, although not requiring IE1-72, are enhanced when both proteins are present (Spector, 1996; Stenberg, 1996). IE1-72 has been shown to associate with, and disrupt the integrity of nuclear domain 10 (ND10) structures (promyelocytic leukemia bodies) (Ahn and Hayward, 1997; Ishov et al., 1997; Kelly et al., 1995; Korioth et al., 1996; Wilkinson et al., 1998). Disruption of ND10 structures is thought to be important in the creation of CMV replication compartments, structures similar to HSV-1 replication compartments that are foci of viral transcriptional activity (Ishov et al., 1997) and DNA replication (Ahn et al., 1999; Maul et al., 1996).

1.4.3 Early Genes:

Based on the kinetics of peak protein expression and sensitivity to inhibitors of viral DNA synthesis such as foscarnet, phosphonoformate (Wahren et al., 1985) or 9-(1,3-dihydroxy-2-propoxymethyl) guanine (DHPG, ganciclovir) (Mar et al., 1983), the E genes are further classified as early (β1) and early-late (β2). Generally, E gene expression commences by 24 hpi, with most E transcripts detectable by 4 to 12 hpi. The true E or β1 genes are transcribed within 4 to 8 hpi and early-late or β2 genes are transcribed within 8 to 24 hpi. Essential for viral replication is the expression of the core CMV replication proteins, including: the viral DNA polymerase (UL54), the tri-partite

helicase-primase complex (UL70, UL102 and UL105), the single stranded DNA binding protein (UL57), and the DNA processivity factor (UL44) (Griffiths, 2000), which are all expressed with E kinetics.

1.4.4 Late Genes:

In addition to having a requirement for the expression of prior kinetic classes of viral genes, as well as contributions from cellular factors, HCMV L gene expression is dependent upon viral DNA replication (Stinski, 1978b). L transcripts are first detectable by 24 hpi, and similar to the E class of viral gene products, the L class is further subdivided into two classes. Leaky-late or γ1 transcription occurs 24-36 hpi and treatment of infected cells with inhibitors of viral DNA replication can modulate their expression (Depto and Stenberg, 1989). True late or γ2 transcription occurs 24-48 hpi and expression of these genes is strictly dependent upon prior viral DNA replication (Adam et al., 1995; Depto and Stenberg, 1989; Meyer et al., 1988). The L genes encode structural proteins that contribute to virion assembly and morphogenesis.

1.4.5 Viral DNA Replication:

Unlike HSV-1, CMVs do not encode functional deoxyribonucleotide biosynthetic enzymes such as thymidine kinase, dihydrofolate reductase, thymidylate synthase or an active ribonucleotide reductase (Chee et al., 1990a; Rawlinson et al., 1996). Therefore, CMVs are dependent upon the host cell for an adequate supply of biosynthetic precursor molecules, and induce the expression of cellular enzymes involved in nucleotide metabolism. HCMV infection has been demonstrated to upregulate the expression of a

multitude of metabolic enzymes, including: thymidine kinase (Estes and Huang, 1977), ornithine decarboxylase (Benson and Huang, 1990; Isom, 1979), topoisomerase II (Benson and Huang, 1990), dihydrofolate reductase (Lembo et al., 1999; Song and Stinski, 2002; Wade et al., 1992), folylpolyglutamate synthetase (Cavallo et al., 2001), thymidylate synthase (Gribaudo et al., 2000; Gribaudo et al., 2002; Song and Stinski, 2002), deoxycytidilate deaminase (Gribaudo et al., 2003) and ribonucleotide reductase (Lembo et al., 2000; Song and Stinski, 2002). To ensure that the virus does not have to compete with the host cell for the use of metabolically important enzymes and biosynthetic precursor molecules, HCMV blocks cell cycle progression at G1-S (Bresnahan et al., 1996; Lu and Shenk, 1996; Lu and Shenk, 1999). In permissive cells that have already entered S or G2, HCMV infection may arrest the cell cycle at other later time points (Jault et al., 1995; Lu and Shenk, 1996; Salvant et al., 1998). The virion tegument protein ppUL69 (Hayashi et al., 2000; Lu and Shenk, 1999) and IE2-86 (Wiebusch and Hagemeier, 1999) have both been demonstrated to contribute to cell cycle dysregulation in HCMV infected cells, with IE2-86 mediating interactions with a number of critical cell cycle related proteins, including: p53 (Muganda et al., 1994; Speir et al., 1995; Speir et al., 1994; Tsai et al., 1996), pRb (Hagemeier et al., 1994; Sommer et al., 1994) and $p21^{cip1}$ (Sinclair et al., 2000).

HCMV contains a single origin of replication (oriLyt) localized adjacent to the UL57 gene locus (Anders et al., 1992; Hamzeh et al., 1990; Masse et al., 1992). The full length oriLyt sequence maps to 90500 to 93930 in the AD169 genome (Anders et al., 1992; Masse et al., 1992; Zhu et al., 1998) and contains numerous transcription factor binding

sites as well as clusters and repeat sequences (Anders et al., 1992; Hamzeh et al., 1990; Masse et al., 1992; Watanabe and Yamaguchi, 1993). Short transcripts that map to the oriLyt region have been detected in purified virions, suggesting that HCMV virions carry oriLyt-specific RNA-DNA hybrids (Prichard et al., 1998). Formation of these RNA-DNA hybrids may provide the initial impetus required to 'melt' adjacent clusters and repeats, thereby promoting the subsequent association of viral replication proteins that prevent re-annealing of DNA strands, thereby facilitating the establishment of the replication fork. Essential HCMV-encoded replication proteins include: the helicase-primase complex (UL70, UL102 and UL105) which unwinds double stranded DNA as the replication fork proceeds; the viral DNA polymerase (UL54); the major DNA binding protein (UL57); and the DNA processivity factor (UL44), which promotes an association between the viral polymerase and the replicating genome by binding double stranded DNA (Gibson, 1983; Gibson et al., 1981), and prevents the dissociation of the viral polymerase from the DNA template (Ertl and Powell, 1992; Weiland et al., 1994).

Transient transfection, DNA replication assays have revealed that in addition to the six core replication proteins described above, the gene products of UL84, UL112-113 and UL114 are required for minimal oriLyt-dependent DNA replication (Anders and McCue, 1996; Pari and Anders, 1993; Prichard et al., 1996; Sarisky and Hayward, 1996). UL84 encodes a 75kd phospho-protein that interacts with IE2-86 and functions as an origin specific initiator of oriLyt-dependent DNA replication (Samaniego et al., 1994; Sarisky and Hayward, 1996; Spector and Tevethia, 1994). The UL112/113 gene locus encodes a family of phospho-proteins (Iwayama et al., 1994; Wright et al., 1988) that localize to

viral DNA replication compartments (Ahn et al., 1999; Penfold and Mocarski, 1997), and contribute to the organization of these structures by recruiting viral DNA replication proteins and regulating the expression of core replication genes (Iskenderian et al., 1996; Pari et al., 1993). UL114 encodes a functional uracil DNA glycosylase that has a role in HCMV replication in post-mitotic cells (Prichard et al., 1996). IE1-72, IE2-86, TRS1/IRS1, and UL36-38 are also required for the transcomplementation of oriLyt-dependent DNA synthesis (Anders and McCue, 1996; Pari and Anders, 1993; Sarisky and Hayward, 1996).

Within 4 hpi the input viral genome circularizes, permitting the binding of key replication proteins and allowing the replication machinery access to the oriLyt. Amplification of the input genome via the birectional theta form of DNA replication is believed to commence by 16 hpi before, switching to late-phase rolling circle DNA replication (LaFemina and Hayward, 1983; McVoy and Adler, 1994). The latter form is responsible for the majority of the output viral genomes. Viral DNA replication peaks between 60 and 80 hpi in HCMV *in vitro* infected fibroblasts.

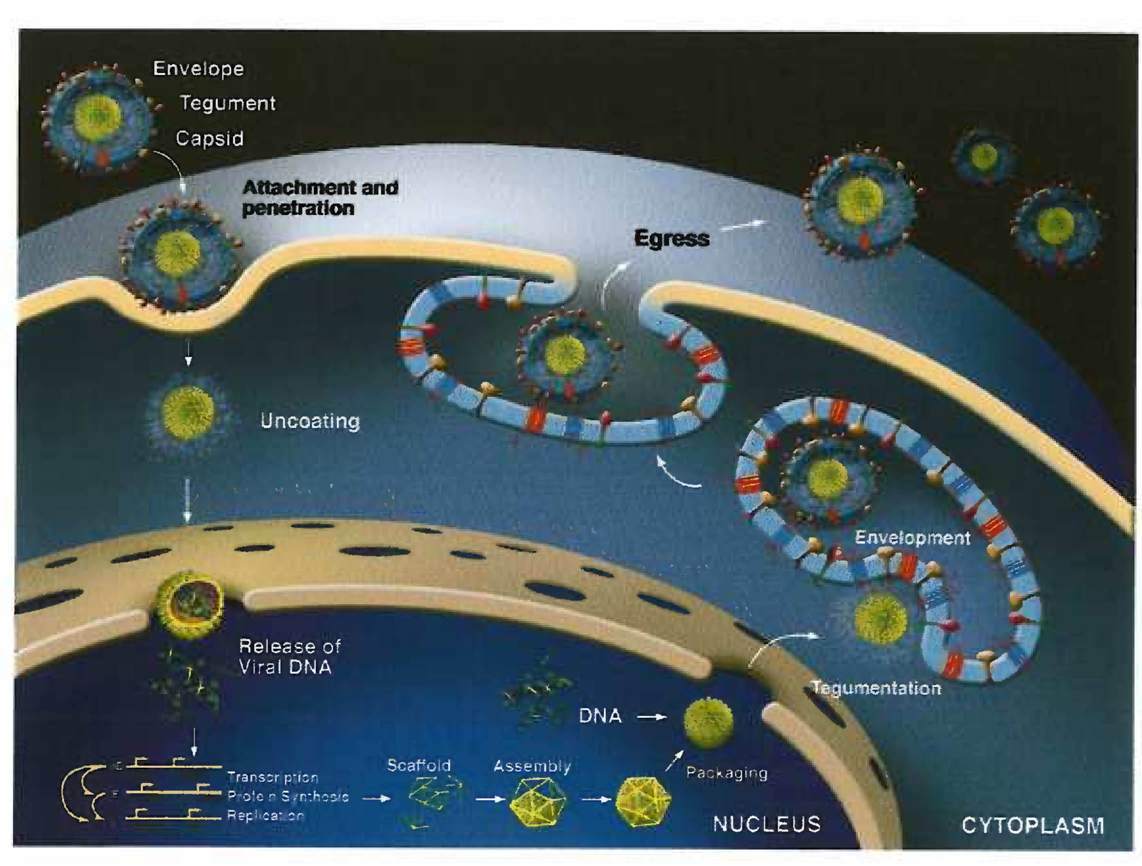
1.4.6 Egress

In the past, one of the more contentious issues in Herpesvirus research has been the nature and location of final capsid envelopment. A model termed the de-envelopment re-envelopment model is now widely accepted as the mechanism of Herpesvirus envelopement (Figure 1.5). Capsid assembly and packaging of the viral genome occurs in the nucleus of the infected cell and tegumentation occurs either in the nucleus or the

cytoplasm. Nucleocapsids are believed to acquire an envelope as they bud through the inner nuclear membrane; however, this envelope is lost when particles fuse with the plasma membrane (de-envelopment). Naked capsids are released into the cytoplasm and amass in the trans-golgi network and endosomes where mature viral-glycoproteins have accumulated. The subsequent budding of virus through the TGN is proposed as the mechanism of final envelopment (re-envelopment). Mature enveloped particles in the TGN are packaged in vescicles/vacuoles that are transported to the plasma membrane. The subsequent fusion of virus containing vacuoles with the plasma membrane mediates the release of infectious virus.

Figure 1.5 Life cycle of Human Cytomegalovirus.

Virus attachment and penetration into the host cell is mediated by an interaction between gB and the EGF recptor. Once in the cytoplasm, the tegumented capsids undergo an uncoating process. Capsids then dock with the nuclear pores and inject their capsid contents into the nucleus. The ensuing IE, E and L gene expression generates the necessary components for viral DNA replication. Once viral DNA replication has begun, capsid scaffold proteins assemble into nucleocapsids. The progeny DNA genomes are packaged into assembled nucleocapsids that may acquire some tegument proteins before budding through the inner and outer nuclear membrane and into the cytoplasm, where the tegument is completed. Budding of the particle into the TGN and/or endosomes is the proposed mechanism of final envelopment. Virus contained in secretory organelles and vacuoles are then transported to the cell surface. Fusion with the plasma membrane releases progeny virus into the extracellular environment (used with permission from J.A. Nelson and D.N. Streblow).


Envelope
Tegument
Capsid
Attachment and penetration
Egress
Uncoating
Envelopment
Release of Viral DNA
Tegumentation
DNA
Transcription
Protein Synthesis
Replication
Scaffold
Assembly
Packaging
NUCLEUS
CYTOPLASM

1.5 Cell type Specificity and Tissue Tropism of HCMV

HCMV has been demonstrated to infect epithelial cells, endothelial cells, SMC, mesenchymal cells, hepatocytes, granulocytes and monocyte-derived macrophages (Dankner et al., 1990; Einhorn and Ost, 1984; Gnann et al., 1988; Howell et al., 1979; Myerson et al., 1984; Schrier et al., 1985; Sinzger et al., 1995; Soderberg et al., 1993; Wiley and Nelson, 1988). Although HCMV does not productively infect all cell types, this pathogen has the capacity to gain entry into most human cell lines, as well as some cell types originating from other species. These observations indicate three things. First of all, the receptor(s) for HCMV is nearly ubiquitously expressed, promoting entry of HCMV into a diversity of different cell types. Second, although virus can gain entry into most cell-types, productive infection does not occur, suggesting that viral-encoded proteins may function as determinants of cellular tropism, thereby permitting virus growth. In conjunction with these viral-encoded factors, cell-type specific factors may also have roles and influence HCMV gene expression and replication. Indeed, variant strains of HCMV display differences in their abilities to productively infect endothelial cells and macrophages (Kahl et al., 2000; MacCormac and Grundy, 1999; Sinzger et al., 2000; Sinzger et al., 1999). Interestingly, most clinical isolates are initially able to infect both endothelial cells and fibroblasts, however, upon serial propagation in fibroblasts, the ability of HCMV to replicate in endothelial cells is lost (Sinzger et al., 1999)

A number of CMV proteins have been identified as determinants of endothelial cell and/or macrophage tropism. M140 and M141, MCMV homologues of HCMV US22 family genes have been shown to be required for normal *in vitro* replication of MCMV in

macrophages (Hanson et al., 1999a; Hanson et al., 2001; Hanson et al., 1999b). A more recent study demonstrated that M139, M140 and M141 interact at the protein level to cooperatively promote MCMV growth in macrophages by an unknown mechanism (Menard et al., 2003). This study also employed a random transposon mutagenesis screen of the MCMV genome in bacterial artificial chromosome (BAC), identifying the anti-apoptotic Bcl-2 homologue, M36, as being necessary for MCMV *in vitro* growth in macrophages but not fibroblasts or an endothelial cell line (Menard et al., 2003). A similar random transposon BAC screen identified the M45 gene, which bears homology to ribonucleotide reductase, as being necessary for the growth of MCMV in cultured endothelial cells (Brune et al., 2001). This gene was also necessary for normal *in vitro* replication of MCMV in macrophages, but not fibroblasts, bone marrow stromal cells or hepatocytes. In contrast, UL45 is dispensable for HCMV-FIX growth in endothelial cells (Hahn et al., 2002). The role of US22 family genes, and UL36 in HCMV macrophage and endothelial cell tropism is not known.

1.6 Cytomegaloviruses and Vascular disease

1.6.1 Vascular Disease

Despite recent advances in molecular medicine, cardiovascular disease remains the leading cause of death in the United States. While atherosclerosis accounts for the majority of deaths due to vascular disease (Colaco et al., 1994), restenosis after coronary angioplasty, and transplant vascular sclerosis (TVS)-the hallmark lesion of chronic solid organ graft rejection-cause significant morbidity and mortality in humans (Heck et al., 1989). Although the initiating circumstances for vascular disease processes differ, the pathogenesis and manifestations are similar, with an end result of vessel narrowing or occlusion, leading to reduced blood supply and end organ ischemia. Numerous risk factors are associated with the development of atherosclerosis including: hypertension, chemicals/tobacco, hyperlipidemia, diabetes, immunological factors and infectious agents of bacterial and viral origin. Indeed, both HCMV and the gram negative bacteria *Chlamydia pneumonia* have been associated with increased incidence and severity of vascular disease (Grayston, 1992; Streblow et al., 2001a; Britt and Alford 1996).

The long-term survival of solid organ allografts has not improved improved in recent years, largely due to chronic rejection (CR). The primary component of CR is an accelerated form of arteriosclerosis, otherwise known as TVS. The incidence of TVS is approximately 10-20% of patients per year, resulting in a prevalence of 60% in patients surviving at least 5 years (Hosenpud et al., 1992; Miller, 1992). Several risk factors have been identified for CR including: donor age, acute rejection, HLA mismatches, hypertension, hypercholesterolemia, and HCMV infection (Almond et al., 1993; Eich et

al., 1991; Isoniemi et al., 1994; Kobashigawa and Kasiske 1997). The high prevalence of CR is of particular concern given that to date the only effective therapy is re-transplantation. TVS is characterized histopathologically by diffuse concentric intimal proliferation that ultimately occludes the vessel (Billingham, 1992). In early or moderate lesions the endothelium is morphologically intact and may appear activated and undamaged. A sparse number of subendothelial cell lymphocytes are seen in earlier lesions, containing macrophages, T-cells, NK cells, and B-cells, while the late lesions are associated with a thickened intima consisting of SMC interspersed with macrophages (Cramer et al., 1992). Important stimuli to the migration and proliferation of SMC are various growth factors, cytokines, and chemokines, which, in response to injury, are produced by platelets, macrophages, and endothelial cells (Clinton and Libby, 1992). The internal elastic lamina remains intact except for small breaks, and the media thickness typically is unaffected by TVS. In addition, TVS is limited to the allograft vessels. Although the pathogenic mechanism of TVS is unknown, the selective involvement of allograft vessels, the diffuse concentric distribution, and histopathologic studies demonstrating a low-grade inflammatory process in the subendothelial layer of vessels, suggest that TVS is, in part, an immune-mediated disease (Billingham, 1992; Hosenpud et al., 1992; Libby et al., 1989). Restenosis following cornonary angioplasty is a vascular pathology initiated by excessive damage to the vascular endothelium during angioplasty procedures. Subsequent inflammatory events, and increased cellular migration and proliferation result in the formation of vascular lesions. Ultimately, all of these vascular pathologies result in vessel narrowing and occlusion and in the TVS, ultimate graft failure.

In general, the initiation and progression of atherosclerosis, TVS and restenosis follow similar developmental stages (Figure 1.6). The first step in the development of vascular disease is an initial insult or injury to the vascular endothelium. In response to this injury, the affected cell and/or adjacent cells release growth factors, chemokines (small chemotactic cytokines) and cytokines that promote the migration of monocyte/macrophages and adherence of platelets to the site of injury. The initial lesion, or the fatty streak, is formed when activated macrophages and T cells accumulate at the site of injury (Ross, 1996). Subsequent platelet adhesion and vascular smooth muscle cell migration and proliferation are observed. Critical to platelet adherence, and the migration and proliferation of both platelets and vascular SMC are the growth factors, chemokines and cytokines secreted by platelets, activated macrophages and endothelial cells. Ultimately, a fibrous plaque composed of SMC, foamy macrophages, and activated T cells embedded in a collagenous matrix of connective tissue is formed. The plaque protrudes into the lumen, obstructing the flow of blood through the vessel, eventually resulting in vessel occlusion.

1.6.2 Viral Association with Vascular Diseases

Epidemiological studies indicate that both bacteria and viruses are associated with increased incidence of vascular disease. While *Chlamydia pneumonia* has been associated with the development of coronary heart disease, carotid stenosis, thrombosis of the lower extremities and aortic aneurysms (Kuo and Campbell, 2000; Kuo et al., 1993; Shor et al., 1992; Taylor-Robinson and Thomas, 2000), infection with HCMV has

been linked to atherosclerosis, TVS following solid organ transplant and restenosis after angioplasty. HCMV seropositivity correlates with a two-fold increase in vascular disease (Melnick et al., 1996; Sorlie et al., 2000), and a two to three-fold increase in coronary artery disease (Muhlestein et al., 2000). The existence of molecular data further exemplifies the association between HCMV and vascular diseases. For example, HCMV antigens and nucleic acids have been detected in the early lesions of diseased vessels (Hendrix et al., 1989; Melnick et al., 1983b; Speir et al., 1994; Zhou et al., 1996b).

A number of recent studies further illustrate an association between HCMV infection and vascular disease. First of all, CMV was detected in the arterial wall of 76% of patients with ischemic heart disease (Horvath et al., 2000). Secondly, HCMV DNA was detected in 53% of carotid artery atherosclerotic lesions (Qavi et al., 2000). Additionally, HCMV was detected in fatty streaks and normal appearing areas of diseased vessels, near intercostal arterial openings, but was rarely detected in late atherosclerotic plaques (Pampou et al., 2000). DNA hybridization studies have revealed the presence of HCMV DNA in endothelial cells and in SMC (in the subendothelium, vessel intima and media). HCMV is also able to productively infect cells of the monocyte/macrophage lineage both *in vitro* and *in vivo* and monocyte/macrophages have been proposed as a site of HCMV latency and/or persistence (Soderberg-Naucler et al., 1997; Soderberg-Naucler et al., 1998). Therefore, HCMV has the ability to infect all the major cell types associated with vascular disease (endothelial cells, macrophages, and SMC), and HCMV antigens and nucleic acids have been detected in diseased vessels as well as early atherosclerotic

plaques, suggesting that HCMV may have a role in the initiation, development and exacerbation of vascular diseases (Figure 1.7).

Figure 1.6 Stages in the development of vascular pathologies.

(A) Healthy vessel.

(B) Initial insult or injury to the vascular endothelium that may be caused by numerous factors including: diet, pathogens, chemicals trauma or an individual's genetics.

(C)) In response to the injury, cytokines, chemokines, and growth factors are released, creating localized chemokine/cytokine gradients. Platelet adhesion is also observed.

(D) The released cytokines and chemokines promote inflammation including the migration of macrophages, T-cells, B-cells and platelets to the site of injury.

(E) The initial vascular lesion or the fatty streak. The Up-regulation of scavenger receptors on macrophages promotes the up-take of lipids, generating foamy cells (foamy macrophages). SMC migration and proliferation are also observed at the site of inflammation.

(F) Ultimately, a fibrous plaque that obstructs blood flow is formed. (adapted from Streblow et al. 2001a)

Stages In the Development of Vasculopathy
(Atherosclerosis, Restenosis, Vascular Sclerosis)

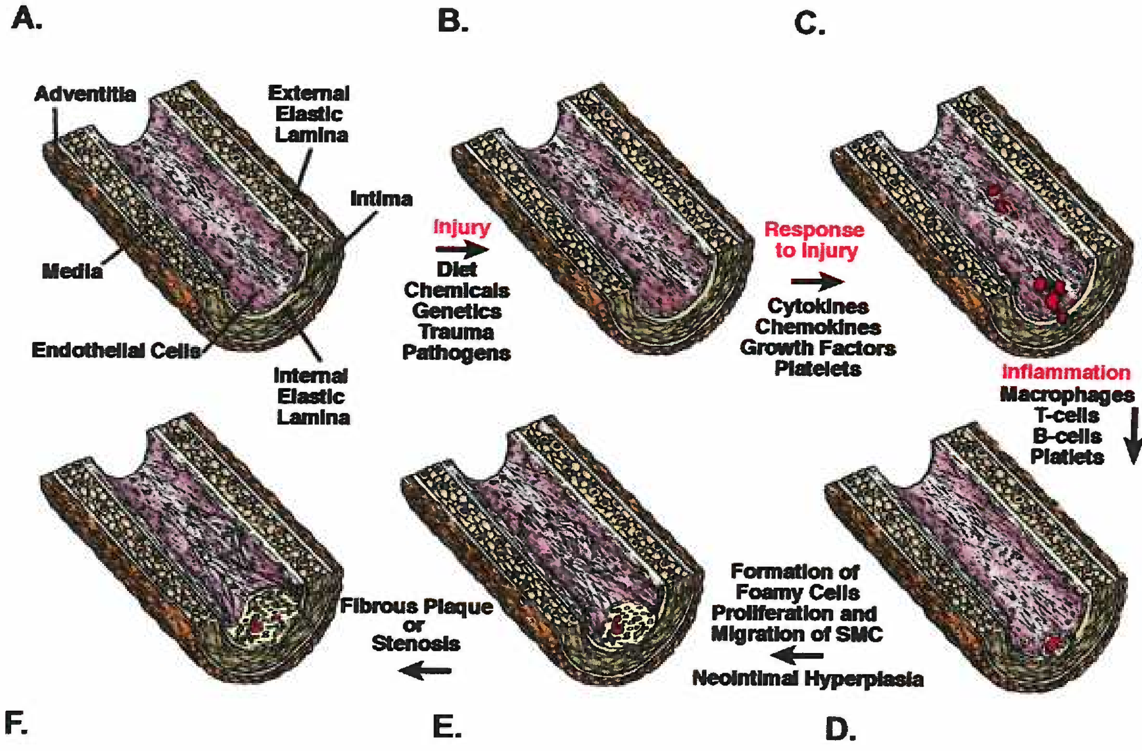
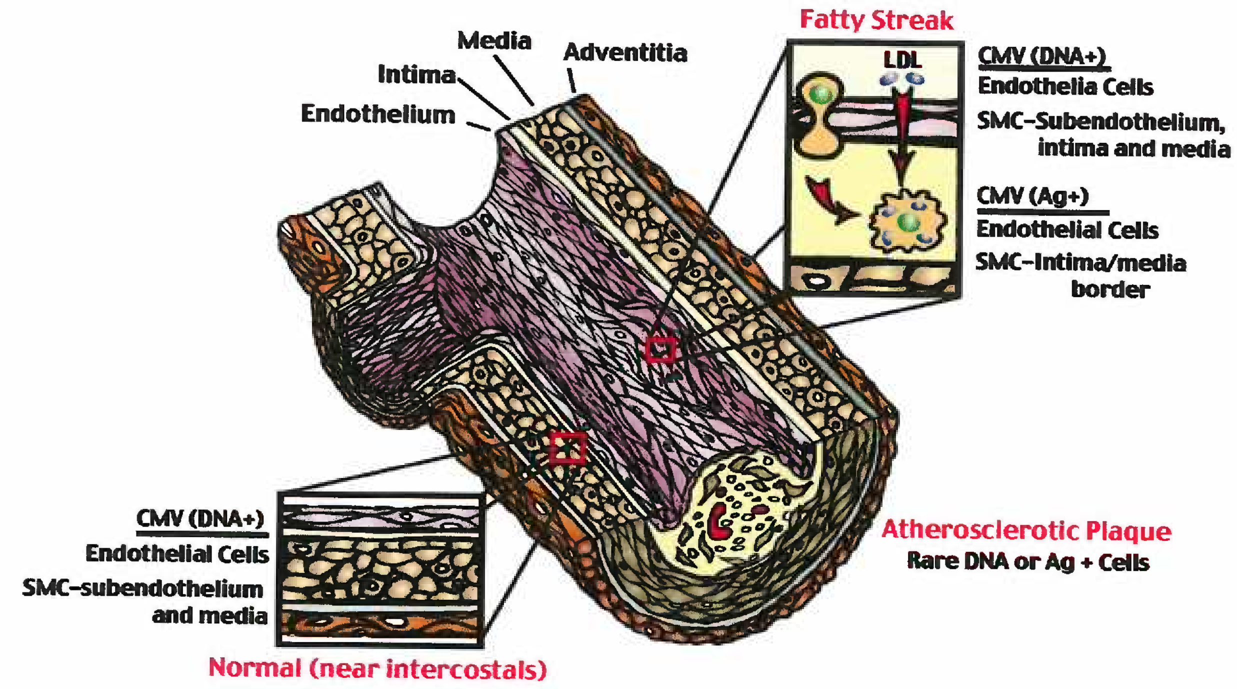


Figure 1.7 Detection of HCMV in the aorta.

HCMV DNA has been detected in endothelial cells and subendothelial, intimal and medial SMC in fatty streaks. HCMV antigens have also been detected in fatty streaks in endothelial cells and SMC at the intima/media border. HCMV nucleic acids have also been observed in SMC in the subendothelium and vessel media, as well as in endothelial cells near intercostals openings. CMV DNA and antigens is rarely detected in atherosclerotic plaques (adapted from Streblow et al., 2001a).

HCMV Detection in the Aorta



1.6.3 Animal Models of Virus-Induced Vascular Disease

Since the genomes and pathogenesis of human, rat and mouse CMVs are similar, animal models provide an ideal model to investigate the association between CMV and vascular disease. Marek's disease virus (MDV), a herpesvirus that infects fowl, was the first etiologic agent found to induce atherosclerosis (Fabricant et al., 1983). MDV infected chickens demonstrate atherosclerotic lesions with similar histologic features to human atherosclerosis and MDV antigens were detected in early lesions and late in SMC at the periphery of vascular lesions. Recently developed mouse models of atherosclerosis have dramatically improved the ability to study lesion formation and development. Crossing Apolipoprotein E deficient (ApoE-/-) mice, which demonstrate an increased incidence of atherosclerosis when fed high fat diets due to increased levels of low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) (Nakashima et al., 1994); with other genetically altered mice has been widely used to study the effects of host proteins in vascular lesion formation. MCMV infection of ApoE-/- mice increases the frequency and severity of lesion formation and accelerates the development of atherosclerosis even when the mice are fed low fat diets (Burnett et al., 2001).

Rat models have also been devised to study the effects of CMV in the development of vascular diseases. Specifically, rat models have been used to study the effects of RCMV on the acceleration of restonosis in a carotid artery balloon angioplasty model. For example, in a rat carotid artery injury model, RCMV-infected animals demonstrated a 40% increase in neointimal formation when compared to controls (Zhou et al., 1995). In rat solid organ transplantation models, infection with RCMV accelerates TVS and graft

failure (Bruning et al., 1994; Lemstrom et al., 1993; Lemstrom et al., 1995). Similarly, in rat heart transplantation and small bowel transplantation models of chronic rejection, acute infection with RCMV increases the degree of TVS in graft vessels, while decreasing the mean time to TVS and graft failure (Orloff, 1999; Orloff et al., 1999).

1.6.4 Role of Chemokines and Chemokine Receptors in Vascular Disease:

Secreted proteins including growth factors, cytokines and chemokines have critical roles in the inflammatory events required for the initiation and development of vascular pathologies. Chemokines are a subgroup of small inducible cytokines that promote cellular migration and activation upon binding 7-transmembrane spanning G-protein coupled receptors (GPCRs) (Rollins, 1997). There are four major chemokine subgroups: CC-, CXC-, -XC and CX_3C-, which differ in the spacing of the first two of four NH_2-terminal cysteine residues. The CXC-chemokines are further subdivided into two groups based on the presence or absence of an NH_2-terminal Glu-Leu-Arg (ELR)-motif prior to the first cysteine residue (Murphy et al., 2000). ELR containing CXC-chemokines are generally regarded as pro-inflammatory and angiogenic, while non-ELR CXC-chemokines are considered angiostatic. Unlike other chemokines which are secreted as soluble molecules, the only identified CX_3C-chemokine, Fractalkine, is generated as a membrane bound molecule with the chemokine domain presented at the apex of a cell-bound mucin like stalk (Bazan et al., 1997; Pan et al., 1997). Soluble Fractalkine is produced by proteolysis by tumor necrosis factor (TNF)-α converting enzyme or ADAM10 (Garton et al., 2001; Tsou et al., 2001). The binding of chemokines to their receptors activates a plethora of cell type specific signal transduction pathways

depending on the chemokine/receptor pair, resulting in the migration of receptor bearing cells towards sites of chemokine secretion, and promoting the additional production of other cytokines, chemokines and/or growth factors that amplify the inflammatory response. Chemokine binding also stimulates the upregulation of numerous proteins that promote cellular adhesion including integrins, growth factors and growth factor receptors.

Numerous CC-chemokines have been detected in human and experimentally induced animal model atherosclerotic plaques, suggesting a strong association between the presence of these secreted molecules, induction of the inflammatory response and vascular disease (Nelken et al., 1991; Pattison et al., 1994; Yla-Herttuala et al., 1991). For example, expression of the CC-chemokine regulated upon activation, normal T expressed and secreted (RANTES) was elevated in kidney allografts of recipients undergoing chronic rejection compared to native kidneys in patients who were chronically rejecting heart allografts (Pattison et al., 1994). Experimental *in vivo* animal models have been particularly useful in elucidating the roles and contributions of chemokines and chemokine receptors in the exacerbation and progression of vascular diseases. Specifically, RANTES has been detected in atherosclerotic plaques and was highly expressed in atherosclerotic lesions associated with heart transplant chronic rejection and vasculopathy in mice (Terkeltaub et al., 1998). Genetically modified mice that are deficient in apolipoprotein E (ApoE-/-) only develop atherosclerotic lesions when fed high fat diets. ApoE-/- mice on high fat diets that were also deficient in monocyte chemoattracttant protein (MCP)-1 or its receptor CC-chemokine receptor (CCR)-2 demonstrate a reduction in the degree of atherosclerosis, indicating that this

chemokine/receptor pair has a significant role in stimulation of the inflammatory response during the development of vascular pathologies (Boring et al., 1998a; Gu et al., 1998). Similarly, rat renal allografts undergoing chronic rejection expressed both MCP-1 and RANTES, and MCP-1 expression was elevated and persisted long term in chronically rejecting rat cardiac allografts in experimentally induced hypertensive rats. (Nadeau et al., 1995; Russell et al., 1993) Furthermore, in a rat cardiac transplantation model, CC- chemokine expression was increased in rat CMV (RCMV)-infected graft recipients compared to uninfected controls (Streblow et al., 2003a). All of these data illustrate the critical role of chemokines and chemokine receptors in the development and progression of vascular pathologies.

1.6.5 Cytomegalovirus-Encoded Chemokine Receptors

Herpesviruses encode numerous proteins with the potential to modulate host inflammatory responses, including molecules with significant homology to chemokines and chemokine receptors. All β-herpesviruses encode homologues of both CC- and CXC-chemokine receptors (Chee et al., 1990a; Chee et al., 1990c; Gompels et al., 1995) and HCMV encodes four putative chemokine receptors: UL33, UL78 (CXC-), US27 and US28 (CC-) (Chee et al., 1990a; Chee et al., 1990c). MCMV, RCMV and guinea pig CMV each encode two chemokine receptor homologues: M33, R33 and GP33 (the positional homologues of UL33 in HCMV), and M78, R78 and GP78 (the positional homologues of UL78 in HCMV) respectively (Rawlinson et al., 1996). Rhesus CMV (RhCMV) encodes as many as seven putative chemokine receptors: Rh33, Rh78 and five US28 homologues designated as RhUS28-1, RhUS28-2, RhUS28-3, RhUS28-4 and

RhUS28-5 (Hansen et al., 2003). The β-herpesviruses Human Herpesvirus (HHV)-6 and HHV-7 also encode two chemokine receptors, U12 (CC-) and U51 (CXC-) (Gompels et al., 1995). *In vitro* binding studies and screening assays that utilize calcium (Ca^{2+}) as a read-out of functional activity indicate that the majority of these viral-encoded GPCRs are functional molecules; however, the role of these molecules in viral growth and pathogenesis is unclear.

Based on their extensive sequence and structural homology to cellular chemokine receptors of known function, CMV-encoded GPCRs have been envisioned to play any number of roles, including modulation of the host inflammatory response. The HCMV-encoded chemokine receptors US27 and US28 have been demonstrated to bind and internalize CC-chemokines (Michelson et al., 1997). By scavenging chemokines, these receptors have been postulated to prevent the recruitment of immune cells to sites of chemokine production, thereby acting as chemokine sinks that prevent further stimulation of the inflammatory response. More recent experimental data does not support this notion, as ligand binding by US28 results in rapid and robust cellular activation (Billstrom et al., 1998), and true chemokine sinks bind and internalize ligands without inducing intracellular signaling events.

Since chemokines and chemokine receptors are required for the formation and maintenance of secondary lymphoid organs, and are necessary for the homing and migration of leukocytes to to inflammatory sites (Muller et al., 2003), CMV-encoded chemokine receptors have been proposed as a means of inducing cellular motility for the

dissemination of virus within the infected host. In this model, inflammatory events that recruit immune cells would also stimulate the migration of HCMV-infected and viral-chemokine receptor expressing cells to the sites of chemokine production and inflammation. At these inflammatory sites HCMV could be transmitted to attracted infection competent cells including macrophages, SMC, and dendritic cells, as well as nearby epithelial or endothelial cells (Streblow et al., 1999). Although deletion of US28 in HCMV has no effect on viral growth and replication *in vitro* (Vieira et al., 1998), *in vivo* infection with MCMV or RCMV in which either M33, M78, R33 or R78 have been disrupted results in reduced viral titers in the salivary glands and other organs compared to infection with wild type virus (Beisser et al., 1999; Beisser et al., 1998; Davis-Poynter et al., 1997; Kaptein et al., 2003; Oliveira and Shenk, 2001). These findings suggest that CMV-encoded chemokine receptors have important roles in CMV pathogenesis and virus dissemination *in vivo*. Viral chemokine receptors have also been postulated to function as co-receptors for HIV. While transient expression of US28 permits the entry of some strains of HIV-1 *in vitro* (Ohagen et al., 2000; Pleskoff et al., 1997), whether US28 or other HCMV-encoded chemokine receptors are able to promote entry of HIV-1 *in vivo* is not known.

1.6.6 Gα-proteins and Cellular Signaling

The seminal event in GPCR-mediated signal transduction is the activation of the heterotrimeric G-proteins. Upon ligand binding, GPCRs undergo structural changes that promote subsequent conformational changes in the receptor associated Gα-subunits of the heterotrimeric G-protein complex. These conformational changes permit the Gα-

subunit to exchange GDP for the physiologically more abundant GTP, thereby activating the Gα-protein, and promoting the release of associated Gβγ-subunits, which activate their own distinct signaling cascades. The Gα-proteins also possess an intrinsic GTPase activity and cleave the γ-phosphate of GTP, attenuating the G-protein signal. Completing the G-protein cycle, GDP associated Gα-proteins readily interact with free βγ-subunits, forming inactive heterotrimeric Gαβγ complexes that reassociate with surface expressed GPCRs (Svoboda et al., 2004).

The distribution of individual Gα-proteins varies in a tissue and cell-type dependent manner, and individual Gα-proteins are known to signal through distinct cellular signaling pathways (Figure 1.8). The pertussis toxin (PTX) sensitive Gαi/o family have a wide tissue distribution and includes: Gαi1, Gαi2, Gαi3, Gαo, and Gαz. The only exceptions are the PTX insensitive Gαo and Gαz, which are limited to neuronal tissues (Gαo and Gαz) and platelets (Gαz only) (Fields and Casey, 1997). Most inflammatory cytokines/chemokines, including chemokines that promote the migration of inflammatory cells, signal through Gαi/o-dependent pathways as is evidenced by their sensitivity to PTX (Fields and Casey, 1997; Murphy, 2001; Thelen, 2001). Signaling pathways activated by the ubiquitously expressed Gαs often stimulate the activation of adenylyl cyclases resulting in the intracellular accumulation of cAMP (Fields and Casey, 1997). Gαq family members activate a plethora of signaling pathways that includes the activation of phospholipase C-β (PLC-β), producing the second messenger molecules (1,4,5)-triphosphate (IP3) and diacylglycerol (DAG), which mediate corresponding increases in cytoplasmic Ca^{2+}. Gαq and Gα11 are ubiquitously expressed, while the

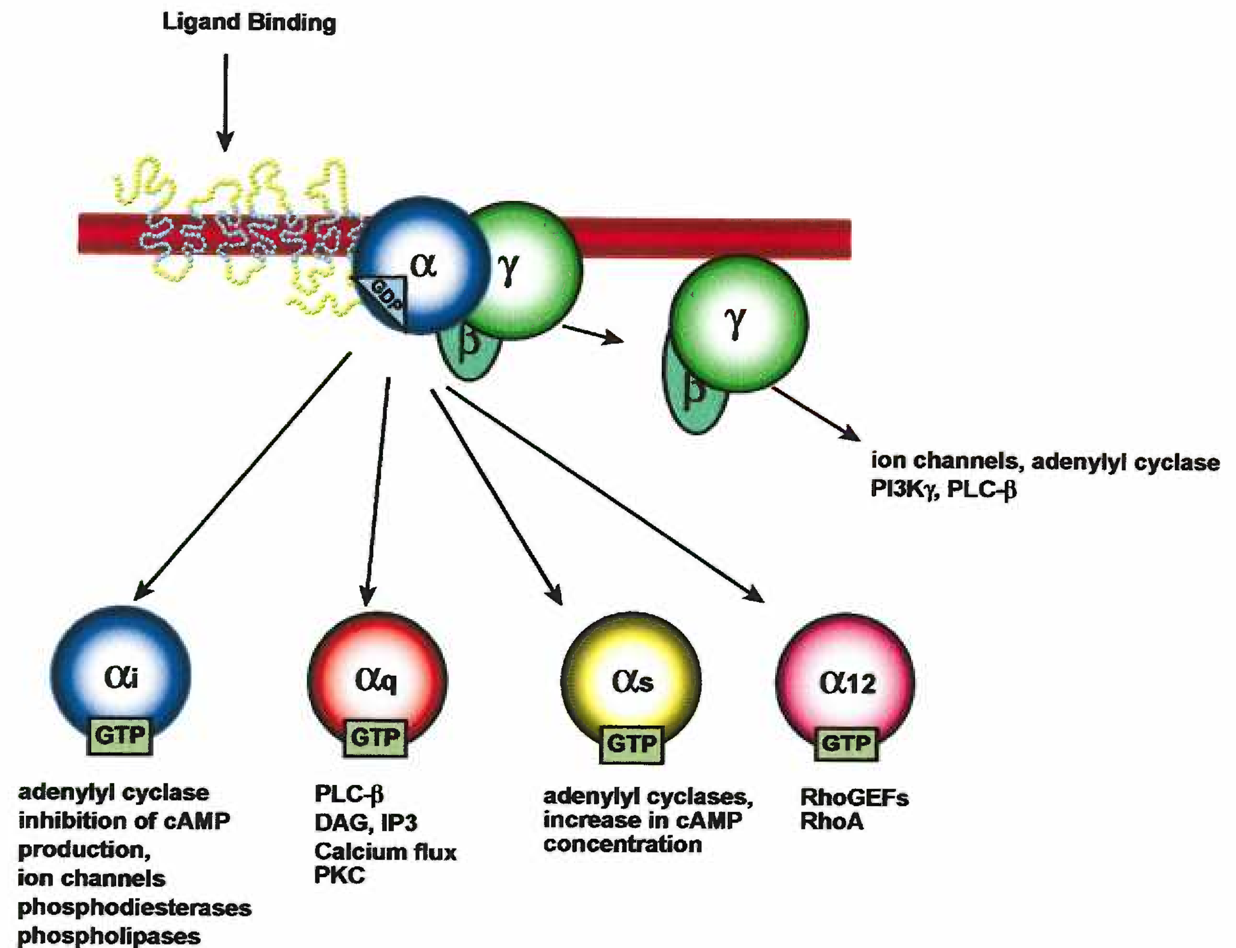
other family members, including: Gα14 (kidney, lung, spleen, testis) and Gα15/16 (myeloid, lymphoid lineage cells) display a more limited tissue distribution (Fields and Casey, 1997). Gα12 family members, Gα12 and Gα13 exhibit a ubiquitous tissue distribution (Fields and Casey, 1997) and interact with a number of Rho guanine nucleotide exchange factors (GEFs), stimulating robust RhoA signaling activity (Buhl et al., 1995; Gohla et al., 1998; Gohla et al., 1999).

Cellular stimulation and activation mediated by chemokine receptors is critical component of a number of physiological responses. Of equal importance is the attenuation of GPCR mediated signal transduction. The inability of cells to terminate GPCR-induced signaling events can result in uncontrolled cellular activation, and ultimately disease. A number of mechanisms have been proposed as means by which the cell can terminate GPCR-mediated signal transduction (Claing et al., 2002; Penn et al., 2000). First of all, agonists may be removed by dilution. Ligand binding by chemokine receptors results in the internalization of the ligand-engaged chemokine receptors, removing ligand from the extracellular environment. In conjunction with receptors binding ligand, transporter proteins and cellular enzymes may promote the internalization of extracellular ligands, and their subsequent enzymatic degradation. Second, GPCR kinases (GRKs) phosphorylate activated receptors, promoting interactions between the newly phosphorylated receptor and β-arrestins, the latter of which interdict interactions with G-proteins and effectively terminate the signal. Although β-arrestins are responsible for rapid signal termination, they do not promote the attenuation of post-G-protein signaling events. Instead, the β-arrestins prevent the further stimulation of Gα-protein

associated effector proteins. Third, ligand binding promotes clathirin-mediated endocytosis, depleting the plasma membrane of receptors, and with fewer receptors on the cell surface the probability of ligand encountering receptor is significantly reduced. Finally, prolonged exposure of cells to agonist leads to surface receptor down-regulation, which is mediated by a reduction in receptor expression and an increase in receptor degradation.

Figure 1.8 Gα-proteins and G-protein mediated signaling pathways.

Signaling pathways stimulated by Gα-proteins. Upon ligand binding, GPCRs undergo structural changes that permit the activation of receptor associated Gα-proteins. GTP binding by Gα-proteins promotes the release of associated βγ-subunits that activate ion channels, phosphoinositide 3-kinase (P3K)γ, PLC-β, and adenylyl cyclases. Gαi G-proteins activate adenylyl cyclases, ion channels, phosphodiesterases, and phospholipases, while inhibiting the production of cAMP. Protein kinase C (PKC) activation is mediated by Gαq G-proteins. These G-proteins also stimulate PLC-β, producing the second messenger molecules IP3 and DAG, leading to increased concentrations of intracellular Ca^{2+}. Stimulation of Gαs results in adenylyl cylcase activity, generating cAMP. RhoGEF and RhoA activation is mediated by Gα12 G-proteins.



Ligand Binding
α
GDP
γ
β
γ
β
ion channels, adenylyl cyclase
PI3Kγ, PLC-β
αi
GTP
αq
GTP
αs
GTP
α12
GTP
adenylyl cyclase
inhibition of cAMP
production,
ion channels
phosphodiesterases
phospholipases
PLC-β
DAG, IP3
Calcium flux
PKC
adenylyl cyclases,
increase in cAMP
concentration
RhoGEFs
RhoA

1.6.7 The HCMV-encoded chemokine receptor US28

US28 is the most well characterized of the CMV-encoded chemokine receptors and shares the greatest sequence homology with the cellular chemokine receptor CCR1 (Figure 1.9) (Gao and Murphy, 1994). US28 is expressed as an unspliced 1.3 kb transcript that has been detected as early as 2 hpi in HCMV infected human foreskin fibroblasts (Bodaghi et al., 1998; Welch et al., 1991; Zipeto et al., 1999). US28-transcripts have also been detected at 4 and 24 hpi in HCMV infected U373MG cells and at 4 hpi in *in vitro* infected myeloid cells (Zipeto et al., 1999). In adddition, US28-transcripts have been observed *in vivo* in peripheral blood mononuclear cells (Patterson et al., 1998). Interestingly, expression of US28 is not sensitive to treatment with cycloheximide, suggesting that US28 is an IE gene; however, the US28 protein is not detectable until 48 hpi (Mokros et al., 2002). Therefore, although the transcripts are expressed with IE kinetics the US28 protein is expressed with E-L kinetics.

Most chemokine receptors only bind a limited subset of chemokines, usually belonging to a single chemokine subfamily. US28 is one of only three chemokine receptors that binds to multiple ligands from different chemokine subfamilies, the other two being the Duffy antigen/receptor for chemokine (DARC-receptor), and the HHV-8-encoded chemokine receptor Orf74 (Lentsch, 2002; Rosenkilde et al., 1999; Szabo et al., 1995). To date, US28 has been shown to bind to multiple CC-chemokines including MCP-1, RANTES, macrophage inflammatory polypeptide (MIP)-1α and MIP-1β, as well as the CX_3C-chemokine Fractalkine with high affinity (Kledal et al., 1998; Kuhn, 1995). US28 binding to most CC-chemokines results in the potent stimulation of a variety of cellular

signaling pathways (Billstrom et al., 1998; Streblow et al., 1999b); while Fractalkine has been exclusively employed as a means of reducing US28-induced constitutive signaling (Casarosa et al., 2001; Fraile-Ramos et al., 2001; Mokros et al., 2002). Interestingly, an US28-expressing murine pre-B cell line but not control cells adhere to a fixed Fractalkine surface, indicating that US28 recognizes plasma membrane associated Fractalkine. This finding also suggests a role for US28 in modulating the trafficking of HCMV-infected cells (leukocyte rolling, adhesion and transendothelial cell migration) to sites of inflammation (Haskell et al., 2000).

Fibroblasts infected with different strains of HCMV demonstrate high levels of PLC-β activation (as measured by intracellular IP3 accumulation) (Minisini et al., 2003). HCMV-mediated PLC-β activation is dependent upon the presence of US28 in the virus and is insensitive to PTX (Gαi/o-independent) (Minisini et al., 2003). Using similar assays, recent findings have suggested that US28 signals in the absence of an exogenous source of ligand, and therefore conclude that US28 is constitutively active. Transient transfection of COS7 cells with US28-expressing plasmid constructs results in significant IP3 production and NF-κB activation (as measured using reporter constructs) in the absence of exogenous ligand stimulation (Casarosa et al., 2001). These signaling activities are enhanced upon expression of Gαq and Gα11, suggesting that US28 couples to Gαq/11 family G-proteins to mediate PLC-β and NF-κB activation. Addition of ligand has varying effects on US28-induced PLC-β and NF-κB activation, with RANTES and MCP-1 acting as neutral antagonists, and Fractalkine functioning as a partial inverse agonist that reduces constitutive signaling activity (Casarosa et al., 2001).

US28 has also been demonstrated to undergo constitutive endocytosis and recycling in transiently transfected HeLa and COS cells (Fraile-Ramos et al., 2001), as well as constitutive receptor phosphorylation in transiently transfected HEK293A cells (Mokros et al., 2002). Fractalkine stimulation of US28 expressing cells diminishes steady state levels of surface receptor (Fraile-Ramos et al., 2001), and reduces constitutive receptor phosphorylation in HEK293A cells without any apparent effect on intracellular signaling activity (Mokros et al., 2002). Endocytosis of US28 does not require β-arrestins and is dependent upon the clathrin mediated pathway for internalization (Fraile-Ramos et al., 2003); however, β-arrestin recruitment and preceding GRK mediated phosphorylation of US28 modulate some constitutive signaling activities (Miller et al., 2003). Interestingly, US28-induced signaling and endocytosis may be uncoupled by deletion of US28's carboxyl (C')-terminal tail (Waldhoer et al., 2003). Specifically, alanine mutagenesis of US28's C'-terminal serine residues uncoupled constitutive receptor phosphorylation and recycling from NF-κB signaling events, demonstrating that turnover of surface US28 and receptor phosphorylation is independent of NF-κB (Mokros et al., 2002).

In addition to signaling in the absence of exogenous ligand, US28 promotes robust cellular activation upon stimulation with chemokines. Both RANTES and MCP-3 induce Ca^{2+} flux and the activation of extra-cellular regulated kinase (ERK)-2 through Gαi/o and Gα16 pathways in US28 expressing 293 cells (Billstrom et al., 1998). We have previously demonstrated a functional role for US28 in the induction of vascular SMC migration in response to an exogenous source of RANTES or endogenously expressed

MCP-1 (Streblow et al., 1999). Ablation of US28 in HCMV results in a significant reduction in cellular motility that could only be rescued by re-introduction of US28, but not the cellular chemokine receptor CCR5. Demonstrating the sufficiency of US28 in HCMV-induced SMC migration, infection of SMC with recombinant adenoviruses that express US28, resulted in SMC migration upon stimulation with RANTES. Interestingly, cellular motility induced by US28 was sensitive to Herbimycin A and not PTX, suggesting the involvement protein tyrosine kinases (PTKs) but not Gαi/o family G-proteins. Therefore, US28 is both necessary and sufficient in promoting the ligand-dependent migration of SMC, thus providing a molecular basis for the correlative evidence that links HCMV to the acceleration and exacerbation of vascular disease.

The fact that all CMVs encode molecules with homology to cellular chemokine receptors, coupled with the *in vivo* growth defects of viruses that lack viral-chemokine receptors, suggests an important role for these proteins in the pathogenesis of CMVs. Moreover, the epidemiological and molecular data that link HCMV to vascular pathologies, and the ability of US28 to promote SMC migration, suggests an important role for HCMV and US28 in vascular disease. To date, SMC motility is the only biological activity associated with US28. Building upon our previous studies of US28 functional activity, the work detailed in this dissertation further elucidates the ligand-dependent mechanisms of cellular activation stimulated by this chemokine receptor with regards to US28-induced SMC migration. Specifically, we identify the G-proteins that couple with US28 to promote cellular migration, as well as the critical effector proteins involved in US28-induced migratory events. We also explore the ligand-specific

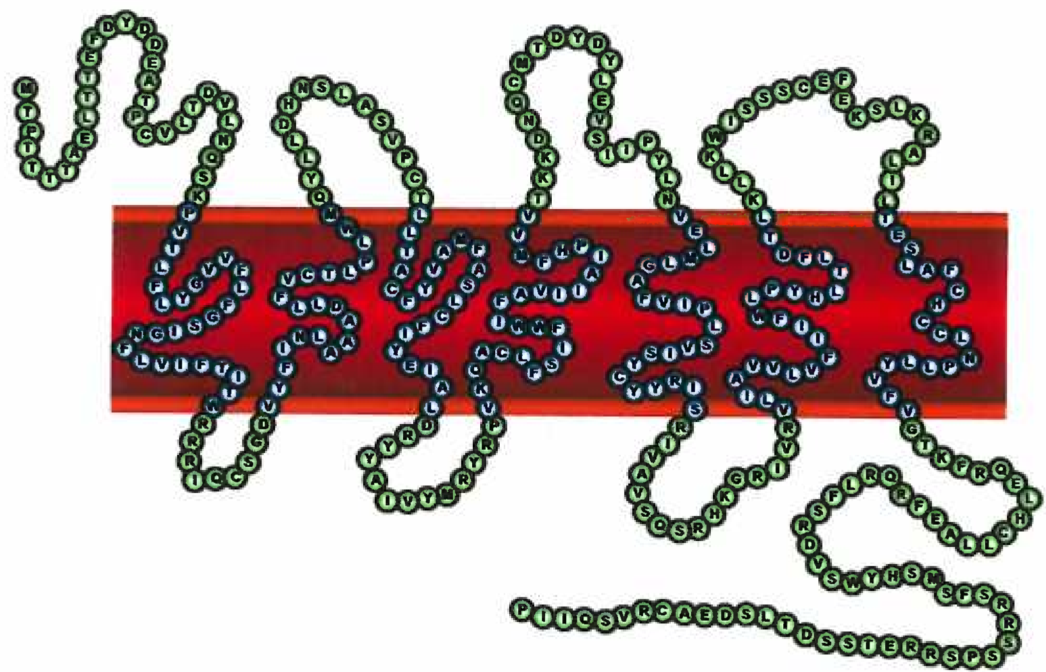
differences in US28-mediated SMC migration and signaling. By examining the pathways of activation, functional activity and transcriptional profiles stimulated by ligands belonging to different chemokine subfamilies, we reveal that in addition to being ligand-dependent, US28-induced SMC migration and signaling are also ligand-specific. Finally, we establish the MCMV-encoded chemokine receptor, M33, as a functional homologue of US28 that is required for MCMV-induced atherosclerosis and the mRANTES enhanced induction of SMC migration.

Figure 1.9 Amino acid sequence and structure of US28.

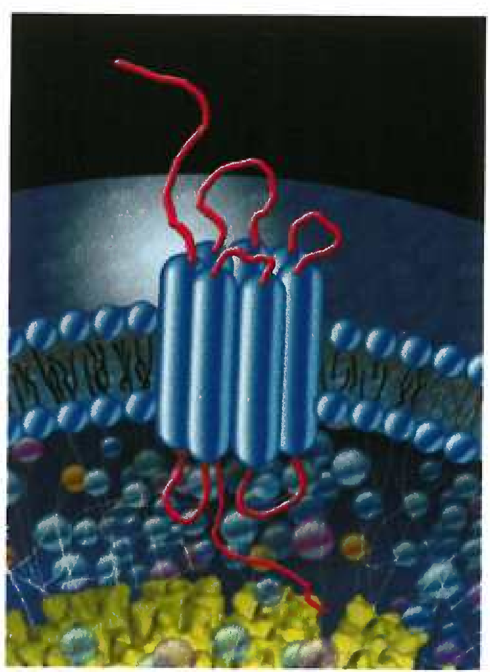
(A) Amino acid sequence and predicted structure of US28. Amino acids in the predicted trans-membrane domains are shown in blue.

(B) Predicted structure of US28 on the plasma membrane.

A.



B.



Chapter 2

The Human Cytomegalovirus Encoded G-protein Coupled Receptor US28 Mediates Smooth Muscle Cell Migration through Gα12

Ryan M. Melnychuk, Daniel N. Streblow, Patricia P. Smith, Alec J. Hirsch, Dora Pancheva and Jay A. Nelson

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In the following chapter, Ryan M. Melnychuk carried out all the experiments in Figures 2.1, 2.3b-c, 2.4 and 2.6a. Patricia Smith performed the smooth muscle cell migration experiments shown in Figure 2.2, 2.3a and 2.6b. Alec Hirsch assisted in the generation of telomerized SMC, and Dora Pancheva contributed to the generation of recombinant adenoviruses and assisted in cell culture.

2.1 Abstract

Coupling of G-proteins to ligand engaged chemokine receptors is the paramount event in GPCR signal transduction. We have previously demonstrated that the HCMV-encoded chemokine receptor US28 mediates human vascular SMC migration in response to either RANTES or MCP-1. In this report, we identify the G-proteins that couple with US28 to promote vascular SMC migration and identify other signaling molecules that have critical roles in this process. US28-mediated cellular migration was enhanced with the expression of the G-protein subunits Gα12 and Gα13 suggesting that US28 may functionally couple to these G-proteins. In correlation with this observation, US28 was able to activate RhoA; a downstream effector of Gα12 and Gα13 in cell types with but not without these G-proteins, and activation of RhoA was dependent upon US28 stimulation with RANTES. In addition, inactivation of RhoA or the RhoA associated kinase p160ROCK with a dominant negative mutant of RhoA or the small molecule inhibitor Y27632, respectively, abrogated US28-induced SMC migration. The data presented here suggests that US28 functionally signals through Gα12 family G-proteins and RhoA in a ligand-dependent manner and these signaling molecules are important for the ability of US28 to induce cellular migration.

2.2 Introduction

HCMV is a ubiquitous β-herpesvirus with an incidence of infection varying between 40-100% depending on age and socioeconomic status. Generally, infection occurs during childhood and after primary infection HCMV establishes a lifelong persistence in the host. HCMV infection of healthy immunocompetent individuals is commonly asymptomatic; however, HCMV may cause life threatening disease in immunocompromised patients (Britt and Alford, 1996) and HCMV is the leading cause of birth defects in viral-infected neonates (Fowler et al., 1997). HCMV infection has also been linked to long-term diseases including: atherosclerosis, arterial restenosis following angioplasty, and TVS associated with chronic allograft rejection (Margulies et al., 1996; Melnick et al., 1998; Melnick et al., 1983a; Peterson et al., 1980; Streblow et al., 2001a; Streblow et al., 2001b). A hallmark of all of these diseases is the migration of arterial SMCs from the vessel media, to the intima, ultimately resulting in vessel occlusion.

HCMV infection of SMC *in vivo* has been linked to a viral etiology of vascular disease. Previously, we have demonstrated that HCMV infection of primary arterial but not venous SMC results in significant cellular migration (Streblow et al., 1999). Similar to other herpesviruses, HCMV encodes chemokine receptor homologues including UL33, UL78, US27, and US28. Ablation of US28 within the virus abrogates SMC migration, which is rescued only by expression of the viral homologue and not the cellular chemokine receptor CCR5 (Streblow et al., 1999). US28 is structurally similar to the human chemokine receptor CCR1 (Gao and Murphy, 1994) and binds chemokines of the β-class (CC-) including: MCP-1, MCP-3, RANTES, MIP-1α, and MIP-1β (Bodaghi et

al., 1998; Kuhn, 1995; Schall et al., 1994), and the CX_3C-chemokine Fractalkine (Haskell et al., 2000; Kledal et al., 1998). Expression of US28 in the presence of either of the CC-chemokines RANTES or MCP-1 was sufficient to promote SMC migration, which was inhibited by PTK inhibitors but not PTX (Streblow et al., 1999). Recently, our group has demonstrated that US28 signals through the non-receptor PTKs Src and FAK and that their activities are necessary for US28-mediated SMC migration (Streblow et al., 2003c). US28-mediated SMC migration was inhibited by treatment with the Src inhibitor PP2, and through the expression of either of two dominant negative inhibitors of FAK. US28 is the first viral-GPCR shown to mediate cellular motility, which is cell type specific and provides a molecular basis for the correlative evidence that link HCMV to the acceleration of vascular disease.

The first link between GPCRs and the intracellular signaling milieu are the heterotrimeric G-proteins, consisting of a complex of α, β and γ subunits. The Gα proteins are subdivided into one of four subfamilies based on sequence similarities: Gαi, Gαq, Gα12 or Gαs. Upon ligand binding, GPCRs undergo conformational changes that promote interactions with the alpha subunits of the heterotrimeric G-protein complex, which causes the exchange of GDP for GTP, and initiates intracellular signaling cascades. The Gαi family (Gαi1, Gαi2, Gαi3, Gαo and Gαz) has a wide tissue distribution and is PTX sensitive. The only exceptions are Gαo and Gαz, which are PTX insensitive and limited to neuronal tissues (both Gαo and Gαz) and platelets (Gαz only) (Fields and Casey, 1997). Numerous hormones and inflammatory cytokines signal through Gαi pathways as is evidenced by their sensitivity to PTX (Fields and Casey, 1997; Murphy, 2001; Thelen,

2001). Signaling pathways activated by the ubiquitously expressed Gαs often stimulate the accumulation of cAMP (Fields and Casey, 1997). Gαq family members activate a diverse array of signaling pathways that includes the activation of PLC-β, producing the second messenger molecules IP3 and DAG. Gαq and Gα11 are ubiquitously expressed, while the other family members Gα14 (kidney, lung, spleen, testis) and Gα15/16 (myeloid, lymphoid lineage cells) display a more limited tissue distribution (Fields and Casey, 1997). Gα12 family members, Gα12 and Gα13 exhibit a ubiquitous tissue distribution (Fields and Casey, 1997) and interact with a number of RhoGEFs stimulating robust RhoA signaling activity (Buhl et al., 1995; Gohla et al., 1998; Gohla et al., 1999).

Cellular migration is an important process in numerous physiological responses including: leukocyte trafficking, the formation of secondary lymphoid organs and remodeling of the vasculature. The most well characterized migratory event is the migration of leukocytes in response to chemokines. Leukocyte migration and homing to secondary lymphoid tissues is highly complex, involving a multitude of chemokine receptors and chemokines from most chemokine subfamilies. In general, leukocyte migration induced by cellular chemokine receptors is sensitive to PTX, suggesting a role for Gαi family G-proteins in this process (Arai and Charo, 1996; Thelen, 2001). US28 has been shown to couple to a variety of G-proteins including Gαi/o, Gα16 and Gαq/11, and different aspects of US28 signaling have been demonstrated to be either sensitive or insensitive to PTX (Billstrom et al., 1998; Casarosa et al., 2001). US28-mediated SMC migration has been shown to be insensitive to PTX, indicating that Gαi G-proteins do not have a role this event (Streblow et al., 1999). Therefore, we examined the signaling

components involved in US28-induced SMC migration. In this paper, we demonstrate that US28-induced SMC migration involves signaling through the Gα12 pathway.

2.3 Results

2.3.1 Gα12 is required for US28-induced SMC migration.

We have previously demonstrated that US28-induces vascular SMC migration through a PTX insensitive pathway (Streblow et al., 1999). A number of studies have revealed that US28 binds multiple chemokines and activates signaling through several G-protein families including Gαi (Billstrom et al., 1998; Casarosa et al., 2001; Gao and Murphy, 1994). Since the majority of cellular migration events are mediated by signaling pathways that involve PTX sensitive Gαi/o, the signaling pathways involved in US28-induced SMC migration are unknown. In this report we sought to identify the G-proteins that functionally couple to US28 to promote SMC migration. Since Gα-protein expression is tissue specific, the Gα-protein expression profiles were determined for a number of cell types including HCMV permissive cells (a neuroglioblastoma cell line (U373MG), pulmonary artery SMC (PASMC), carotid artery SMC (CASMC) and normal human dermal fibroblasts (NHDF)), as well as cells commonly used in signaling studies: COS7 and rat aortic SMC (rat AoSMC). Cellular lysates were probed by western blotting for the expression of a number of different Gα-protein subunits. Representative blots probed for Gαi1/2, Gαq, Gα11, Gα12, and Gα13 from PASMC and rat AoSMC are shown in Figure 2.1A. The ubiquitous Gαi1/2, Gαq/11 families of G-proteins were detected in all of the cell types examined (Figure 2.1A&B). Interestingly, Gα12 and Gα13 expression was readily identifiable in rat AoSMC, PASMC and U373MG but not in COS7 cells or NHDF. Thus, G-protein expression profiles vary in a cell type-dependent manner.

Figure 2.1. Gα-protein detection in human vascular SMC and Rat Aortic SMC.

(A) Representative western blots of Gα-protein expression in human pulmonary artery SMC (PASMC) and rat aortic SMC (rat AoSMC).

(B) A table showing the presence of Gα-subunits expressed in various HCMV-permissive cell-types including PASMC, coronary artery SMC (CASMC), U373MG, Normal Human Dermal Fibroblasts (NHDF) as well as cell types commonly used in signal transduction studies: COS7 and rat AoSMC.

$G_{\alpha}i1/2$ $G_{\alpha}q$ $G_{\alpha}11$ $G_{\alpha}12$ $G_{\alpha}13$

PA-SMC

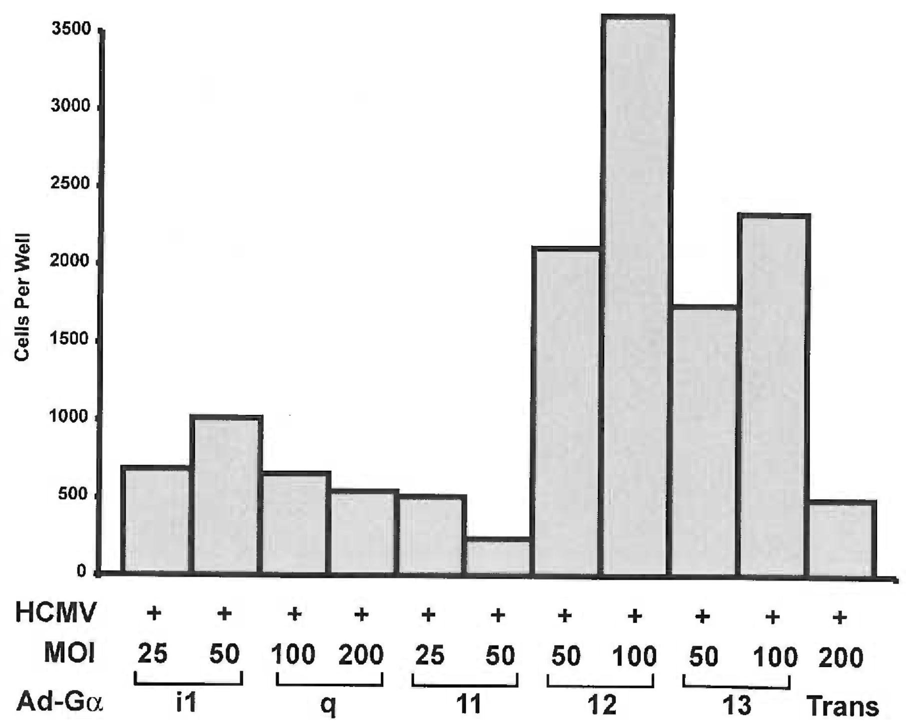
RatSMC

	$G_{\alpha}i1/2$	$G_{\alpha}q$	$G_{\alpha}11$	$G_{\alpha}12$	$G_{\alpha}13$
PA-SMC	+	+	+	+	+
CA-SMC	+	+	+	+	+
U373	+	+	+	+	+
NHDF	+	+	+	-	-
RatSMC	+	+	+	+	+
Cos	+	+	+	-	-

Over-expression of individual Gα-proteins has been used to enhance or modulate GPCR-mediated signal transduction. An enhanced response resulting from over-expression of a single alpha subunit suggests the involvement of that Gα-protein in signaling events initiated by the GPCR. In contrast, a reduced response may suggest that that particular Gα-protein is not involved in the signaling pathway (Lee et al., 1992; Lee et al., 1998). To identify individual Gα-subunits that may enhance SMC migration induced by US28, a panel of adenoviruses expressing different individual Gα-subunits was constructed. Adenoviruses expressing Gαq, Gα11, Gα12, Gα13, and Gαi1 were used to infect PASMC to determine if any individual Gα-proteins could enhance US28-mediated SMC migration. For these experiments, altering the moi according to western blotting results for each specific adenovirus normalized Gα-protein expression in PASMC. PASMC were co-infected with adenoviruses expressing different individual Gα-proteins and HCMV, and then cellular motility was assessed using a modified Boyden migration assay (Streblow et al., 1999). As shown in Figure 2.2, expression of Gα12 promotes US28-mediated SMC migration and migration is further enhanced with increasing levels of Gα12 expression. Gα13, another member of the Gα12 family of G-proteins also promoted HCMV-mediated SMC migration, although to a lower extent. Conversely, Gαi1, and Gαq/11 family G-proteins failed to promote US28-mediated SMC migration. In the absence of HCMV infection expression of these G-proteins did not induce SMC migration. These data suggest that Gα12 family G-proteins functionally couple with US28 to promote SMC migration.

Figure 2.2. Gα-12/13 enhances US28 mediated SMC migration.

PASMC were infected with HCMV and adenoviruses expressing Gαq, Gα11, Gα12, Gα13, or Gαi1. Such that equivalent amounts of protein were expressed, expression of the Gα-proteins was normalized by western blotting and adjusting the moi accordingly. The total number of cells migrating from the upper to lower chamber were enumerated at 48-72 hours post-infection.


3500
3000
2500
2000
1500
1000
500
0
Cells Per Well
HCMV + + + + + + + + + + +
MOI 25 50 100 200 25 50 50 100 50 100 200
Ad-Gα i1 q 11 12 13 Trans

2.3.2 US28-induced SMC migration is mediated through Gα12 activation of RhoA.

Signaling assays have a tendency to be very sensitive, and even small amounts of ligand can stimulate robust signaling activity. Previous observations indicate that *in vitro* cultured PASMC constitutively produce the US28 ligand MCP-1, as addition of MCP-1 neutralizing antibodies abrogated US28-induced PASMC migration (Streblow et al., 1999). Therefore, the majority of our signaling studies were performed in rat AoSMC and U373MG cells. Signaling molecules including Gα-proteins, small G-proteins (ie. RhoA, Rac and CDC42) and PTKs (ie. Src, FAK, and Pyk2) are highly conserved in mammals and similar to human SMC, US28 induces the migration of rat AoSMC in response to recombinant human RANTES (Figure 2.3A). Interestingly, rat RANTES also induced US28-mediated SMC migration; however, mouse RANTES (mRANTES) failed to elicit the same functional response.

Since US28-mediated SMC migration is enhanced by Gα12 and Gα13, and this family of G-proteins is known stimulate stress fiber formation, as well contribute to migratory events through RhoA, we examined the ability of US28 to activate RhoA (Buhl et al., 1995; Gohla et al., 1999). To assess the ability of US28 to stimulate RhoA through Gα12 family G-proteins, active RhoA pull down assays using GST-tagged Rhotekin (GST-fused to the RhoA binding domain of Rhotekin) were performed. This fusion protein only binds to RhoA in the active GTP-bound state (Ren et al., 1999). For these experiments, serum-starved rat AoSMC expressing US28 and/or tet-transactivator were stimulated with either 1 or 50ng/ml RANTES for 0 (unstimulated), 5, 10 or 30 minutes. Cells lysates were administered to glutathione-linked Sepharose beads baited with GST-

RhoTekin and then probed for RhoA. To ensure that equivalent amounts of proteins were used in each experiment, aliquots of cellular lysates were analyzed by western blotting for input RhoA. Rat AoSMC infected with adenoviruses expressing a Flag-tagged version of US28 and treated with RANTES display a dose-dependent and kinetic activation of RhoA (Figure 2.3B). Control cells infected with adenoviruses expressing the tet-transactivator alone and treated with RANTES failed to activate RhoA. US28-mediated activation of RhoA was most pronounced at 10 and 30 minutes post-ligand stimulation (50ng/ml RANTES).

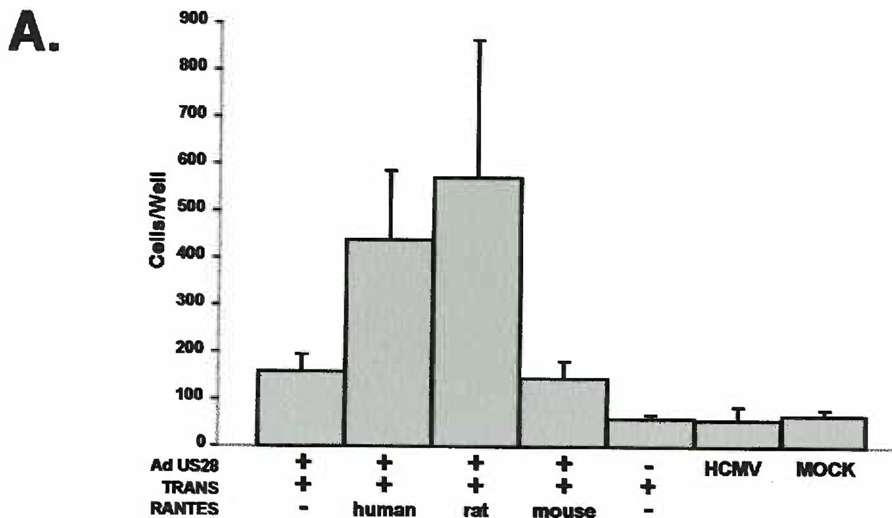
In similar experiments performed in U373MG cells, the ligand-dependent activation of RhoA was observed in cells expressing US28. In these experiments, US28-mediated activation of RhoA peaked at 30 minutes post-RANTES addition (1ng/ml) (Figure 2.3C). When higher concentrations of RANTES (50ng/ml) were administered to US28-expressing cells, peak RhoA stimulation occurred earlier, reaching maximal levels at 10 minutes post-ligand stimulation. The slight differences in the timing of US28-induced RhoA activation in U373MG cells versus rat AoSMC may be due to cell type specific differences in the ability to respond or variations in the surface expression of US28. Regardless of the kinetics, ligand binding by US28 induces the activation of RhoA in two distinct cell types (rat AoSMC and U373MG).

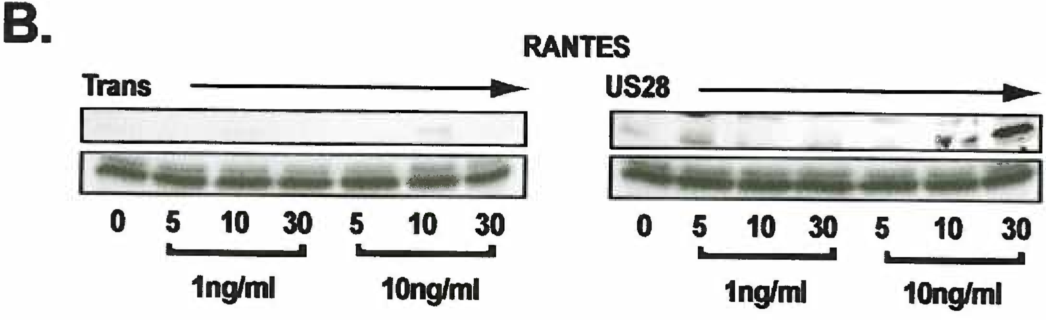
Figure 2.3. US28 Activates RhoA in a ligand-dependent manner.

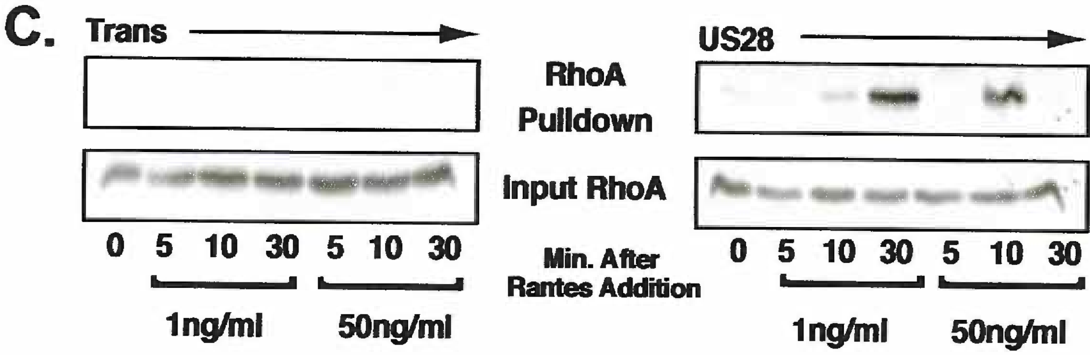
(A) The ability of US28 to induce the migration of rat AoSMC was determined by infecting cells with adenoviruses expressing US28 in the presence of 10ng/ml human, rat or mouse RANTES. The number of migrating cells was determined by microscopic enumeration at 48-72 hours post infection.

(B) Rat AoSMC were serum starved for 16-18 hours and subsequently infected with adenoviruses expressing US28. US28 expressing cells were either left unstimulated or were stimulated with 1 or 50ng/ml RANTES at 16-18 hours post-infection. At the indicated times, cells were harvested and lysates applied to GST-Rhotekin baited beads in order to precipitate active RhoA. The amount of bound/active RhoA was determined by western blotting using an anti-RhoA polyclonal antibody. To ensure equal loading, lysates were also probed for total RhoA prior to being applied to GST-RhoTekin beads (lower panel).

(C) US28-induced RhoA activity assay performed in U373MG cells as described above for assays in rat AoSMC.


A.
Cells/Well
900
800
700
600
500
400
300
200
100
0
Ad US28
TRANS
RANTES
+ + + + - HCMV MOCK
+ + + + +
- human rat mouse -


B.
RANTES
Trans
US28
0 5 10 30 5 10 30
1ng/ml
10ng/ml
0 5 10 30 5 10 30
1ng/ml
10ng/ml


C.
Trans
US28
RhoA
Pulldown
Input RhoA
0 5 10 30 5 10 30
Min. After
Rantes Addition
0 5 10 30 5 10 30
1ng/ml
50ng/ml
1ng/ml
50ng/ml

Since COS7 cells and NHDF lack detectable Gα12 and Gα13 protein expression, these two cell types provide the ideal background to test the necessity of Gα12 family G-proteins in US28's ability to activate RhoA. Therefore, active RhoA pull-down assays were performed in these two cell types. In both COS7 (Figure 2.4A) and NHDF (Figure 2.4B), US28 does not display any activation of RhoA either in the presence or absence of ligand. Although US28 does not induce the activation of RhoA in either of these cell types, both COS7 and NHDF are fully capable of activating RhoA, as serum starved NHDF and COS7 cells demonstrate RhoA activity when treated with 1% serum (Figure 2.4A&C).

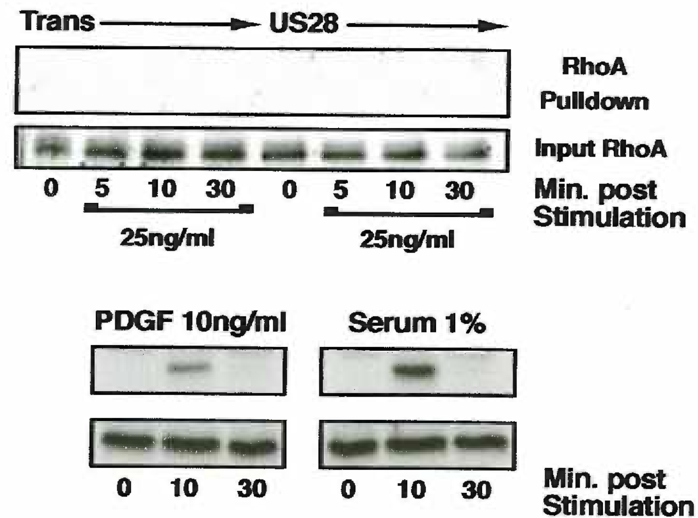
Since US28 was unable to stimulate RhoA activity in cell types that lack detectable Gα12 and Gα13 expression, experiments were performed to determine whether reconstituting NHDF with Gα12 would restore US28's ability to activate RhoA. Gα12 expression levels were titered in to reflect the normal Gα12 expression observed in PASMC. NHDF do not display detectable Gα12 (Figure 2.4C), and upon expression of Gα12 in the absence of US28 or in the presence of US28 without ligand, there was no observable RhoA stimulation. However, ten minutes post-RANTES stimulation, cells co-infected with adenoviruses expressing both US28 and Gα12 induced >10-fold activation of RhoA compared to untreated Gα12/US28 expressing cells. Therefore, the US28-RhoA signaling pathway can be functionally restored in NHDF by reconstituting these cells with Gα12. These data suggest that US28 activation of RhoA is cell-type specific and dependent upon the compliment of endogenously expressed G-proteins.

Figure 2.4. Reconstitution of NHDF with Gα12 restores US28-induced RhoA activation.

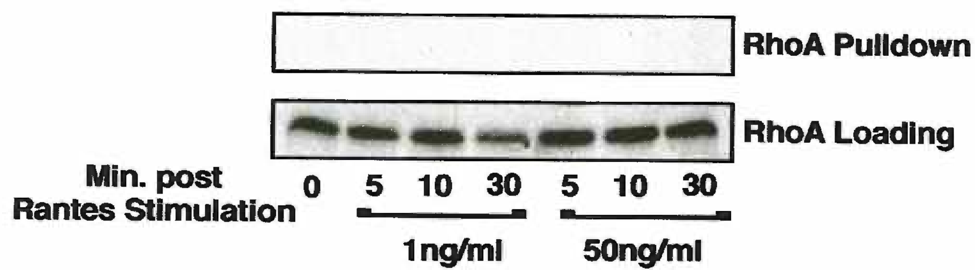
(A) COS7 cells were serum starved for 18 hours and then infected with adenoviruses expressing US28 and/or Ad-trans. Cells were stimulated at 18 hours post-infection with 25ng/ml RANTES for 0 (unstimulated), 5, 10 or 30 minutes. Alternatively, serum starved COS7 cells were stimulated with 10ng/ml PDGF-BB or 1% serum for 10 or 30 minutes. Active RhoA assays were performed and RhoA was visualized by SDS-PAGE followed by western blotting. To ensure equivalent loading, total input RhoA was determined by western blotting (lower lanes).

(B) NHDFs were serum starved and infected as above. Infected cells were stimulated with 1-50ng/ml RANTES for 0, 5, 10 or 30 minutes, or with 1% serum for 10 minutes (positive control) and RhoA activity determined as described above.

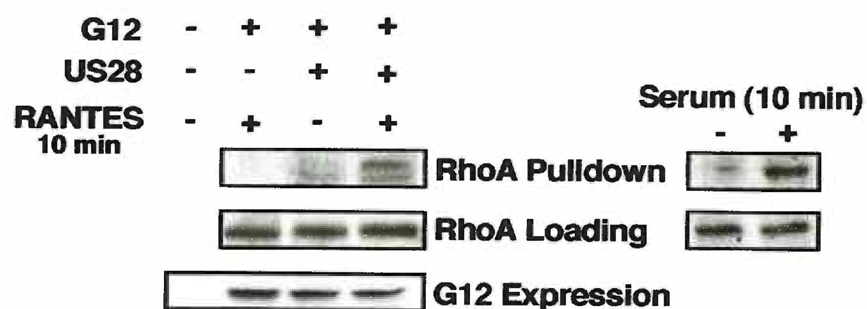
(C) NHDFs were infected with adenoviruses expressing either Gα12 and/or US28 and then treated with 10ng/ml RANTES for 10 minutes.



B.



C.



2.3.3 RhoA activity is required for US28-mediated SMC migration.

Since US28-induced SMC migration is enhanced by Gα12 and Gα13, and Gα12 is required for US28 to activate RhoA, the necessity of RhoA in US28-mediated SMC migration was determined. Adenoviruses expressing either wild type or dominant negative (DN) RhoA (a RhoA mutant that is unable to exchange GDP for GTP) were assessed in migration assays. Protein expression (which was determined by western blotting for HA-tagged RhoA DN and WT) was normalized in SMC by adjusting the moi accordingly. Expression of wild-type RhoA consistently enhanced HCMV-mediated SMC migration (Figure 2.5), whereas expression of DN-RhoA abrogated US28-induced SMC migration. The inability of US28 to promote SMC migration in the presence of DN-RhoA indicates that RhoA activity is required for US28-mediated SMC migration. Thus, US28 coupling with Gα12 family G-proteins activates RhoA, which is functionally important for US28 to induce cellular migration.

The small G-protein RhoA is capable of activating a number of factors that contribute to migratory processes. Dia and Rho-associated kinases (ROCKs), including p160ROCK, are downstream effectors of active RhoA (Ridley, 2001). ROCK activation leads to the phosphorylation and activation of myosin light chain (MLC) kinase, by both inhibiting MLC phosphatases and phosphorylating MLC, ROCK activity is critical for the re-organization of the actin cytoskeleton (Amano et al., 2000; Kaibuchi et al., 1999). Expression of US28 in PASMC resulted in actin cytoskeletal rearrangements and altered cellular morphology (Figure 2.6A). To examine the contribution of RhoA activated ROCKs in this process, PASMC were infected with adenoviruses expressing US28 and

then treated with a p160ROCK inhibitor Y-27632. When US28 expressing PASMC were treated with Y-27632, US28-induced actin cytoskeletal rearrangements were inhibited compared to untreated control cells (Figure 2.6A). These results suggest that RhoA activated ROCKs may also have an essential role in US28-induced migratory processes. To assess the importance of RhoA activated ROCKs in US28-mediated SMC motility cellular migration experiments were performed in the presence or absence of Y-27632. This inhibitor effectively blocked US28-induced cellular migration, indicating that p160ROCK activity is essential in US28-mediated cellular migration (Figure 2.6B). Therefore, US28-induced SMC migration is ligand-induced, and dependent upon the Gα-12 mediated activation of RhoA, which subsequently activates the Rho-associated kinase p160ROCK.

Figure 2.5. RhoA activity is essential for US28-induced SMC migration.

PASMC were infected with HCMV along with adenoviruses expressing either WT or DN RhoA at the indicated moi. Cellular migration was determined by microscopy at 48-72 hours post-infection. Cellular migration is expressed as a percentage when compared to the number of cells migrating upon co-infection with HCMV and Ad-Trans.

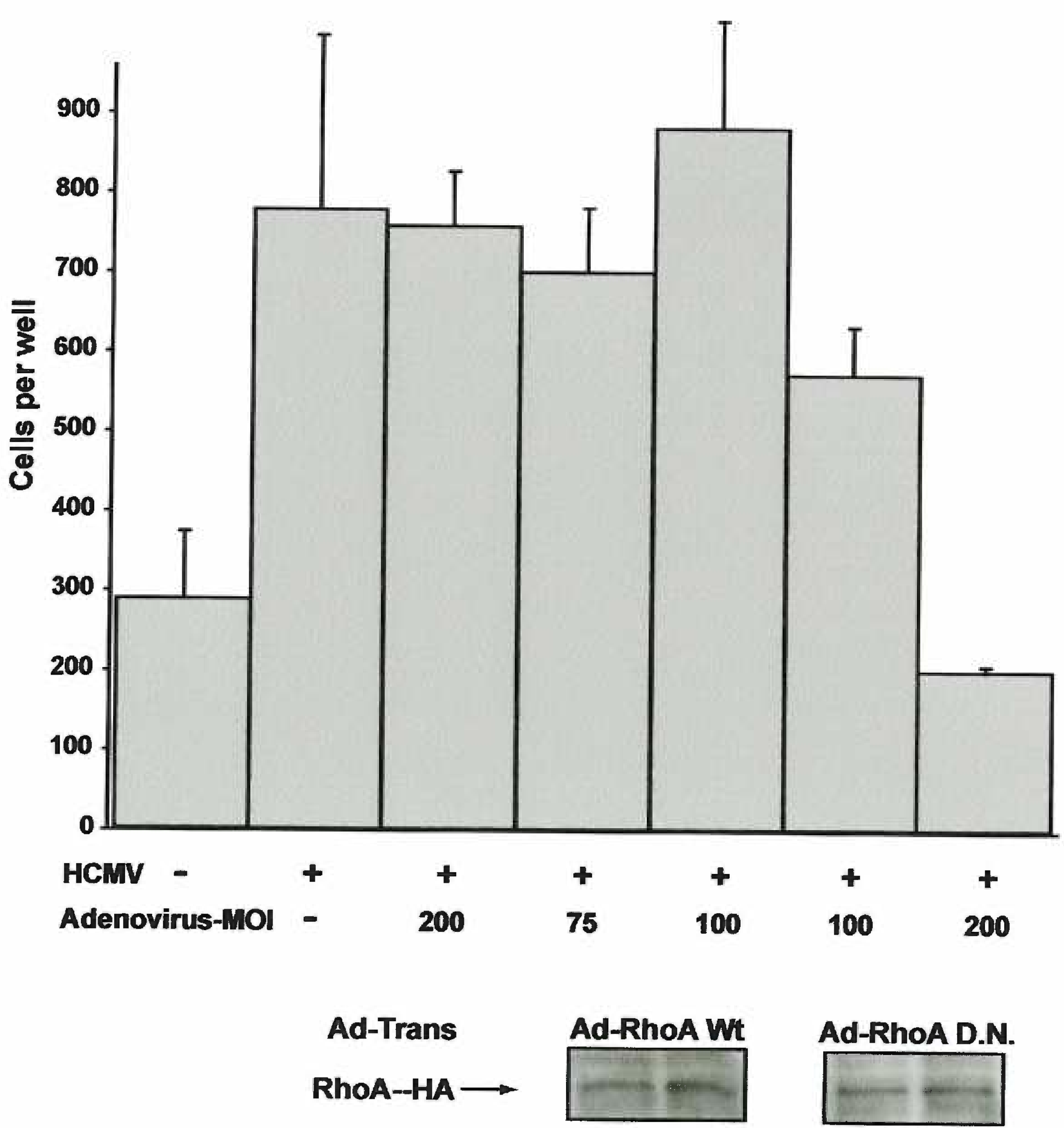
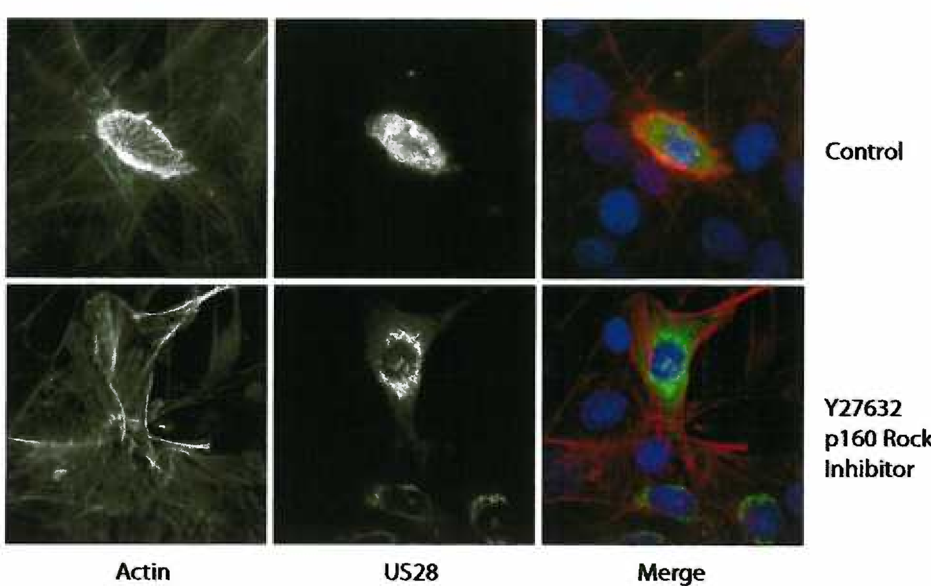

Cells per well
900
800
700
600
500
400
300
200
100
0
HCMV - + + + + + +
Adenovirus-MOI - 200 75 100 100 200
Ad-Trans
Ad-RhoA Wt
Ad-RhoA D.N.
RhoA--HA

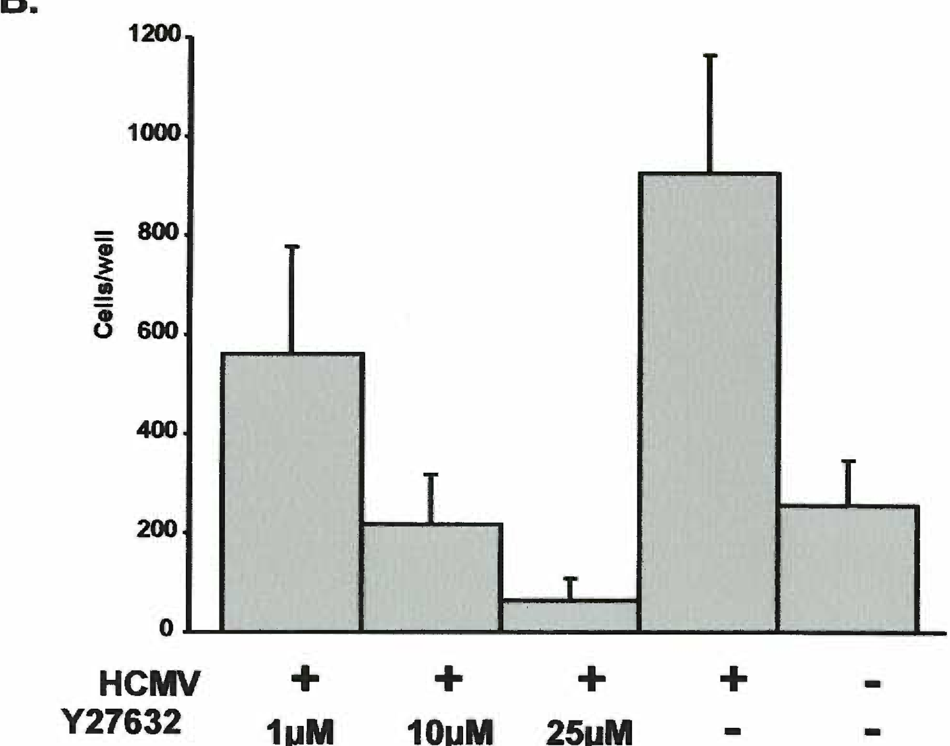
Figure 2.6. p160ROCK activity is important for US28-mediated actin cytoskeleton reorganization and cellular migration.

(A) PASMC expressing US28 were left untreated or were treated with the p160ROCK inhibitor Y27632. RANTES treated cells were fixed 4 hours post-ligand addition. Cells were stained for actin with phalloidin (red), the US28 flag epitope, and nuclei with Hoechst DNA staining dye.

(B) PASMC were infected with HCMV and then treated with increasing concentrations of the p160ROCK inhibitor Y-27632. The number of cells migrating from the upper to lower chamber were enumerated by microscopy and expressed as a total number of cells migrating from the upper to lower chamber.



Control
Y27632
p160 Rock
Inhibitor
Actin
US28
Merge



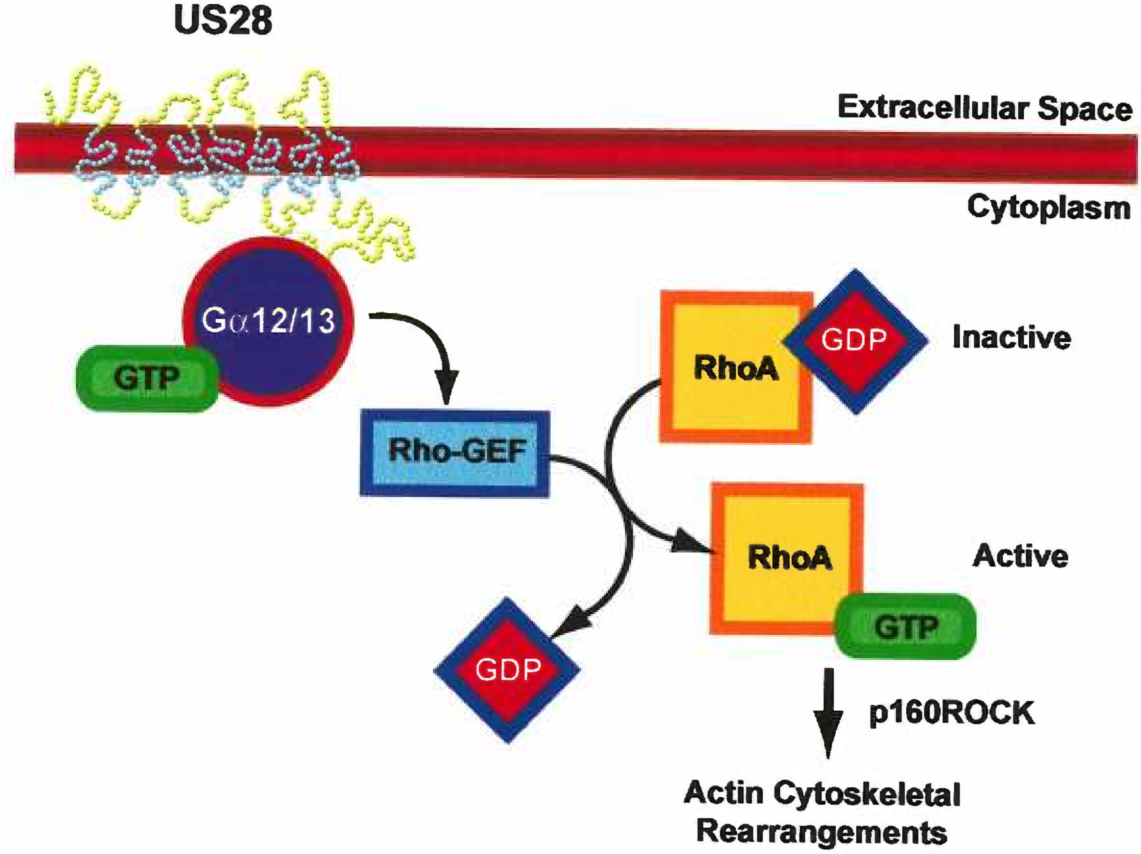
1200
1000
800
600
400
200
0
Cells/well
HCMV + + + + -
Y27632 1μM 10μM 25μM - -

2.4 Discussion

In the current report, we demonstrate that US28 signaling through Gα12 family G-proteins is important for the induction of vascular SMC migration. Migration was enhanced in the presence of Gα12 family G-proteins but not in the presence of G-proteins from either the Gαi or Gαq/11 families. This finding supports our previous findings that US28-induced SMC migration occurs in a PTX insensitive manner. Consistent with the activation of the Gα12 family members, was our finding that US28 promoted RhoA activation. RhoA is a small GTPase of critical importance in the signaling events that mediate cytoskeletal rearrangements associated with cellular migration. Importantly, US28 failed to stimulate RhoA activity in fibroblasts and COS cells that lack Gα12 protein expression. However, US28's RhoA stimulating potential can be restored in fibroblasts by introduction of Gα12 using adenovirus vectors, indicating that activation of this pathway is Gα12-dependent. Subsequent US28-mediated signaling events induced actin-cytoskeletal rearrangements in SMC, and these morphological changes were inhibited by treating the cells with an inhibitor to the RhoA effector, p160ROCK, thus identifying this molecule as having an essential role downstream of RhoA in US28-induced SMC migration (pathway summarized in Figure 2.7). In this report, we provide the first evidence that US28 signals in a ligand-dependent manner through Gα12 family G-proteins.

Figure 2.7. US28 Signaling Through Gα12 Family G-proteins.

Upon ligand binding, US28 couples with Gα12/13 in vascular SMC. Gα12 family G-proteins stimulate RhoGEFs, which activate RhoA by promoting the exchange of GDP for GTP. Active RhoA plays a critical role in the actin cytoskeleton rearrangements that are necessary for US28-induced SMC migration. RANTES stimulation of US28 also leads to the activation of the RhoA effector p160ROCK, which is essential in US28-mediated SMC migration.


US28
Extracellular Space
Cytoplasm
Gα12/13
GTP
Rho-GEF
RhoA
GDP
Inactive
RhoA
Active
GTP
GDP
p160ROCK
Actin Cytoskeletal
Rearrangements

2.4.1 US28 G-protein coupling and usage.

Why has US28 acquired the ability to bind multiple G-proteins? Under physiological conditions GPCR/G-protein coupling is restricted and highly regulated. This regulation occurs at multiple levels, including the ability of the receptor to specifically couple to different G-protein family members and at the level of differential G-protein expression within tissues and cell types. While most, but not all, host chemokine receptors are expressed in cells of hematopoietic origin, US28 can be expressed in a wide variety of cell types because HCMV productively infects hematopoietic cells, endothelial cells, epithelial cells, neuronal-derived cells, and vascular SMC. All of these cells are important in HCMV pathogenesis; however, we do not yet know in which of these cell types US28 is functionally required *in vivo*. Since each of these cell types express varying profiles of G-proteins, and the virus-encoded chemokine receptor must be able to function in each cell type, US28 has acquired the ability to couple to multiple G-proteins.

In general, hematopoietic cell migration induced by the chemokine receptors CCR1, CCR2, CCR5 and CXCR4 is PTX sensitive, suggesting the involvement of Gαi family Gα-proteins, which are highly expressed in these cells (Arai and Charo, 1996; Thelen, 2001). Interestingly, CCR2 can couple to multiple G-proteins including Gαi, Gαq, and Gα16, although Gαi coupling in hematopoietic cells is dominant since CCR2-mediated migration is sensitive to PTX (Arai et al., 1997). Vascular SMC migration induced by the angiotensin II Type 1 receptor is mediated by receptor coupling to Gαq and Gα12, which are highly expressed in these cells (Touyz and Schiffrin, 2000). Interestingly, CCR2 expression can be induced in SMC, which may be important under inflammatory

conditions and may explain why CCR2 can functionally couple to more than one G-protein. Together these cell type differences in the receptor/G-protein coupling used to mediate migration suggest that G-protein expression is an important determinant in GPCR-signaling. Thus the ability of US28 to bind multiple G-proteins may have been acquired through selection in order to allow US28 to signal in the presence of different G-protein environments.

2.4.2 Signaling through US28.

Previously, US28 has been demonstrated to stimulate intracellular signal transduction through a number of Gα-proteins. US28 coupling to Gαi and the Gαq family member Gα16 promotes ERK-2 activation and Ca^{2+} flux (Billstrom et al., 1998). Other Gαq family members Gαq and Gα11 also couple with US28, promoting PLC-β and NF-κB signaling activity (Casarosa et al., 2001). However, the significance and biological outcome resulting from signaling through these G-proteins has not been established. There exists a clear relationship between Gαi and hematopoietic cell migration, which when viewed with the ability of US28 to signal through Gαi pathways, suggests that this viral-GPCR has the inherent capacity to induce the migration of cell types other than SMC such as monocyte/macrophages. Enhancing or altering the migration potential of these cells would be important for virus escape and dissemination.

In this report, we demonstrate for the first time that US28 couples with G-proteins from the Gα12 family, which is important for the induction of SMC migration. Expression of other G-proteins (Gαi and Gαq/11 family members) in vascular SMC did not enhance

US28-mediated migration, which is consistent with our earlier findings showing that US28-induced migratory events operate through PTX-insensitive G-proteins (Streblow et al., 1999). This finding also suggests that even in the presence of other G-proteins that couple to US28 such as Gαi or Gαq, Gα12 coupling is dominant in SMC. We also demonstrate that US28 activates RhoA in a Gα12-dependent manner, as is evidenced by the reconstitution of fibroblasts with Gα12, which restored the capacity of US28 to activate RhoA. Activation of Gα12 family members can directly stimulate RhoGEFs that then promote RhoA activity by stimulating the release of bound GDP for the physiologically more abundant GTP. Recently, the non-receptor PTK FAK was demonstrated to bind and phosphorylate p190RhoGEF upon stimulation through growth factor receptors or integrins (Zhai et al., 2003). We have recently reported that US28 activates FAK in a ligand-dependent manner suggesting that US28 may promote RhoA activation through direct activation of Gα12 family G-proteins or through FAK (Streblow et al., 2003d). Regardless of the mechanism of activation, RhoA activity is critical for US28-induced SMC migration since expression of a dominant negative inhibitor of RhoA prevented vascular SMC motility. In addition, treatment of cells with an inhibitor to a downstream effector of RhoA, p160ROCK, also blocked migration, further supporting our findings and demonstrating the importance of RhoA in US28-induced migration.

Endothelial cells, SMC, epithelial cells, fibroblasts, and monocyte/macrophages are all important cell types in the pathogenesis of HCMV, and defining the signaling characteristics of US28 in these cell types is critical to understanding how the virus interacts, and persists in the host. In this report, we further characterize the signaling

pathway stimulated by US28 and required for the induction of vascular SMC migration. The intracellular signaling cascade initiated by US28 binding to RANTES leading to SMC migration is dependent upon the G-protein Gα12 and the activation of RhoA. Importantly, our results demonstrate that ligand-dependent US28 signaling occurs in a cell-type specific manner, which minimally depends on the cellular G-protein environment.

2.5 Materials and Methods

Reagents. RANTES was purchased from R&D Systems (Minneapolis, MN). Antibodies specific for Gαi1 (I-20) sc-391, Gα12 (S-20) sc-409, Gα13 (A-20) sc-410, Gαq (E-17) sc-393, Gα11 (D-17) sc-394, Gαz (I-20) sc-388, and anti-RhoA rabbit polyclonal antibody sc-179 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-flag M2 monoclonal antibody (F-3165) was purchased from Sigma (St. Louis, MO). Anti-mouse and anti-rabbit HRP-conjugated secondary antibodies used for western blotting were purchased from Amersham (NA934V and NA931V respectively; Piscataway, NJ). The p160ROCK inhibitor Y-27632 was purchased from Calbiochem (San Diego, CA).

Cells. Normal human dermal fibroblasts (NHDF), U373MG (neuroglioblastoma cell line) and COS-7 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin-L-glutamine (PSG). Primary Rat aortic SMC (ratAoSMC) were isolated from F344 rats and maintained in DMEM supplemented with 10% FCS and PSG. We used pulmonary artery SMC (PASMC) for experiments involving human arterial SMC (Clonetics, Walkersville, MD). These cells were life-extended by genetically engineering them to constitutively express the human telomerase gene. The telomerase gene was introduced into these cells at passage 6 using a retroviral vector system (pLXSN from BD Sciences Clontech, Palo Alto, CA). Cells expressing the telomerase gene were selected for G418 resistance and then screened for telomerase activity using the Trapeze telomerase activity kit (Intergen, Toronto, Canada). PASMC cells were maintained in Medium 199 supplemented with

20% FCS and PSG. These cells exhibited SMC characteristics and morphology when maintained in culture beyond passage 60. Carotid artery SMC (CASMC; Clonetics) were maintained in Medium 199 supplemented with 20% FCS and PSG.

Adenovirus Construction. Adenoviruses expressing US28 were previously described (Streblow et al., 1999). Human HA-tagged RhoA cDNA constructs (DN and WT) in pCDNA3.1 (generously provided by Dr. P. Stork, OHSU) were subcloned into the EcoRI and XbaI sites within pADtet7 (Hsia et al., 2003). Gα-subunit cDNA constructs in pCDNA3 were obtained from the Gutherie Institute (Sayre, PA) and subcloned into pADtet7 under a constitutively expressed HCMV-MIEP or tet-responsive promoter. The pAdTet7 plasmid contains Tet-responsive enhancer sequences within a minimal CMV promoter followed by SV40 late poly(A) cassette, as well as adenovirus E1A, and a single loxP site to increase recombination frequency. Recombinant adenoviruses were produced by co-transfection of Cre-recombinase expressing 293 (293-Cre) cells with pADtet7-RhoA DN/WT, or pADtet7-Gα-protein i1, q, 11, 12, 13, and z along with adenovirus DNA (Ad5-ψ5) that contains an E1A/E3-deleted adenovirus genome (Hsia et al., 2003). Recombinant adenoviruses were expanded on 293-Cre cells and the bulk stocks were titered on 293 cells by limiting dilution. Expression of US28, the Gα-proteins and DN and WT-RhoA were driven by co-infection with adenoviruses expressing the Tet-off transactivator (AdTrans) as described (Streblow et al., 1999). Cells were infected at moi =10 or 30 pfu/cell for Ad-Trans and/or Ad-RhoA (WT or DN) and analyzed for protein expression and for their effects on US28-mediated migration.

RhoA Activation Assay. The Rho binding domain of Rhotekin was expressed as a fusion protein with GST (GST-Rhotekin, kindly provided by Dr. J. Scott, OHSU) in the E.coli strain BL21 as previously described (Diviani et al., 2001; Ren et al., 1999). Bacteria were grown until an OD_{600}=0.6-0.8 was achieved at which time the bacteria were induced overnight with IPTG (1mM). After IPTG induction the bacteria were lysed and the clarified supernatants were bound to Glutathione-linked 4B-CL Sepharose beads (Amersham) overnight at 4°C. The beads were washed 3 times with PBS followed by 3 washes in RhoA wash buffer (50mM Tris pH 7.2, 1% Triton-X100, 150mM sodium chloride, and 10mM magnesium chloride) with a final equilibration wash using RhoA lysis buffer (50mM Tris pH 7.2, 1% Triton-X100, 500mM sodium chloride, 10mM magnesium chloride, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). For these assays, cells were plated onto 15cm tissue culture dishes and at 75% confluence the cells were serum-starved for 24 hours. The cells were infected with Ad-Trans and Ad-US28 at different moi depending upon the ability of each cell type to be infected: NHDF=2000, COS7 cells=500, U373MG cells=500 and ratAoSMC=1000. Sixteen hpi the cells were stimulated with RANTES (ranging from 1-50ng/ml) and then scraped in RhoA lysis buffer at 0 (unstimulated), 5, 10 or 30 minutes post-ligand stimulation. Uninfected serum-starved cell cultures treated with 1% serum served as positive controls. Cell lysates were incubated with GST-Rhotekin baited beads (100μl of a 1:1 slurry) for 45 minutes at 4°C, then washed 4 times with 1ml of RhoA wash buffer. The final pellet was resuspended in 60μl of 2x Laemmli's sample buffer, boiled and then run on 10% SDS-PAGE. The gels were transferred to Immobilon-P membranes and the blots were blocked overnight in 3% milk in phosphate buffered saline (PBS). All primary antibody

incubations (1:1000) were carried out in 1% bovine serum albumin, 0.01% polyvinylpyrrolidone, 0.01% sodium azide) for 3 hours at room temperature. The blots were then washed 4 times with TBS-T buffer (10mM Tris pH 7.2, 100mM sodium chloride, 0.1% Tween-20). The secondary antibody (goat anti-rabbit conjugated to horseradish peroxidase) was added at 1:2000 dilution in TBS-T buffer and the incubation was carried out for 1 hour at room temperature. After washing 3 times with TBS-T buffer, once in TBS, and incubation with Western Lightning chemiluminescent reagents (Perkin Elmer), the blots were visualized by autoradiography on Kodak MR or Biomax Light film.

SMC migration assay. Cell migration assays were performed as previously described (Streblow et al., 1999). Briefly, cells were added to the upper well of a transwell (12 mm diameter, 3.0 μm pore size, Costar Corning, Cambridge, MA) at $1x10^5$ cells per well. Cells were serum starved for 24 hours and then incubated with HCMV at a moi =10 for 2 hours. The inserts were washed and transferred to fresh 12-well plates. Cells migrating to the lower chamber were enumerated at 48-72 hrs post-infection using a Nikon TE300 microscope at magnification 10X. Experiments were done in at least triplicate wells, and ten random readings of each well were made for each well. The average number of cells per well was determined by multiplying the average number of cells per 10X field by the number of fields per well. Mean and standard deviation were calculated from at least triplicate wells.

For SMC migration studies involving RhoA and Gα-proteins, SMC were infected with HCMV (moi=10) for 2 hrs followed by co-infection of cells with Ad-Trans and Ad-RhoA-DN (moi=100-200) or Ad-RhoA-WT (moi=75-100) for an additional 2 hrs. Subsequently, the transwells were transferred to fresh 12-well plates. Cellular migration was determined as described above. In migration experiments involving G-proteins, cells were co-infected with HCMV and Ad-Trans and adenoviruses expressing either Gαi1 (moi=25 and 50), Gα12 (moi=50 and 100), Gα13 (moi=50 and 100), Gαq (moi=100 and 200) and Gα11 (moi=25 and 50) and the experiment carried out as described above. Prior to their use in these experiments the expression levels of all recombinant Adenoviral-derived proteins were equalized in SMC by adjusting the moi as determined by Western blotting using antibodies directed against the various G-protein subunits.

For SMC migration experiments involving the p160ROCK inhibitor Y-27632, SMC were infected with HCMV as described above and treated with increasing concentrations of Y-27632 or left untreated. Cellular migration was determined as described above.

Immunofluorescence. PASMC were grown in 4-well chamber slides (Nalge-Nunc, Seattle, WA). Adenovirus vectors were used to express US28 as described above. Cells were untreated or treated with the p160ROCK inhibitor Y27362 at two hours post-infection. At 20 hours post-infection, the cells were washed with PBS and fixed in 1% paraformaldahyde for 10 minutes at room temperature. The samples were then permeabilized and blocked in 0.3% Triton-X-100 in PBS with 10% FCS and 0.1%

sodium azide. The cells were incubated with antibodies directed against US28-flag epitope in a 1:200 dilution for 1 hour at room temperature. Cells were washed 3 times in PBS and binding of the primary antibody was detected with a fluorescein-isocyanate-tetramethyl (FITC) conjugated goat anti-mouse antibody for 1 hour at room temperature. At this time, the cells were also stained for actin cytoskeleton using Phalloidin (Molecular Probes, Eugene, OR) to monitor US28-induced alterations in the actin cytoskeleton. Fluorescence positive cells were visualized on an inverted Nikon Fluorescent microscope using a 60X objective.

2.6 Acknowledgements

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Chapter 3

Ligand-specific Smooth Muscle Cell Migration and Signaling Induced by The Human Cytomegalovirus Chemokine Receptor US28

Ryan M. Melnychuk, Patricia P. Smith, Jennifer Vomaske, Laurel Hall, Victor DeFillipe, Klaus Fruh, Martine Smit, David D. Schlaepfer, Jay A. Nelson and Daniel N. Streblow

In the following chapter, I carried out the experiments in Figures 3.2-3.6. Patricia Smith performed the cellular migration experiments shown in Figure 3.1. Victor DeFilipe and Klaus Fruh assisted with the generation and interpretation of microarray data. Martine Smit provided the US28 inhibitor VUF2274. David Schlaepfer provided FAK contructs used to generate FAK expressing adenoviruses. Laurel Hall assisted in cloning contructs utilized in RT-PCR experiments and Jennifer Vomaske assisted in culture of FAK -/- cells and in optimization of immunoprecipitation reactions.

3.1 Abstract

While most chemokine receptors fail to cross the chemokine class boundary with respect to the ligands that they bind, the HCMV-encoded chemokine receptor US28 binds multiple CC-chemokines, and the CX_3C-chemokine Fractalkine. We have previously demonstrated that US28 is both necessary and sufficient for HCMV-mediated induction of vascular SMC migration in response to the CC-chemokines MCP-1 and RANTES. In this report, we reveal that in addition to being ligand-dependent, US28 signaling and the induction of SMC migration are also ligand-specific. We demonstrate that the US28 ligands MCP-1, RANTES and Fractalkine mediate activation of FAK, Grb2, and ERK-1/2; however, only RANTES and MCP-1 promote SMC migration and activate RhoA. While US28 binding to RANTES induces cellular migration and signaling, not all RANTES binding receptors mediate these effects, as evidenced by differences observed between CCR1, which induces SMC migration and RhoA activation, and CCR5, which does not. Similar to the US28 activation profile induced by binding to Fractalkine, the US28 inhibitor of constitutive activity VUF2274 activates FAK in US28 expressing cells but fails to promote the stimulation of RhoA or induce SMC migration. Interestingly, ligand-specific US28-induced SMC migration and signaling are paralleled by a similar up-regulation of cellular genes involved in cellular motility (p160Rock, actin-related protein 2/3, and gravin). These findings demonstrate that US28-mediated signaling and SMC migration are ligand-specific, which has important implications in the role of US28 in HCMV pathogenesis.

3.2 Introduction

All β and γ-herpesviruses encode molecules with the potential to modulate the host immune response, including chemokines and/or chemokine receptor homologs. The β-herpesvirus HCMV encodes a CXC-chemokine (UL146), a putative CC-chemokine (UL128), and four potential chemokine receptors (US27, US28, UL33 and UL78) with the most characterized being US28 (Akter et al., 2003; Chee et al., 1990b; Chee et al., 1990c; Penfold et al., 1999). Chemokines are small, inducible cytokines that have critical roles in the induction and promotion of cellular migration and activation upon binding 7-transmembrane spanning GPCRs. There are four major chemokine subfamilies that are categorized according to the spacing of the first two of four conserved NH_2-terminal cysteine residues: CC-, CXC-, CX_3C- and XC-. The CXC-chemokines are further subdivided based on the presence or absence of a Glu-Leu-Arg (ELR)-motif (Murphy et al., 2000). ELR containing CXC-chemokines are generally pro-inflammatory and angiogenic, while non-ELR CXC-chemokines are considered angiostatic. The CC- and CX_3C-chemokines have major roles in inflammation, orchestrating the migration and activation of immune cells, and binding to their respective receptors stimulates the cell type-dependent activation of a plethora of cellular signaling pathways depending on the chemokine/receptor pair. Functional outcomes of chemokine binding include: the migration of receptor expressing cells toward sites of inflammation; the additional production of other cytokines, chemokines and/or growth factors that amplify the inflammatory response; and the up-regulation of integrins, growth factor receptors and other proteins involved in cellular adhesion and activation.

Most chemokine receptors bind a limited subset of ligands belonging to a single subfamily. The ability to bind multiple ligands from different chemokine subfamilies is unique to a select few receptors including DARC-receptor, and the HHV-8-encoded chemokine receptor Orf74. These receptors have been reported to bind to both CC- and CXC-chemokines (Lentsch, 2002; Rosenkilde et al., 1999; Szabo et al., 1995). Another chemokine receptor known to bind multiple ligands from different subfamilies is US28. This receptor contains homology to CC-chemokine receptors, with the greatest homology to CCR1 (Gao and Murphy, 1994) and binds to a broad spectrum of CC-chemokines with high affinity including: RANTES, MCP-1, MIP-1α and MIP-1β (Kuhn et al., 1995). Interestingly, US28 also binds the CX_3C-chemokine Fractalkine with greater affinity than CC-chemokines. Since Fractalkine binding is not competed with saturating quantities of selected CC-chemokines, Fractalkine is predicted to bind unique regions of US28 compared to the CC-chemokines (Kledal et al., 1998).

The CC-chemokines are known to be potent stimulators of cellular activation through US28. In 293 cells, RANTES binding to US28 activates ERK-1/2 pathways through the G-proteins Gαi1 and Gα16 (Billstrom et al., 1998). We have previously demonstrated that US28-mediated SMC migration is a ligand-dependent event requiring either exogenously added RANTES or endogenously expressed MCP-1 (Streblow et al., 1999). This migratory process is not blocked by treatment with pertussis toxin (PTX), a Gαi/o inhibitor, suggesting that other G-proteins are involved in this event. Subsequent studies revealed that US28 couples with Gα12/13 promoting SMC migration and ligand-dependent signaling through the small G-protein RhoA (Chapter 2). US28-mediated

SMC migration is sensitive to treatment with PTK inhibitors, and the PTKs FAK and Src are activated in US28 expressing cells upon RANTES binding (Streblow et al., 2003c). Dominant negative inhibitory FAK molecules blocked US28-induced SMC migration suggesting that FAK activation is critical for US28-mediated SMC motility.

In contrast, addition of Fractalkine to US28 expressing cells has been employed as a means of reducing constitutive signaling activity induced by US28. Treatment of US28 expressing cells with Fractalkine or the US28 synthetic inverse agonist VUF2274 leads to substantial decreases in the ability of US28 to promote the Gαq/11 dependent constitutive activation of PLC-β and NF-κB, where as MCP-1 and RANTES have only negligible effects on constitutive signaling levels (Casarosa et al., 2001; Casarosa et al., 2003). Additionally, Fractalkine treatment of US28 expressing HEK293A cells reduces constitutive US28 phosphorylation (Mokros et al., 2002) and steady state levels of surface US28, but has little influence on the rapid endocytosis observed in HeLa cells (Fraile-Ramos et al., 2001). Together these data illustrate some of the ligand-dependent activities and cell type-specific effects associated with US28 signaling.

The ability of US28 to efficiently bind ligands from multiple chemokine subfamilies coupled with the vastly different signaling responses elicited by divergent ligands is intriguing and suggests that US28 signaling is not only ligand and cell-type dependent, but also ligand-specific. Although US28 binding to CC-chemokines leads to the activation of a multitude of cellular signaling pathways, the only activities associated with US28 binding to Fractalkine involve the modulation of constitutive signaling

activity. Here we investigate the signaling potential of US28 upon stimulation with CC-chemokines compared to the CX_3C-chemokine Fractalkine and demonstrate that RANTES but not Fractalkine promotes the US28-dependent migration of SMC, RhoA activation, and induction of host genes involved in cellular motility. The signaling activities associated with US28 binding to RANTES are similar to those observed in CCR1-expressing cells; however, RANTES binding to CCR5 does not promote the same signaling activities even though CCR5 binds RANTES with similar affinity as US28 and CCR1. RANTES, MCP-1, Fractalkine and VUF2274 (US28 nonpeptigenic inverse agonist) binding to US28 induced similar levels of FAK activation, promoting FAK association with the adaptor protein Grb2. US28 stimulated FAK activation is PTX resistant ($G\alpha i$/-independent) and independent of $G\alpha 12$ mediated signaling pathways. These results suggest that US28-signaling is ligand-specific, and that RANTES and Fractalkine promote differential G-protein coupling leading to the activation of alternative signaling pathways depending on the cell-type and the compliment of endogenously expressed G-proteins.

3.3 Results

3.3.1 Ligand-specific US28-mediated smooth muscle cell migration and transcriptional activation.

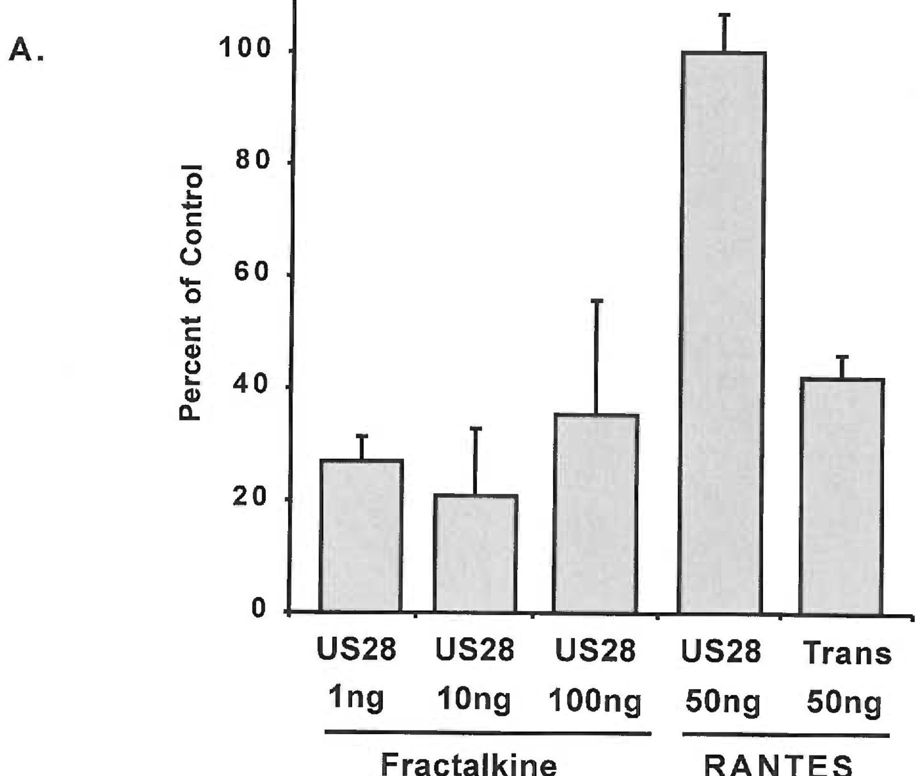
US28 binds multiple CC-chemokines including MCP-1 and RANTES (Billstrom et al., 1998; Bodaghi et al., 1998; Kuhn, 1995; Schall et al., 1994), as well as the CX_3C-chemokine Fractalkine with high affinity (Haskell et al., 2000; Kledal et al., 1998). The unique ability of US28 to bind both CC- and CX_3C-chemokine ligands raises the question of whether US28 signaling is not only ligand-dependent, but also, and more importantly ligand-specific. To determine whether US28 signaling and SMC migration are ligand-specific, SMC migration and signaling assays were performed in the presence of RANTES, MCP-1 or Fractalkine. First, the ability of Fractalkine to promote SMC migration through US28 was assessed. While addition of RANTES readily induced US28-mediated SMC migration, increasing concentrations of Fractalkine failed to stimulate cellular motility above Ad-Trans infected and RANTES stimulated controls, indicating that not all US28 ligands evoke the same functional response (Figure 3.1A). We also compared the ability of three known RANTES receptors, CCR1, CCR5 and US28 to promote SMC migration upon RANTES binding. Both CCR1 and US28 induced similar levels of migration in response to RANTES, where as RANTES stimulation of CCR5 expressing SMC failed to promote migration, indicating that not all chemokine receptors elicit equivalent biological responses upon treatment with the same ligand (Figure 3.1B).

Figure 3.1. US28-mediated SMC migration is ligand-specific.

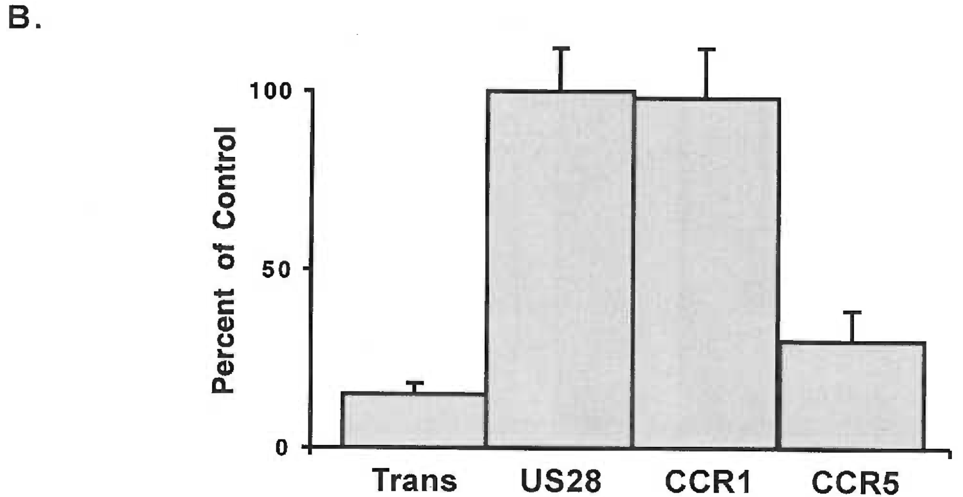
(A) SMC migration assays were performed on US28-expressing cells treated with either RANTES or Fractalkine at the indicated concentrations.

(B) SMC migration assays were performed on cells expressing US28, CCR1, or CCR5. Cells were treated with RANTES (50ng/ml) in the lower well.

A.



B.



Since RANTES but not Fractalkine caused the migration of US28 expressing SMC, we hypothesized that the difference in the ability to promote motility occurred at the level of signaling. Similarly, the capability of both CCR1 and US28 to promote cellular migration in response to RANTES but not CCR5 also suggests the differences are at the level of cellular signaling. To determine whether there exists a gross difference in the ability of these chemokine receptors/ligands to induce migration, host transcriptional profiles were examined using DNA microarrays. Human vascular SMC expressing US28, CCR1, CCR5 and/or Trans were stimulated with RANTES or Fractalkine (US28 expressing cells only). Four hours post-ligand stimulation RNA was prepared using the Trizol method, labeled, and then analyzed using Affymetrix human U95A microarray chips containing about 12,000 cellular genes. RNA samples from Trans expressing SMC independently stimulated with either RANTES or Fractalkine served as baseline controls for the appropriate comparisons. Addition of RANTES to US28 expressing SMC resulted in the up-regulation of 136 cellular genes and the down-regulation of 51 genes (totals from Figure 3.2A), while RANTES stimulation of CCR1 and CCR5 expressing SMC resulted in the up-regulation of 151 and 107 genes, and the down-regulation of 67 and 63 genes respectively. Overall, 49 common genes were up-regulated and 7 cellular genes down-regulated by each of CCR1, CCR5 or US28 upon stimulation with RANTES. As depicted by the regions of the circles that intersect between US28 and CCR1, US28 and CCR5, and CCR1 and CCR5, and in accordance with Figure 3.1B, the two chemokine receptors that promote SMC migration upon RANTES binding, US28 and CCR1, display similar gene expression profiles.

The gene expression profile of US28-expressing SMC stimulated with RANTES differs from the profile obtained upon stimulation with Fractalkine (Figure 3.2B- left). The fold changes above Trans infected (control) SMC stimulated with RANTES or Fractalkine are shown for each of the ligand treatments. In all, there were 112 common genes that were differentially regulated in US28 expressing cells stimulated with RANTES or Fractalkine. As depicted in the region of intersection between the two circles, 8 genes were up-regulated and 11 genes down-regulated by both ligands (Figure 3.2B-right). Interestingly, most of the genes that were up-regulated upon RANTES stimulation were down-regulated by Fractalkine, and overall, RANTES induced cellular gene expression through US28 (63 genes were up-regulated, and only 15 down-regulated), where as Fractalkine down-regulated cellular gene expression (67 genes were down-regulated and only 9 genes were up-regulated). Many of the cellular genes up-regulated in US28-expressing SMC treated with RANTES have roles in cellular migration, actin-cytoskeletal rearrangements, and signal transduction. These findings indicate that there are ligand-specific differences in US28 signaling that parallel the ability of either RANTES or Fractalkine to promote SMC migration through this receptor.

To confirm the up-regulation of cellular genes observed in the microarray experiments, RT-PCR TaqMan was performed on selected cellular genes. In three independent experiments, human vascular SMC were infected with adenoviruses expressing US28 and/or Trans. At 16 hpi US28 expressing SMC were stimulated with RANTES. Four hours post-RANTES stimulation RNA was prepared and RT-PCR TaqMan was performed utilizing primers directed against: p160Rock, FAK, human protein tyrosine kinase, γ-isomer from PP2A, gravin, rab5, and actin-related protein 2 (Arp2). RNA levels

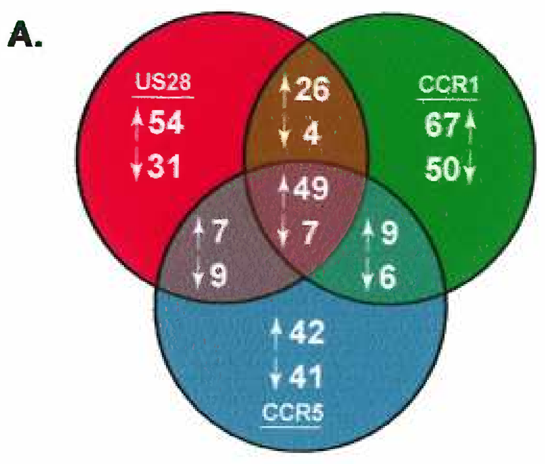
were normalized against the ribosomal protein L32. The number of copies induced in RANTES stimulated SMC expressing US28 were compared to the copy number in Trans infected and RANTES treated controls. Data are represented as a fold-increase above Trans infected and ligand stimulated controls (Figure 3.2C). All of the genes examined were at least 2-fold induced in RANTES stimulated SMC expressing US28. Interestingly, two of the genes found to be up-regulated upon RANTES treatment of US28 expressing SMC were the RhoA effector protein p160ROCK, and FAK. We have previously demonstrated a role for these proteins in RANTES induced US28-mediated SMC migration (Chapter 2; Streblow et al., 2003c). Treatment of US28 expressing SMC with a p160ROCK inhibitor or expression of dominant negative FAK mutants significantly reduced cellular motility stimulated by RANTES, as well as US28-mediated actin-cytoskeletal re-arrangements. These results confirm that RANTES binding to US28 leads to increases in cellular gene expression, and many of these genes have roles in cellular migration, actin-cytoskeleton rearrangements and signal transduction.

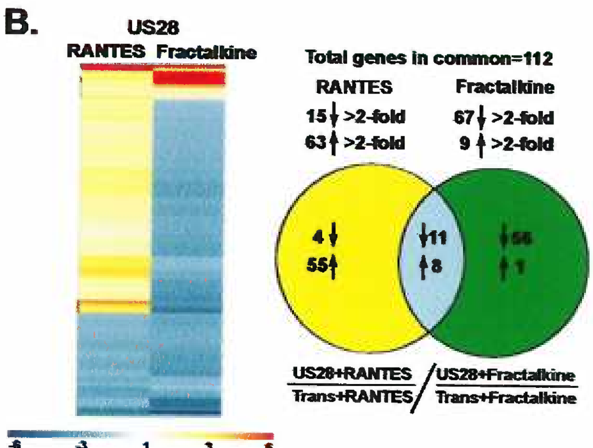
Figure 3.2. Induction of SMC cellular gene expression is receptor and ligand specific

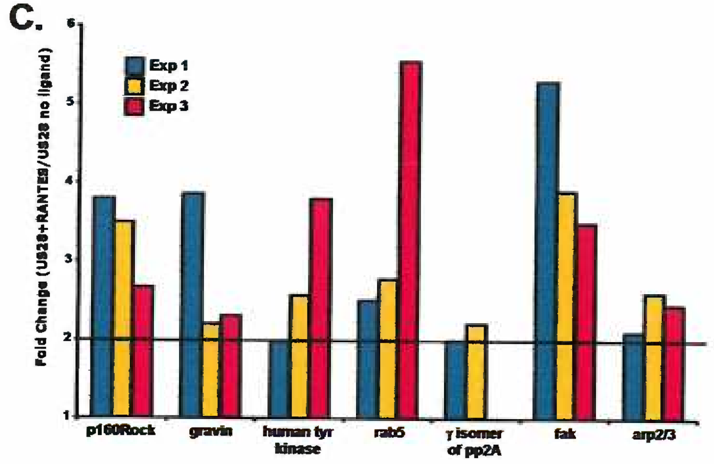
(A) Profiles of cellular genes induced by US28, CCR1, and CCR5 treated with RANTES.

(B) Comparison of the gene profile induced by treatment of US28 expressing SMC with either RANTES or Fractalkine.

(C) Genes induced by US28 in the presence of RANTES were confirmed by RT-PCR TaqMan. Shown are the fold increases in copy number compared to control cells treated with RANTES. Levels of mRNA were normalized using primers directed against the gene for the ribosomal protein L32.


A.
US28
54
31
26
4
CCR1
67
50
49
7
7
9
9
6
42
41
CCR5


B.
US28
RANTES Fractalkine
Total genes in common=112
RANTES
Fractalkine
15 >2-fold
63 >2-fold
67 >2-fold
9 >2-fold
4
55
11
8
US28+RANTES
Trans+RANTES
US28+Fractalkine
Trans+Fractalkine
-6
-3
1
3
6


C.
Fold Change (US28+RANTES/US28 no ligand)
Exp 1
Exp 2
Exp 3
p160Rock
gravin
human tyr kinase
rab5
γ isomer of pp2A
fak
arp2/3

3.3.2 Ligand-specific signaling mediated by US28.

Differential transcriptional activity induced by GPCRs is often the result of alterations in cellular signaling. To determine if the differential up-regulation of cellular genes induced by RANTES or Fractalkine binding to US28 is reflected in differences at the level of signal transduction, the ability of these two chemokines to activate the small G-protein RhoA was examined. RhoA pull down assays utilizing the RhoA binding domain of RhoTekin (which only binds RhoA in the active GTP bound state) fused to GST were performed on lysates from cells expressing US28 and treated with RANTES (10ng/ml), MCP-1 (10ng/ml) or Fractalkine (10ng/ml). In accordance with the migration (Figure 3.1A), microarray (Figure 3.2B) and gene expression data (Figure 3.2C) both RANTES and MCP-1 binding to US28 promoted RhoA activation, while Fractalkine failed to stimulate RhoA activity (Figure 3.3). In addition, CCR1 and US28, but not CCR5 induced the activation of RhoA in response to RANTES (Figure 3.3), paralleling the migration and microarray data. These results indicate that cells expressing different chemokine receptors, stimulated with the same ligand may result in the activation of alternative signaling pathways, ultimately resulting in differential biological responses. Furthermore, stimulation of US28 with chemokine ligands belonging to different subfamilies results in vastly different functional outcomes (RANTES and MCP-1 induced SMC migration and RhoA activation versus the failure of Fractalkine to promote either cellular migration or RhoA activity through US28), while displaying differential signaling characteristics.

Figure 3.3. US28 activation of RhoA is ligand-specific.

RhoA activation assays using Rhotekin-conjugated GST beads were performed in SMC expressing US28, CCR1, or CCR5. Cells expressing the Tet transactivator (Trans) were used as a control. Cells were treated with RANTES (10ng/ml), MCP-1 (10ng/ml) or Fractalkine (10ng/ml).

trans US28

0 5 10 30 0 5 10 30 min.

RANTES

MCP-1

Fractalkine

CCR5 CCR1

0 5 10 30 0 5 10 30

RANTES

We have previously demonstrated that RANTES binding by US28 stimulates the activation of FAK, promoting an active association between FAK and the adaptor protein Grb2 (Streblow et al., 2003c). To determine the ability of CC-chemokines and the CX_3C-chemokine Fractalkine to promote US28 mediated FAK activation and formation of active Grb2/FAK complexes, FAK-/- cells expressing US28 alone or in combination with FAK were stimulated with RANTES, MCP-1 or Fraktalkine (40ng/ml) for 0 (unstimulated), 5, 10, 15 or 30 minutes. Grb2 was immunoprecipitated and active FAK associated with Grb2 visualized by western blotting for phospho-FAK (Streblow et al., 2003c). RANTES, MCP-1 and Fractalkine all promoted FAK activation and formation of Grb2/FAK complexes through US28, although the kinetics and magnitude of activation differed slightly with each ligand (Figure 3.4A). Interestingly, while Fractalkine failed to promote SMC migration or RhoA activation, this US28 ligand potentiated the most robust signaling through FAK of any of the three ligands tested.

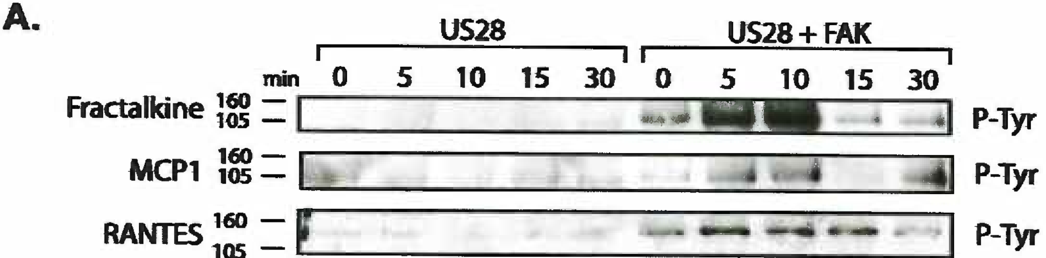
Ligand-induced signaling through US28 also promotes pronounced actin-cytoskeletal changes in multiple cell types (Chapter 2; Streblow et al., 1999; Streblow et al., 2003c). Therefore, we assessed the ability of these three ligands to promote actin-cytoskeletal re-arrangements through US28 in FAK-/- fibroblasts. FAK-/- cells infected with adenoviruses expressing US28 and FAK were stimulated with RANTES, MCP-1, or Fraktalkine (20ng/ml). Two hours post-ligand stimulation, fixed and permeabilized cells were incubated with antibodies directed against the Flag (US28) and HA (FAK) epitopes, and actin was visualized by staining with Phalloidin. While RANTES, MCP-1, and Fractalkine failed to stimulate morphological changes in the absence of US28 each of the

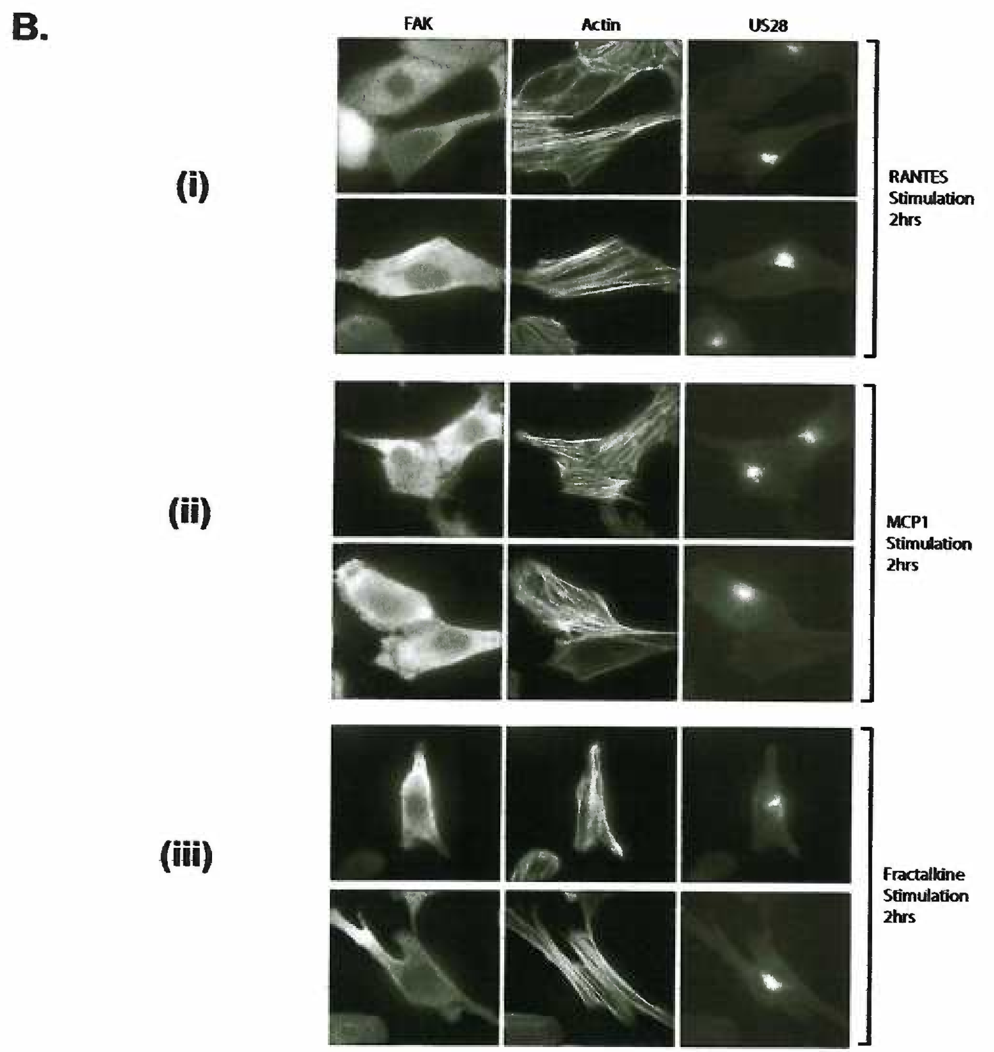
three ligands readily promoted actin-cytoskeletal re-arrangements in US28 expressing cells (Figure 3.4B). These are the first data to demonstrate the activation of specific signaling pathways mediated by US28 binding to Fractalkine. Although RANTES, MCP-1 and Fractalkine differ with respect to their ability to promote cellular migration through US28, all are capable of promoting FAK activation and formation of active Grb2-FAK complexes, as well as re-organization of the actin-cytoskeleton.

Figure 3.4. All US28 ligands are capable of activating FAK and inducing Actin Stress Fiber Formation in reconstituted FAK-/- cells.

(A) FAK activation was determined by Grb2/FAK co-immunoprecipitation reactions on reconstituted FAK-/- cells expressing US28 that were treated with RANTES, Fractalkine, MCP-1. Cells were harvested in modified RIPA buffer at 0 (unstimulated), 5, 10, 15, and 30 minutes post addition of ligand. Active FAK associated with Grb2 was visualized by western blotting for phospho-FAK.

(B) FAK null cells expressing US28 were reconstituted with Wt FAK. RANTES (i), MCP-1 (ii), Fractalkine (iii) treated cells were fixed two hours post addition of ligand. Cells were stained for actin with phalloidin (actin) and FAK using antibodies directed against the FAK-N'terminal HA-tag, and US28 using antibodies directed against the N-terminal Flag epitope present on US28.


A.
US28
US28 + FAK
min
0
5
10
15
30
Fractalkine
MCP1
RANTES
160
105
P-Tyr


B.
FAK
Actin
US28
(i)
(ii)
(iii)
RANTES Stimulation 2hrs
MCP1 Stimulation 2hrs
Fractalkine Stimulation 2hrs

3.3.3 VUF2274 Displays Similar US28 Cellular Signaling Activities as Fractalkine

Recently, a family of 4-hydroxypiperidine analogs (VUF2274 and related compounds) were reported to act as inverse agonists and block US28 constitutive signaling activity (Casarosa et al., 2003). These molecules were originally discovered as inhibitors of CCR1, the most closely related cellular chemokine receptor to US28 (Hesselgesser et al., 1998). Interestingly, while these molecules displace RANTES from US28, they also exhibit non-competitive binding characteristics in saturation binding assays. These findings suggest that displacement of RANTES is the result of a change in receptor conformation allosterically induced by VUF2274 (Casarosa et al., 2003). To assess the ability of VUF2274 to promote cellular activation through US28, Grb2-FAK co-immunoprecipitation reactions were performed in FAK-/- cells reconstituted with FAK. US28 expressing and FAK reconstituted FAK-/- cells were stimulated with VUF2274 (10μM) for 0 (untreated), 5, 10, 15 or 30 minutes and cells were harvested in modified RIPA buffer. Active FAK associated with Grb2 was visualized by western blotting as described above. Surprisingly, VUF2274 readily stimulated US28-mediated activation of FAK and formation of Grb2-FAK complexes, with peak activation occurring 15 minutes post-VUF2274 stimulation (Figure 3.5A). VUF2274 treatment of reconstituted FAK-/- cells expressing US28 also resulted in profound actin-cytoskeletal rearrangements (Figure 3.5B). The observed changes in the organization of the actin-cytoskeletal resembled those induced by treatment of US28 expressing cells with MCP-1, RANTES or Fractalkine. The ability of VUF2274 to stimulate the activation of ERK-1/2 through US28 was also determined. Western blots were performed on lysates from wt-FAK reconstituted FAK -/- cells expressing US28 and stimulated with either RANTES

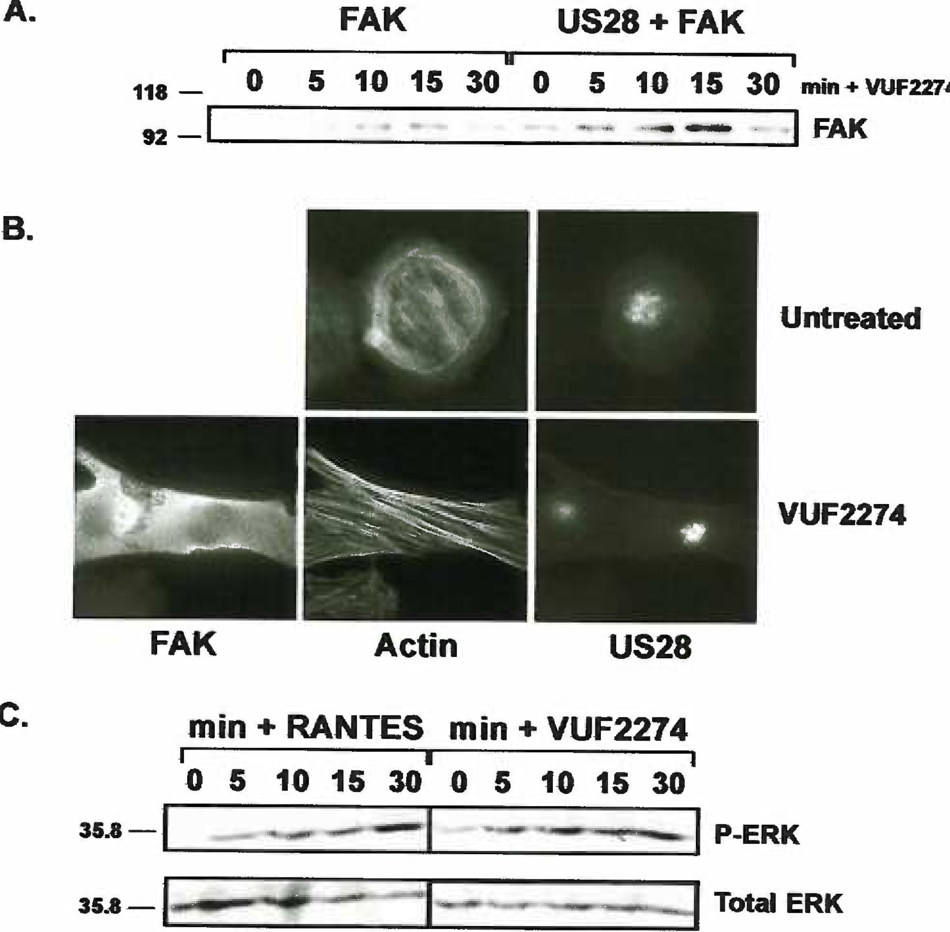
(10ng/ml) or VUF2274 (10μM). Both RANTES and VUF2274 promoted the activation of ERK-1/2 through US28 (Figure 3.5C). Similar to Fractalkine, VUF2274 binding to US28 did not induce the activation of RhoA, and the ability of VUF2274 to promote SMC migration could not be assessed because of drug toxicity. Therefore, the US28 inverse agonist VUF2274 is also a potent agonist, stimulating Grb2-FAK association, FAK activation, actin-cytoskeletal rearrangements, ERK-1/2 activation, and exhibits signaling characteristics similar to Fractalkine.

Figure 3.5. US28 Activation of FAK by treatment with VUF2274.

(A) US28 and/or FAK expressing FAK -/- cells were stimulated with VUF2274 (10μM) and cell lysates were analyzed for FAK activation by Grb2-FAK co-immunoprecipitation assays. Immunoprecipitated FAK associating with Grb2 was visualized by western blotting for phospho-FAK (p-Tyr).

(B) VUF2274 treatment of US28 expressing reconstituted FAK-/- cells induces actin-cytoskeletal rearrangements.

(C) VUF2274 induces the phosphorylation of ERK-1/2 in US28 expressing cells.



A.
FAK
US28 + FAK
118
92
0 5 10 15 30 0 5 10 15 30 min + VUF2274
FAK
B.
Untreated
VUF2274
FAK
Actin
US28
C.
min + RANTES
min + VUF2274
0 5 10 15 30 0 5 10 15 30
35.8
P-ERK
35.8
Total ERK

3.3.4 Fractalkine induced FAK activation through US28 does not require Gαi/o and is blocked by Gα12 G-proteins.

We have previously determined that US28-mediated SMC migration requires the Gα12/13-dependent activation of RhoA (Chapter 2). To identify the G-proteins involved in RANTES and Fractalkine stimulated FAK activation through US28, Grb2-FAK co-immunoprecipitation reactions were performed on lysates from US28 expressing reconstituted FAK-/- cells. Cells were pre-treated with the Gαi/o inhibitor PTX or were left untreated and then stimulated with either RANTES or Fractalkine (10ng/ml) and Grb2/FAK co-immunoprecipitations were visualized by western blotting. Pre-treatment with PTX significantly enhanced both Fractalkine and RANTES mediated activation of FAK through US28, suggesting that both ligands promote coupling to G-proteins other than Gαi/o family G-proteins to induce FAK activation (Figure 3.6A). Interestingly, stimulation of US28 expressing cells with either RANTES or Fractalkine led to the PTX resistant activation of ERK-1/2. Unlike US28-mediated FAK activation, which was enhanced by PTX, ERK-1/2 activation was not affected by PTX pre-treatment. Therefore, US28-mediated activation of ERK-1/2 in reconstituted FAK -/- cells is independent of Gαi/o family G-proteins, and differs from PTX sensitive MCP-3 and RANTES induced ERK-2 activation observed in 293 cells (Billstrom et al., 1998). Since RANTES and Fractalkine induced activation of FAK through US28 is independent of Gαi/o family G-proteins, and US28 is known to signal through Gα12 to promote cellular migration, we assessed the role of Gα12 in RANTES and Fractalkine mediated activation of FAK. Reconstituted FAK -/- cells expressing US28 and Gα12 were pre-treated with PTX and then stimulated with either RANTES or Fractalkine. FAK activation was

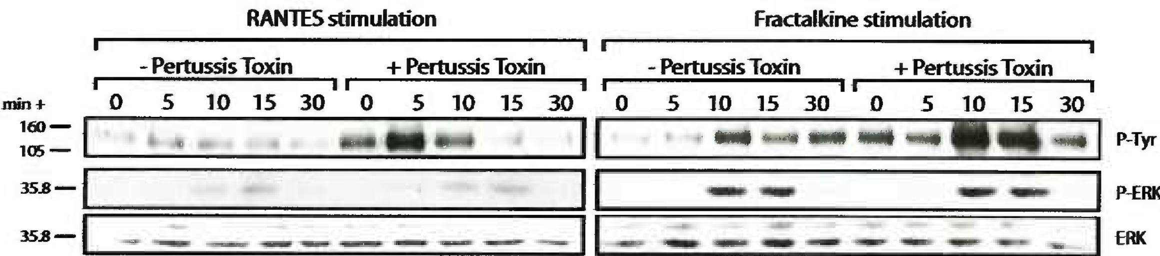
determined using Grb2-FAK co-immunoprecipitation reactions as described above. Introduction of Gα12 to PTX treated cells had little influence on the kinetics of FAK activation by RANTES, but significantly delayed and reduced FAK activation by Fractalkine (Figure 3.6B). These findings combined with our previous findings indicate that US28 G-protein coupling occurs in a ligand specific manner wherein RANTES promotes US28 coupling to Gαi/o, Gα16 (Billstrom et al., 1998), and Gα12/13 (Chapter 2), where as Fractalkine promotes US28 coupling to G-proteins other than Gαi/o and Gα12/13.

Figure 3.6. Pertussis Toxin Increases RANTES and Fractalkine activation of FAK

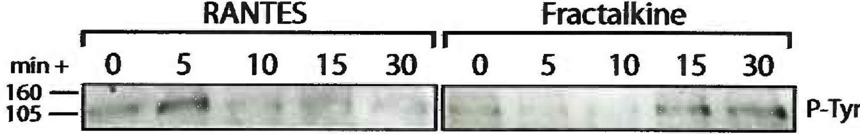
(A) FAK activity in FAK -/- cells expressing both US28 and wt-FAK in response to either Fraktalkine or RANTES and in the presence or absence of pertussis toxin was assessed by Grb2/FAK co-immunoprecipitation reactions. Cells were harvested in modified RIPA buffer at 0 (unstimulated), 5, 10, 15, and 30 minutes post addition of ligand. Active FAK associated with Grb2 was visualized by western blotting for phospho-FAK.

(B) The ability of $G\alpha12$ to enhance or abrogate RANTES and Fraktalkine mediated activation of FAK through US28 was assessed by expressing $G\alpha12$ in FAK -/- cells. FAK -/- cells expressing US28, wt-FAK and $G\alpha12$ were stimulated with either RANTES or Fraktalkine in the presence or absence of pertussis toxin. As in (A), FAK activity was assessed by Grb2/FAK co-immunoprecipitation reactions and active FAK associated with Grb2 was visualized by western blotting for phospho-FAK.

A.



B.



3.4 Discussion

In the current report, by examining the functional response, signaling characteristics, and transcriptional profiles induced by US28 upon binding a diversity of ligands, we demonstrate that not only is US28-signaling ligand and cell-type dependent but also ligand specific. The ability of US28 to promote cellular migration and RhoA signaling occurred in RANTES and MCP-1 (CC-chemokines) treated cells but not CX_3C-chemokine Fractalkine treated cells. Similarly, RANTES but not Fractalkine increased the transcription of genes involved in cellular migration. Interestingly, while these ligands display differential signaling characteristics with respect to SMC migration, all are capable of inducing FAK activation and association with Grb2 in fibroblasts. The ability of Fractalkine to induce US28-dependent FAK activity in fibroblasts was abrogated in the presence of Gα12 but not by PTX suggesting that Fractalkine induced-US28 signaling occurs in a Gαi/o, Gα12-independent manner. This pathway of activation is different in RANTES stimulated SMC and fibroblasts, which can occur in a Gα12-dependent manner. Ultimately, our findings indicate that US28 binding to RANTES or Fractalkine results in differential G-protein coupling/activation leading to unique functional consequences.

3.4.1 Multiple chemokine family binding receptors.

While most chemokine receptors of either viral or cellular origin bind a limited subset of chemokines from a single chemokine subfamily, there are three examples of chemokine receptors that bind chemokines from multiple subfamilies: the DARC-receptor, Orf74 of HHV-8, and US28 (Kledal et al., 1998; Lentsch, 2002; Rosenkilde et al., 1999; Szabo et

al., 1995). To date DARC, which binds both CC- and CXC-chemokines (CCL2, CCL5, CXCL1, and CXCL8), is the only true chemokine sink because this receptor binds and internalizes these ligands without inducing signaling events. Orf74 has also been demonstrated to bind both CC- and CXC-chemokines; however, there is a significant difference in the affinity of individual ligands for this receptor. Despite being referred to as an IL-8 receptor, Orf74 has a far greater affinity for GRO peptides (α, β, γ) than for IL-8 (Rosenkilde et al., 1999). In competition binding assays with IL-8, Orf74 binding to the CC-chemokines MIP-1α, MIP-1β, MCP-1 and RANTES is virtually undetectable, while MCP-3 and aminooxypentane (AOP)-RANTES display affinities in the 200nm range. Interestingly, the structurally distinct, non-ELR containing CXC-chemokines IP-10 and SDF-1α can displace IL-8 binding, and function as efficient inverse agonists of Orf74 signaling at nanomolar concentrations (Rosenkilde et al., 1999). Although Orf74 binds to chemokines from multiple chemokine subfamilies, Orf74 signaling only occurs in the presence of ELR, pro-inflammatory/angiogenic chemokines, whereas the angiostatic non-ELR CXC-chemokines function as efficient inverse agonists. Unlike Orf74, US28 binds multiple ligands from different chemokine subfamilies with near equal affinity (Kledal et al., 1998; Kuhn et al., 1995), and as we demonstrate in the current report, these distinct ligands promote cellular activation upon binding US28. Therefore US28 is the only proven chemokine receptor capable of signaling upon binding ligands from multiple chemokine subfamilies.

3.4.2 US28 ligand-specific signaling.

We have previously demonstrated that both MCP-1 and RANTES promote US28-mediated SMC migration (Streblow et al., 1999), and in the current report we have shown that both of these ligands induce RhoA activation through US28. While Fractalkine is a known modulator of US28-induced constitutive signaling activity (Casarosa et al., 2001; Mokros et al., 2002), this ligand does promote SMC migration or the activation of the small G-protein RhoA upon binding US28 (Figures 3.1B & 3.3). In accordance with these ligand-specific functional responses, microarray analysis of US28-expressing SMC stimulated with either RANTES or Fractalkine revealed profound differences at the level of gene induction. Whereas RANTES stimulation resulted in the up-regulation of cellular genes with roles in signal transduction, actin-cytoskeletal rearragements and cellular motility, Fractalkine down-regulated many of these same genes. Our gene expression data differs from recently published findings that suggest that US28 is not responsible for cellular signaling activity late during HCMV infection of fibroblasts (Hertel and Mocarski 2004). While numerous genes were influenced by HCMV infection, whether US28 was responsible for any of the changes is unclear, as the virus used in these studies lacked both US28 and the putative CC-chemokine receptor US27. Furthermore, the authors failed to stimulate cells with ligand. Thus, the absence of allterations in transcripitional activity may be the result of a failure to stimulate US28 expressing cells. Finally, as we have previously demonstrated, US28-induced signaling activity varies in a cell-type dependent manner and is influenced by endogenously expressed G-proteins (Chapter 2). Importantly, we have observed that cultured fibroblasts lack Gα12 family

G-protein expression, and that these G-proteins are critical mediators of signaling events involved in US28-induced SMC migration.

Our findings that Fractalkine modulates cellular activation are consistent with previous signaling activities associated with Fractalkine binding to US28 (Casarosa et al., 2001; Fraile-Ramos et al., 2001; Mokros et al., 2002). Although Fractalkine fails to induce RhoA activity or SMC migration, and reduces the expression of cellular genes induced by RANTES, we demonstrate for the first time that Fractalkine is a potent agonist capable of inducing cellular activation upon binding US28. RANTES, MCP-1, Fractalkine and the US28 inhibitor VUF2274 all display similar abilities to induce ERK-1/2, FAK, actin cytoskeletal rearrangements and formation FAK-Grb2 complexes. Pre-treatment with PTX enhanced Fractalkine mediated FAK activation through US28, while introduction of Gα12 delayed and reduced FAK activity. This observation can be construed in one of two manners. First, Fractalkine binding to US28 may promote coupling to Gα12. However, Gα12 may not signal through FAK, thus preventing activation by Fractalkine. Alternatively, expression of Gα12 may act as a dominant inhibitory molecule that prevents G-proteins that activate FAK by Fractalkine from coupling to US28. Our data supports the latter hypothesis since we do not observe Gα12-dependent activation of RhoA or migration of SMC by Fractalkine, suggesting that Fractalkine mediates the activation of FAK through G-proteins other than Gαi/o and Gα12. Expression of Gα12 in the presence of PTX had little influence on RANTES mediated activation of FAK, suggesting that RANTES-induced FAK activation through US28 is also independent of Gαi/o and Gα12. Therefore, RANTES stimulates varying signaling pathways through

different G-proteins in SMC (Gα12-dependent) and fibroblasts (Gαi/o independent). Together, these findings demonstrate that not only is US28 signaling ligand-dependent and ligand-specific, but also G-protein specific.

Expression of membrane-bound Fractalkine can be induced on endothelial cells by numerous cytokines including IFN-γ, TNF-α and IL-1, resulting in the recruitment of inflammatory cells and contributing to chronic inflammatory vascular diseases such as atheroscleorosis, restenosis following angioplasty and transplant vascular sclerosis (Bazan et al., 1997). Unlike other chemokines which are secreted as soluble molecules that must associate with proteoglycans and other components of the extracellular matrix to establish chemokine gradients (Tanaka et al., 1993), Fractalkine is generated as a membrane bound ligand with the chemokine domain presented at the top of the cell-bound mucin-like stalk (Bazan et al., 1997; Pan et al., 1997). The generation of soluble Fractalkine requires proteolysis by TNF-α-converting enzyme or ADAM10 (Garton et al., 2001; Tsou et al., 2001) and Fractalkine is a potent chemoattractant. In many instances this ligand is more effective than other ligands in promoting leukocyte activation and migration. Thus, our findings demonstrating that Fractalkine binding to US28 down-modulates cellular genes involved in cellular motility was surprising and further exemplify the uniqueness of US28. However, Fractalkine can also function as an adhesion molecule similar to vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1 and E-selectin, and CX_3CR1 expressing cells have been demonstrated to adhere to the vascular endothelium without receptor bearing cells dislodging under normal physiological vascular flow conditions (Fong et al., 1998; Goda

et al., 2000; Imai et al., 1997). Interestingly, an US28 expressing murine pre-B cell line has been demonstrated to adhere to fixed Fractalkine surfaces (Haskell et al., 2000), suggesting that US28-mediates the adhesion of receptor expressing cells to extracellular-associated Fractalkine. Thus, the inherent ability of US28 to adhere to mobilized Fractalkine coupled with our finding that this chemokine reverses transcriptional activation required for cellular migration (ie. RANTES-induced up-regulation of cellular genes involved in motility and signaling versus the down-regulation of these same genes by Fractalkine), suggests that Fractalkine may arrest US28-induced SMC migration and promote the subsequent adhesion of US28 expressing cells to the vascular endothelium. The migration of HCMV infected and US28 expressing SMC from the vessel media to inflammatory sites in the vessel intima and the subsequent adhesion and accumulation of SMC in the vessel intima may have important implications in the dissemination and *in vivo* pathogenesis of HCMV, as well as in the exacerbation of vascular disease.

Prior to this study, Fractalkine had been employed as a modulator of US28-mediated constitutive signaling activity. In this report we demonstrate that similar to RANTES and MCP-1, Fractalkine is a potent US28 agonist that promotes robust signaling through FAK and ERK1/2 and induces actin cytoskeletal rearrangements. Similarly, the US28 inverse agonist of constitutive activity, VUF2274, promoted US28-mediated activation of FAK and ERK-1/2. Unlike RANTES and MCP-1, Fractalkine fails to activate RhoA, induce SMC migration, or increase expression of cellular genes involved in motility and signaling. Thus demonstrating that US28 signaling is ligand specific. In addition, the US28 ligand-specific and cell-type dependent activation of differential signaling

pathways suggest that this chemokine receptor has the capacity to couple to different G-proteins depending upon the ligand bound and cellular G-protein environment. Therefore, US28 binds to a diversity of chemokines, which promote US28 coupling to multiple G-proteins, eliciting functional signaling through these various G-proteins. HCMV encounters and infects a multitude of distinct cell types *in vivo* including fibroblasts, monocyte/macrophages, endothelial cells and SMC that may differ substantially with respect to the G-proteins that they express. The ability of US28 to couple to multiple G-proteins may have important implications in the persistence and pathogenesis of HCMV in these different cell-types.

3.5 Methods and Materials

Cell Lines. The life-extended human pulmonary artery smooth muscle cell line, PAT1 (Chapter 2) were maintained in Medium 199 supplemented with 20% FCS and PSG. For migration and microarray experiments described below, PAT1 cells were utilized between passage 5 and 30 post-telomerization. Mouse FAK-/- fibroblasts were maintained on gelatin coated culture dishes in DMEM supplemented with 10% FCS, PSG, non-essential amino acids (Cellgro), and G418 (Sigma; 500μg/ml) as previously described (Ilic et al., 1995; Sieg et al., 1998). FAK-/- cells used in experiments were between passage 5 and 15. U373MG cells were maintained in DMEM supplemented with 10% FCS and PSG.

Reagents. RANTES, MCP-1, and Fractalkine were purchased from R&D Systems (Minneapolis, Minn). The US28 antagonist VUF2274 was previously described (Casarosa et al., 2003). Anti-Grb2 (C-7), anti-phosphotyrosine (PY99), anti-RhoA (sc-179) and anti-HA (F-7) antibodies were purchased from Santa Cruz Biotechnology. Phospho-specific antibodies to ERK-1/2 (Thr202/Tyr204) and total ERK-1/2 were from Cell Signaling Technologies. Anti-M2-Flag antibody (F-3165) was purchased from Sigma. Secondary anti-mouse and anti-rabbit HRP-conjugated antibodies (NA934V and NA931V) were purchased from Amersham (Piscataway, N.J.).

RhoA Activation Assay. The RhoA binding domain of Rhotekin was expressed as a fusion protein with GST (GST-rhotekin, kindly provided by Dr. J. Scott, OHSU) in the E.coli strain BL21, as previously described (Chapter 2). After IPTG induction the bacteria were lysed and the clarified supernatants were bound to Glutathione-linked 4B-

CL Sepharose beads (Amersham) overnight at 4°C. The beads were washed 3 times by pelleting in PBS followed by 3 washes in RhoA wash buffer (50mM Tris pH 7.2, 1% Triton-X100, 150mM NaCl, and 10mM $MgCl_2$) with a final equilibration wash using RhoA lysis buffer (50mM Tris pH 7.2, 1% Triton-X100, 500mM NaCl, 10mM $MgCl_2$, 0.5% sodium deoxycholate, and 0.1% SDS). For these assays U373MG cells were plated onto 15 cm culture dishes, and at 75% confluence the cells were serum-starved for 24 hours. The cells were infected with Ad-Trans and Ad-US28, Ad-CCR1 or Ad-CCR5 MOI 500 and placed in serum free medium. After 16 hours, the cells were stimulated with RANTES (10ng/ml), MCP-1 (10ng/ml) or Fractalkine (10ng/ml) and then scraped in RhoA lysis buffer at 0 (unstimulated), 5, 10, 15, or 30 minutes post-stimulation. Cell lysates were incubated with GST-Rhotekin beads (100μl of a 1:1 slurry) for 45 minutes at 4°C, then washed 4 times with 1 ml of RhoA wash buffer. The final bead pellet was resuspended in 60μl of 2x Laemmli's sample buffer, boiled and then run on 10% SDS-PAGE. The gels were transferred to Immobilon-P membranes for Western blotting. Blots were blocked in blocking buffer (1% bovine serum albumin, 0.01% polyvinylpyrrolidone, 0.01% sodium azide) for 1 hour at 4°C, and the primary anti-RhoA rabbit polyclonal antibody was added at 1:1000 dilution in blocking buffer overnight at 4°C. The blots were washed 4 times with TBS-Tween buffer (10mM Tris pH 7.2, 100mM NaCl, 0.1% Tween-20). The secondary antibody (goat anti-rabbit conjugated to HRP) was added at 1:2000 dilution in TBS-Tween buffer. After washing 3 times with TBS-Tween buffer and incubation with ECL reagents, the blots were visualized by autoradiography.

Immunoprecipitation Reactions. FAK-/- cells were plated in 10cm culture dishes and serum starved for 6 hrs upon achieving 75% confluence. The cells were co-infected with Ad-Trans and/or Ad-US28 and/or Ad-FAK WT at MOI 50. After 16 hrs the cells were stimulated with RANTES (40ng/ml), Fractalkine (40ng/ml), MCP-1 (40ng/ml), or VUF22784 (10μM) and then harvested at times 0 (unstimulated), 5, 10, 15, and 30 minutes post ligand addition. Cells were lysed in RIPA lysis buffer and total Grb2 was immunoprecipitated and samples analyzed by western blotting using antibodies directed against phospho-Tyr (Streblow et al., 2003c). Co-precipitation of FAK-HA was demonstrated by stripping the blots in buffer containing 0.1M Tris pH 6.8, 1% SDS, and 1% 2-β-mercaptoethanol and staining using antibodies directed against HA. Prior to immune-complex reactions, a total of 50μl of cellular lysate was assayed by SDS-PAGE/western blotting for the presence of input US28 and FAK using antibodies directed against the HA-epitope present on both recombinant proteins.

SMC migration assay. Cell migration assays were performed as previously described (Streblow et al., 1999). Briefly, cells were added to the upper well of a transwell (12 mm diameter, 3.0 μm pore size, Costar Corning, Cambridge, MA) at $1x10^5$ cells per well. Cells were serum starved for 16-24 hrs. HCMV at MOI 10 was added to the upper well for 2 hours. After infection the inserts were washed and transferred to fresh 12-well plates. Cells migrating to the lower chamber were counted at 48-72 hrs pi using a Nikon TE300 microscope at magnification 10X. Experiments were done in at least triplicate wells. Ten random fields were read in each well. The average number of cells per well

was determined by multiplying the average number of cells per 10X field by the number of fields per well. Mean and standard deviation were calculated.

For SMC migration studies involving US28, CCR1, and CCR5 adenovirus constructs, SMC were co-infected with Ad-Trans and the adenovirus expressing the chemokine receptor at MOI of 1,000 for 2 hrs. Subsequently, the transwells were transferred to fresh 12-well plates. Cellular migration was determined as described above in the presence of RANTES or Fractalkine (1-100ng/ml). Recombinant protein levels were monitored by western blotting and equalized by adjusting the adenoviral vector MOI accordingly.

Adenovirus Construction. Adenoviruses expressing Gα12, WT-FAK, US28-Flag, US28-HA, and CCR5 were previously described (Chapter 2; Streblow et al., 1999; Streblow et al., 2003c). Adenovirus vectors expressing CCR1 were constructed by subcloning the DNA fragment into pAdTet7. This vector contains the tet-responsive enhancer within a minimal CMV promoter followed by the SV40 late poly(A) cassette, adenovirus E1A, and a single loxP site to increase recombination frequency. Recombinant adenoviruses were produced by pAd-CCR1 construct co-transfection of 293 cells expressing the Cre-recombinase with adenovirus DNA (Ad5-ψ5) that contains an E1A/E3-deleted adenovirus genome (Hsia et al., 2003). Recombinant adenoviruses were expanded on 293-Cre cells and the bulk stocks were titered on 293 cells by limiting dilution. Gene expression was driven by co-infection with Ad-Trans expressing the Tet-off transactivator as previously described (Streblow et al., 1999).

Immunocytochemistry. $FAK^{-/-}$ fibroblasts were grown in 0.1% gelatin coated 4-well chamber slides (Nalge-Nunc). US28 and/or FAK was expressed using the adenovirus vectors described above and were left untreated or were treated with MCP-1, RANTES or Fractalkine (20ng/ml) for 2 hours. The cells were washed in PBS and fixed in phosphate buffered 1% paraformaldehyde (PFA) for 10 minutes at room temperature, then permeabilized and blocked with 0.3% Triton X-100 in PBS with 10% FCS and 0.1% sodium azide. Thereafter, the cells were incubated with antibodies against US28-Flag epitope or FAK-HA epitope in a 1:200 dilution for 1 hr at room temperature. Cells were washed three times in PBS and binding of the primary antibody was detected with a fluorescein isothiocyanate-tetramethyl (FITC) conjugated goat anti-mouse or rhodamine conjugated goat anti-rabbit antibody for 1 hr at room temperature. At this time the cells were also stained for actin using Phalloidin (Molecular Probes, Eugene, OR) to monitor alterations in cellular actin cytoskeleton induced by US28 and FAK. Fluorescence positive cells were visualized on an inverted Nikon fluorescent microscope.

Microarray analysis. PAT1 SMC expressing US28, CCR1, or CCR5 were stimulated with RANTES or Fractalkine (50ng/ml). Total RNA was extracted from $1x10^6$ SMC using the Trizol method (Life Technologies). Labeled RNA was analyzed using Affymetrix human U95A microarray chips. RNA samples from Trans expressing SMC stimulated independently with either RANTES or Fractalkine were used as the baseline controls for the appropriate comparisons.

TaqMan RT-PCR detection of Cellular Genes Upregulated by US28. Total RNA was extracted from 1x10^6 PAT1 SMC using the Trizol method (Life Technologies). cDNA was generated using the Omniscript RT kit (Qiagen) and analyzed by TaqMan PCR techniques using primer sets recognizing human FAK, Gravin, p160Rock, Arp2/3, and the γ-isomer of PP2A. The cellular mRNA levels normalized using primers directed against the ribosomal protein L32. Primers used were L32 Forward (5'-GGC ACC AGT CG CCG ATA TG); L32 Reverse (5'-CTT GAA TCT TCT ACG AAC CCT GTT G); FAK Forward (5'-GAT GTG AGA CTC TCT CGA GGC A), FAK Reverse (5'-GGA TCT GGT TTA CCC ACA GGC); Gravin Forward (5'-ACA GGG CCC GAC TGT CAG); Gravin Reverse (5'-TGG CAA GCA ACC TGC TCA), p160Rock Forward (5'-CCA AAA TCA CAA AGG CCA TGA); p160Rock Reverse (5'- TCT TCG ACA CTC TAG GGC AGG); RAB5 Forward (5'-ATG GCT AGT CGA GGC GCA); RAB5 Reverse (5'-GAT TTG CCA ACA GCG GAC TC) Arp2/3 Forward (5'-ACT GGA TAT TGC TGG GAG GGA), Arp2/3 Reverse (5'- TGC GAA CCG TTT CAA AAT CAG), γ-PP2A Forward (5'-CAG CCC ACA CTT CCA GGT G); and γ-PP2A Reverse (5'-GAA GGA AAC ATG ATG GGC AGA). RT-PCR reactions were set up using the TaqMan Universal PCR Master Mix (Applied Biosystems) according to manufacturer's specifications. Following thermal activation of AmpliTaq Gold (10 min. at 95°C), a total of 40 cycles were performed (15 sec. at 95°C and 1 min. at 58°C) using Prism 7700 TaqMan apparatus (Applied Biosystems). Plasmid clones containing the gene amplicons for each of the genes were used as positive controls and quantitation standards. TaqMan results were analyzed using ABI Prism 7700 Sequence Detector Software. The sensitivity of detection of this assay was <100 copies. The normalized FAK expression for US28-

expressing PAT1 cells treated with RANTES was compared to RANTES treated control cells using Student's *t* test. *P* values <0.05 were considered statistically significant.

3.6 Acknowledgements

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Chapter 4

Mouse Cytomegalovirus M33 is both Necessary and Sufficient in Virus-induced Vascular Smooth Muscle Cell Migration

Ryan M. Melnychuk, Patricia P. Smith, Craig N. Kreklywich, Franziska Ruchti, Jennifer Vomaske, Laurel Hall, Lambert Loh, Jay A. Nelson, Susan L. Orloff and Daniel N. Streblow

In the following chapter, I performed the experiments in Figures 4.1, 4.2a-c, 4.3, 4.4, 4.5a and 4.6. Patricia Smith conducted the SMC migration experiments shown in Figures 4.2d and 4.5b. Craig Kreklywich assisted with animal studies. Franziska Ruchti generated the MCMV-FlagM33GFP virus. Jennifer Vomaske and Laurel Hall assisted in cell culture and in optimization of siRNA conditions. Lambert Loh provided M44 (pp50) monoclonal antibodies.

4.1 Abstract

Mouse cytomegalovirus encodes two potential 7-transmembrane spanning proteins with homology to cellular chemokine receptors, M33 and M78. While these viral-encoded chemokine receptors are necessary for the *in vivo* pathogenesis of MCMV, the function of these proteins is unknown. Since vascular SMC migration is of critical importance for the development of atherosclerosis and other vascular diseases, the ability of M33 to promote SMC motility was assessed. Similar to HCMV, MCMV induced the migration of mouse AoSMCs, but not mouse fibroblasts. To demonstrate whether M33 was required for MCMV-induced SMC migration, we employed interfering RNA (RNAi) technology to specifically knockdown M33 expression in the context of viral infection. Knockdown of M33 resulted in the specific reduction of M33 protein expression and ablation of MCMV-mediated SMC migration, but failed to reduce viral growth in cultured cells. Adenovirus vector mediated expression of M33 was sufficient to promote SMC migration, which was enhanced in the presence of recombinant mRANTES. In addition, M33 promoted the activation of Rac1 and ERK-1/2 upon stimulation with mRANTES. These findings that mRANTES is a ligand for this chemokine receptor and that the activation of M33 occurs in a ligand-dependent manner. Thus, M33 is a functional homologue of US28 that is required for MCMV-induced vascular SMC migration.

4.2 Introduction

HCMV is a ubiquitous β-herpesvirus that establishes a life-long latent/persistent infection after primary infection. Although anti-viral therapy has significantly reduced HCMV related disease in individuals suffering from AIDS, HCMV infection is still a significant problem in congenital disease and transplant patients (Pass, 2001). HCMV infection has been associated with a number of vascular diseases including atherosclerosis, restenosis following angioplasty, chronic rejection associated with solid organ transplantation and more recently malignancies (Cobbs et al., 2002). However, the mechanisms involved in CMV-associated development of vascular disease are unknown (Melnick et al., 1998; Melnick et al., 1983a; Speir et al., 1994).

The most convincing evidence demonstrating that herpesvirus infections exacerbate vascular disease is exemplified in animal models. Marek's Disease Virus (MDV), a herpesvirus that infects fowl, was the first etiologic agent found to induce atherosclerosis (Fabricant et al., 1983; Fabricant et al., 1978). MDV-infected chickens develop atherosclerotic lesions with comparable histological features to human vascular disease, which includes the finding of MDV antigens in early vascular lesions, and late in SMC at the periphery of the plaque. The advent of mouse models of atherosclerosis has dramatically improved the ability to study the effects of CMV infection on vascular lesion development. While wild type mice appear to be resistant to the development of atherosclerosis, ApoE-/- mice are prone to develop disease when fed a high fat diet (Nakashima et al., 1994). MCMV infection of ApoE-/- mice accelerates the development of atherosclerosis by increasing the frequency of lesion formation and the severity of the atherosclerotic plaques (Berencsi et al., 1998; Burnett et al., 2001; Hsich et al., 2001;

Vliegen et al., 2002). Crossing of ApoE-/- mice with other genetically altered mice have been employed to study the roles of host proteins in lesion formation. For example, MCP-1 and the receptor for this chemokine, CCR2, are important regulators of monocyte infiltration involved in the formation of atherosclerotic plaques (Boring et al., 1998b; Gu et al., 1998). In a rat heart transplantation model, RCMV-induced acceleration of chronic rejection is associated with increased infiltration of immune cells and enhanced chemokine expression (Streblow et al., 2003a). These and other similar findings suggest an important role for CMVs, chemokines and chemokine receptors in the development of vascular disease.

All β-herpesviruses encode proteins with homology to chemokines and/or chemokine receptors. For example, HCMV encodes four putative chemokine receptors: UL33, US27, US28, and UL78, with US28 being the most characterized (Chee et al., 1990c). US28 is necessary and sufficient to induce the ligand-dependent migration of vascular SMCs (Streblow et al., 1999), which involves the activation of the small G-protein RhoA (Chapter 2) and the PTKs FAK and Src (Streblow et al., 2003c). US28 was the first viral-GPCR shown to mediate cellular motility, which is cell-type specific and provides a molecular basis for the correlative evidence that link HCMV to the acceleration of vascular disease. RCMV and MCMV each encode two putative chemokine receptor homologues, R33 and R78, and M33 and M78, respectively. M33 is predicted to be a CC-chemokine receptor, while M78 is predicted to be a CXC-chemokine receptor and disruption of either M33 or M78 in MCMV results in reduced viral titers in salivary glands compared to infection with wild-type virus (Davis-Poynter et al., 1997; Oliveira

and Shenk, 2001). These findings and the conservation of viral-encoded chemokine receptors amongst β-herpesviruses suggest an important role for these molecules in the biology of these viruses.

Since vascular SMC migration is crucial for the development of atherosclerosis, and an animal model is unavailable for the study of HCMV, the ability of MCMV to induce the migration of vascular SMC was determined. In this report we demonstrate that although MCMV encodes two chemokine receptors (Rawlinson et al., 1996), the putative CC-chemokine receptor M33 is both necessary and sufficient in MCMV-induced SMC migration. While M33 has been shown to constitutively signal through PLC-β and NF-κB (Waldhoer et al., 2002), we report that recombinant mRANTES enhanced M33-induced SMC migration and triggered the activation of the small G-protein Rac1 as well as ERK-1/2, demonstrating for the first time that mRANTES is a ligand of M33. Therefore, both MCMV and HCMV encode chemokine receptors that share a similar ability to induce the ligand-dependent migration of SMC.

4.3 Results

4.3.1 The MCMV-encoded chemokine receptor M33 mediates mouse SMC migration.

We have previously demonstrated that the HCMV-encoded chemokine receptor US28 is capable of inducing SMC migration, thus providing a molecular link between HCMV and the acceleration of vascular disease (Streblow et al., 1999). Since SMC migration is a fundamental process in the development of vascular diseases, we determined whether MCMV infection of SMC resulted in cellular migration similar to SMC motility induced by HCMV (Streblow et al., 1999). To accomplish this, AoSMC were isolated from p53-/- mice and to confirm their phenotype, cells were stained with antibodies directed against α-SMC actin (Figure 4.1A). Although the isolated SMC stained α-SMC actin, staining for the endothelial cell marker von Willibrand's Factor yielded negative results, indicating that the SMC population was not contaminated with endothelial cells. Subsequently, AoSMC were cultured in transwell dishes for migration assays, and infection with MCMV induced their migration (Figure 4.1D). MCMV encodes two chemokine receptors M33 and M78, and M33 is similar to the HCMV-encoded chemokine receptor US28 (Rawlinson et al., 1996). Like US28, M33 is predicted to bind CC-chemokines. Thus, an adenovirus vector expressing a C'terminal flag-tagged version of M33 (Ad-M33Flag) was generated to determine whether M33 was responsible for MCMV-induced AoSMC migration. When expressed in mouse SMC, M33 localized to intracellular vesicles similar to US28 (Figure 4.1B) (Streblow et al., 1999; Streblow et al., 2003c). Ad-M33Flag expression in mouse SMC resulted in cellular migration comparable to MCMV infected cells (Figure 4.1C). These results indicate that M33 is sufficient for SMC migration and that this chemokine receptor is a functional homologue

of US28 (Figure 4.1D). The induction of SMC migration by M33 was independent of an exogenous source of chemokine ligands, suggesting that this chemokine receptor is either constitutively active or that SMC produce ligands for M33. Previous experimental evidence supports the latter hypothesis, as *in vitro* cultured human SMC constitutively produce the US28 ligand MCP-1 which is capable of promoting migration in the absence of exogenously added chemokines. Addition of neutralizing MCP-1 antibodies abrogates US28-mediated SMC migration, and the migration phenotype can be rescued upon addition of RANTES (Streblow et al., 1999). Similarly, a consistent dose-dependent increase in the migration of M33 expressing mouse AoSMC was observed with the addition of recombinant mRANTES to the lower chamber, indicating that M33 responds to ligand stimulation to promote cellular motility.

As an alternative approach to demonstrate that M33 is the only MCMV gene involved in promoting SMC migration, we used silencing inhibitory RNA (siRNA) technology to block expression of M33 in the context of full virus replication. Two different siRNA molecules were designed against the MCMV-M33 sequence and designated as M33-345 and M33-387 based on their relative position in the M33 gene. In order to optimize siRNA transfection procedures, we also obtained a fluorescent (FITC) tagged control oligo to the cellular protein Lamin A/C, which was efficiently transfected into >90% of the cells.

Use of inhibtors, including siRNA, can have deleterious effects on viral gene expression and replication. To test whether siRNA transfection negatively impacted MCMV infection, cells were transfected with either the Lamin A/C control siRNA or one of the

two siRNAs directed against M33, and then infected with MCMV-GFP at a moi of 1. As assessed by fluorescence, the siRNA molecules did not affect the ability of MCMV to infect mouse fibroblasts. These findings were confirmed by determining MCMV replication in the presence of each of the siRNAs using multi-step growth analysis (Figure 4.2). MCMV replicated to equivalent levels in mouse fibroblasts treated with any of the siRNA reagents compared to mock-transfected cells, thus providing a means of specifically knocking-down genes during viral infection without affecting *in vitro* MCMV replication.

Currently, antibodies directed against M33 are not available. Thus, in order to monitor M33 protein expression during MCMV infection, a recombinant version of the Smith strain of MCMV containing a Flag-tagged version of M33 and expressing GFP under control of the constitutive EF-1α promoter was constructed (MCMV-M33FlagGFP). MCMV-M33FlagGFP displays replication characteristics that parallel MCMV-GFP. To determine the kinetics of M33 protein expression, NIH-3T3s were infected with MCMV-M33FlagGFP at a moi of 1. Cells were harvested at the indicated times and expression of IE1, the early protein M44 (pp50) and M33 (Flag) was assessed by western blot analysis (Figure 4.3). As expected, robust IE1 expression commenced by 4 hpi and was sustained for the duration of the experiment. The DNA processivity factor M44 and M33 were expressed with similar early kinetics (detectable by 12 hpi).

The efficacy of siRNA as a means of reducing protein expression varies depending on the target gene, cell type, and individual siRNAs. To determine whether siRNA directed against M33 would block protein expression, mouse fibroblasts were transfected with

varying concentrations (0, 5, 10 or 25 nm) of Lamin A/C, M33-345 or M33-387 siRNAs. Cells were then infected with MCMV-M33FlagGFP for 24 hours. Western blotting for Flag-M33 protein expression was quantitated and normalized to the protein levels of M44 (pp50, MCMV early protein). In accordance with our findings that MCMV replication was not affected by siRNA transfection, the levels of pp50 in the siRNA treated samples was the same as those detected in mock-transfected and MCMV infected cells. Interestingly, the two oligos specific for M33 differed in their ability to block M33 protein expression. The M33-387 oligo was more effective in blocking M33 protein expression than was the M33-345 oligo, with the former reducing M33 protein expression levels to 40% versus 100% of the Lamin control (Figure 4.4A). To confirm our findings that M33 was responsible for SMC migration induced by MCMV, siRNA molecules were tested in SMC migration assays. AoSMC were transfected in triplicate with either control Lamin-A/C, M33-345, or M33-387 siRNAs as described above. Cells were then infected with MCMV-M33FlagGFP, and 16 hpi, the cells were divided and transferred into 12-well transwell dishes. Treatment with the M33-387, but not the M33-345 or Lamin A/C control siRNA abrogated MCMV-M33 mediated SMC migration (Figure 4.4B). The effects of M33-387 on MCMV-M33 mediated SMC migration were consistent with our findings that this oligo effectively blocked M33 protein expression. Therefore, M33 mediates SMC migration and is a US28 functional homologue in MCMV.

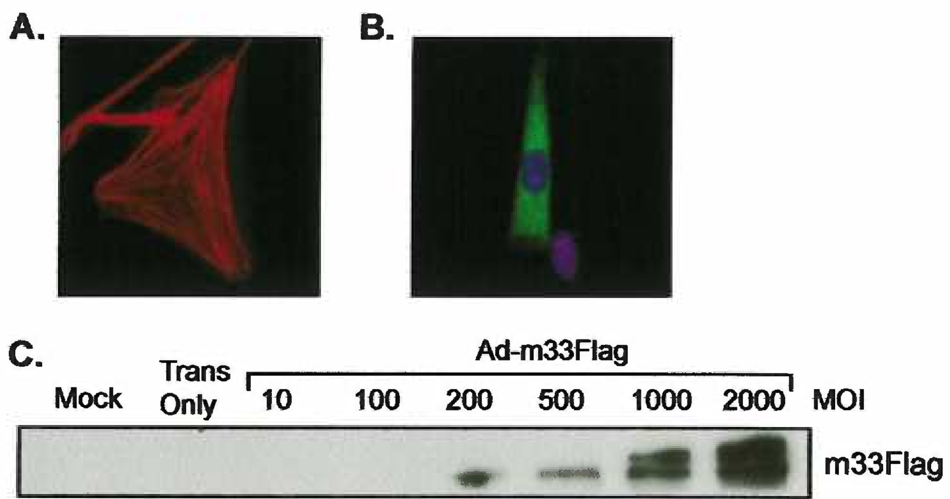
Figure 4.1. MCMV-M33 induces vascular smooth muscle cell migration.

(A) Mouse AoSMC stained for α-actin (red).

(B) Expression of Ad-M33Flag (green) in mouse SMC, nuclei were stained with Hoescht stain (blue).

(C) AoSMC were infected with various concentrations of Ad-M33Flag. Cell lysates were probed by western blotting for M33Flag.

(D) SMC migration assay; mouse aortic SMC or NIH3T3 cells were plated onto transwells and infected with either MCMV or Ad-M33Flag. Ad-M33Flag infected cells were treated with increasing concentrations of RANTES in the lower well. Migrating cells were enumerated by microscopy 48-72 hpi.


A.
B.
C.
Ad-m33Flag
Mock
Trans Only
10
100
200
500
1000
2000
MOI
m33Flag

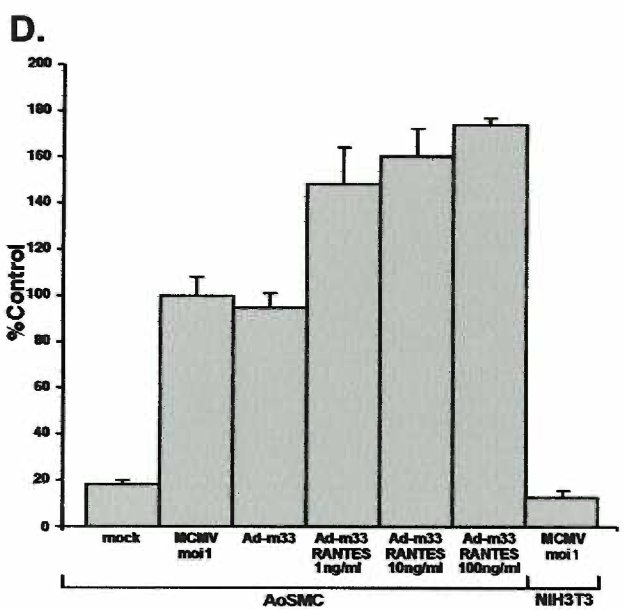

D.
%Control
mock
MCMV moi1
Ad-m33
Ad-m33 RANTES 1ng/ml
Ad-m33 RANTES 10ng/ml
Ad-m33 RANTES 100ng/ml
MCMV moi1
AoSMC
NIH3T3

Figure 4.2. siRNA transfection does not interfere with MCMV replication.

MCMV multi-step growth curves were performed on mouse fibroblasts transfected with M33-345, M33-387, or lamin A/C siRNA and then infected with MCMV-GFP at 24 hours post-transfection. Cell supernatants were analyzed by plaque assays for the presence of infectious MCMV.

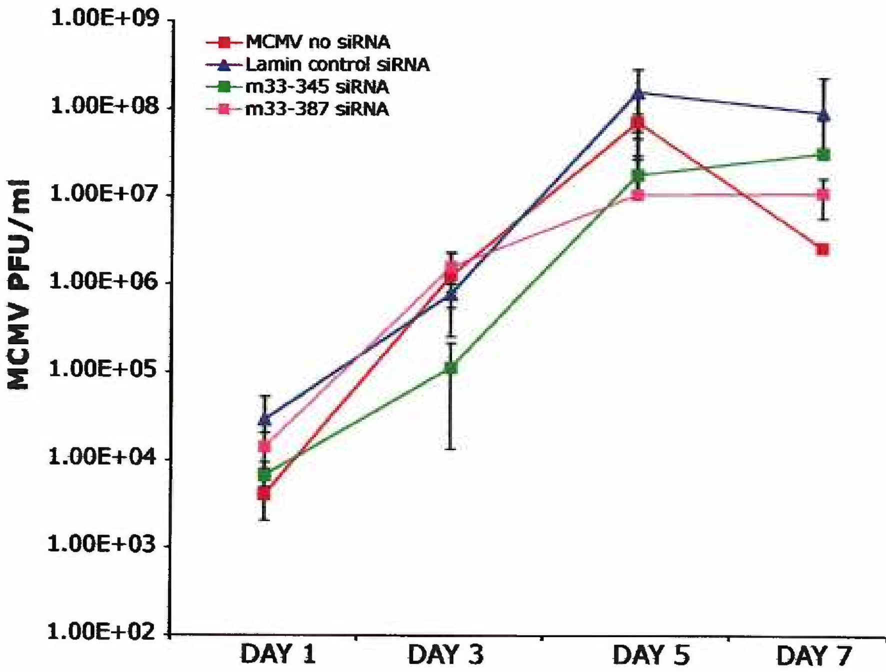

MCMV no siRNA
Lamin control siRNA
m33-345 siRNA
m33-387 siRNA
MCMV PFU/ml
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
DAY 1
DAY 3
DAY 5
DAY 7

Figure 4.3. The kinetics of M33 protein expression by infection with MCMV-GFPM33Flag.

Mouse fibroblasts were infected with MCMV or MCMV-GFPM33Flag at moi=1. Protein expression in MCMV infected cell lysates was determined by western blotting for M33Flag, immediate early 1 protein (IE-1), and the early protein pp50 (M44).

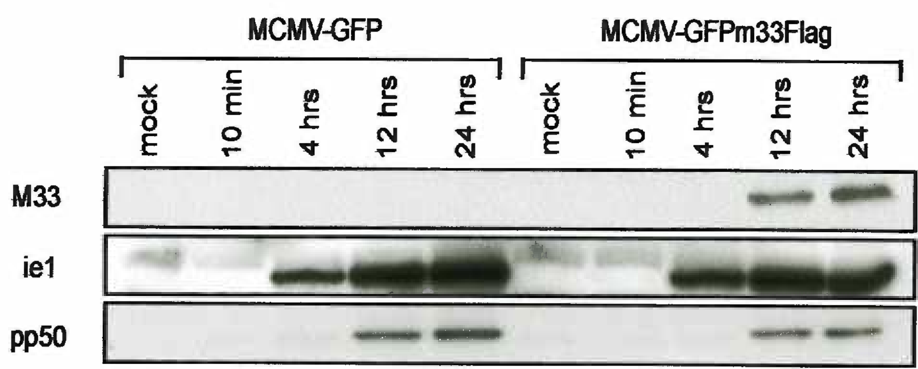
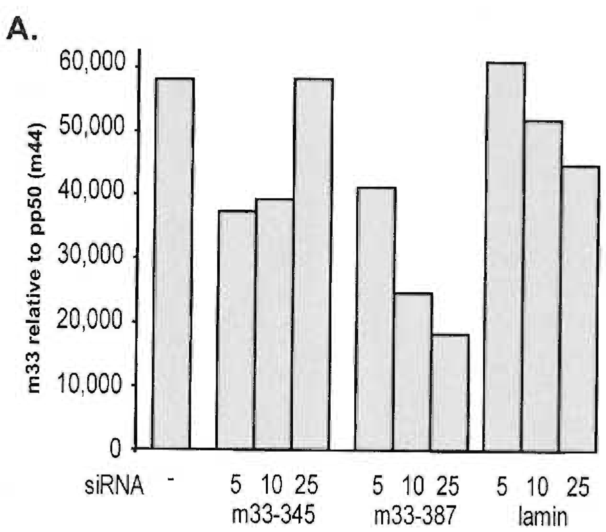

MCMV-GFP
MCMV-GFPm33Flag
mock
10 min
4 hrs
12 hrs
24 hrs
mock
10 min
4 hrs
12 hrs
24 hrs
M33
ie1
pp50

Figure 4.4. M33-specific siRNA blocks protein production and vascular SMC migration.

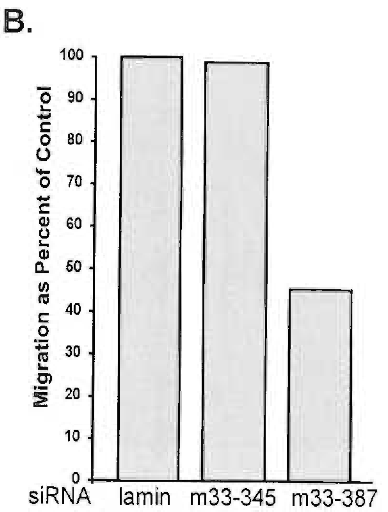
(A) Inhibition of M33 protein synthesis in MCMV-GFPM33Flag infected mouse fibroblasts transfected with 5, 10, or 25ng siRNA (lamina, M33-345, or M33-387). M33 protein (y-axis) was measured relative to pp50.

(B) MCMV infected AoSMC were treated with siRNA (lamina, M33-345, or M33-387) and subjected to migration assays.

A.



B.

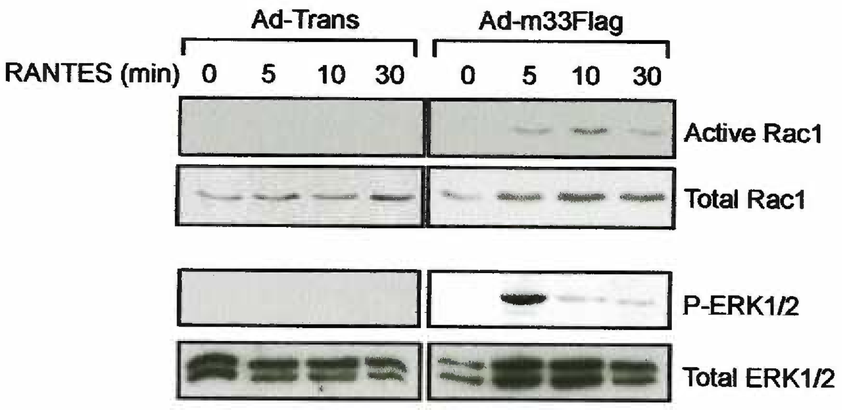


4.3.2 M33 Activation of Rac1 and ERK1/2

Although we have demonstrated a function for M33, the signaling pathways activated by M33 and the ligands that bind this viral GPCR are unknown. We have recently demonstrated that US28 signaling through RhoA is critical for the ability of US28 to induce SMC migration (Chapter 2). Rac1, an additional member of the Rho-like family of small G-proteins, is a key mediator of cellular migration (Gismondi et al., 2003). To assess the ability of M33 to activate Rac1, active Rac pull down assays were performed using Glutathione-linked Sepharose beads bound with GST-fused to the Rac/CDC42 binding domain of Pak1 (GST-CRIB). This fusion protein only binds Rac1 in the active GTP-bound state (Knaus et al., 1998; Manser et al., 1994). Serum-starved rat AoSMC expressing M33 and/or the tet-transactivator were stimulated with 10ng/ml of recombinant mRANTES for 0 (unstimulated), 5, 10, or 30 minutes. GST-CRIB was administered to pre-cleared cell lysates and active Rac1 associated with GST-CRIB was determined by western blotting for Rac1. To ensure that equivalent amounts of cellular proteins were used in each assay, pre-cleared lysates were analyzed by western blotting for the presence of Rac1 prior to the addition of GST-CRIB. SMC expressing M33 and treated with recombinant mRANTES demonstrated a kinetic activation of Rac1, with peak activation occurring at 10 minutes post-ligand stimulation (Figure 4.5). Similarly, addition of mRANTES to M33 expressing cells but not control AoSMC induced the phosphorylation/activation of ERK-1/2, as determined by western blotting for ERK-1/2 using phospho-specific antibodies. Therefore, similar to US28, M33-mediates the ligand-dependent activation ERK-1/2, as well as small G-proteins that are known to have important roles in cellular migration.

Figure 4.5. RANTES stimulation of M33 activates Rac1.

AoSMC expressing M33 and/or Trans were stimulated with 10ng/ml recombinant mRANTES. Lysates were baited with GST-CRIB for active Rac1 pull-down assays. M33-induced Rac1 activation was determined by western blotting for Rac1. ERK-1/2 phosphorylation/activation was determined by western blotting using phospho-specific antibodies. Total input protein was detected by western blotting for Rac1 and ERK-1/2.


Ad-Trans
Ad-m33Flag
RANTES (min) 0 5 10 30 0 5 10 30
Active Rac1
Total Rac1
P-ERK1/2
Total ERK1/2

4.4 Discussion

In this report we demonstrate that similar to our findings with HCMV and the HCMV-encoded chemokine receptor US28, MCMV infection of vascular SMC induces their migration (Streblow et al., 1999). We used siRNA to knockdown M33 protein expression, which had no affect on *in vitro* viral growth kinetics, representing a viable mechanism for specific gene knockdown during viral replication. While treatment with M33-specific siRNA blocked AoSMC migration in MCMV-infected cells, adenovirus mediated expression of M33 was sufficient to induce migration to levels similar to MCMV infection. Addition of mRANTES to M33 expressing SMC enhanced migration and stimulated the activation of the small G-protein Rac1. To our knowledge, this is the first demonstration of a ligand for M33 and that this viral-GPCR signals in a ligand-dependent manner. Together these findings suggest that M33 is a functional homologue of US28 and that M33 is required for MCMV-induced SMC migration.

Atherosclerosis is a complicated disease process that involves many different cells types including macrophages, endothelial cells, and SMC. CMV is capable of infecting and altering the cellular physiology of each of these cells types. For instance, one of the first observations supporting a role for infectious agents in atherosclerosis was the finding that arterial vessels from MDV-infected chickens demonstrated increased lipid deposition (Fabricant et al., 1981; Hajjar et al., 1985). The accumulation of lipids is thought to be both an initiator and accelerator of atherosclerotic plaque formation. CMV is also capable of altering lipid metabolism in infected SMC and macrophages by increasing expression of scavenger receptors, thus enhancing lipid deposition in these cells (Zhou et al., 1996a). CMV infection also increases endothelial expression of chemokines and

adhesion molecules (VCAM-1 and ICAM-1) enhancing transendothelial migration of inflammatory cells (Steinhoff et al., 1995; Summersgill et al., 2000).

SMC migration from the vessel media into the neointimal space is a hallmark of vascular lesion formation, suggesting that pathogen-mediated acceleration of vascular disease involves enhanced accumulation of SMC in the lesion. A reduction in apoptosis caused by HCMV infection of SMC, could lead to accumulation of these cells at sites of vascular injury. CMV infection of HeLa cells inhibits TNF-α induced apoptosis (Zhu H et al., 1995). An explanation for this block in apoptosis is the finding that the HCMV IE1-72 binds and inactivates the tumor suppressor gene p53 (Speir et al., 1994). Another mechanism of cellular accumulation occurs through SMC proliferation at the site of vascular injury. CMV infection of endothelial cells induces the release of growth factors and cytokines including fibroblast growth factor (FGF) and platelet derived growth factor (PDGF-BB), which are potent stimuli of SMC proliferation (Srivastava et al., 1999). Furthermore, HCMV infection up-regulates expression of the CC-chemokine RANTES in SMC and fibroblasts (Michelson et al., 1997; Streblow et al., 1999). We have previously demonstrated that infection of human SMC with HCMV induces their migration, which is dependent upon expression of the viral-encoded chemokine receptor US28, and binding of the CC-chemokines RANTES or MCP-1 (Streblow et al., 1999). Similarly, in the current report, we have shown that MCMV-M33 induces mouse SMC migration. We hypothesize that HCMV infection preferentially enhances SMC migration towards sites of vascular injury due to expression of viral-encoded chemokine receptors. The resultant SMC accumulation in the vessel intima leads to neointimal hyperplasia and vessel narrowing. siRNA-mediated ablation of M33 protein expression during MCMV

infection confirmed the requirement of this chemokine receptor in MCMV-induced SMC migration.

4.4.1 β-Chemokine Receptors in β-herpesviruses

While others have demonstrated that M33 can signal independent of exogenous ligands (Waldhoer et al., 2002), we demonstrate that mRANTES binding to M33 enhances SMC migration and activates Rac1 and ERK-1/2. These findings indicate that RANTES is a potent ligand for M33. The ligands for HCMV UL33, the M33 positional homologue, are unknown; however, deletion of US27 and US28 is required to prevent RANTES binding in HCMV-infected fibroblasts, and HCMV-induced PLC-β signaling, suggesting that UL33 does not bind RANTES (Bodaghi et al., 1998). Interestingly, the requirement for a β-chemokine receptor is a common feature of all β-herpesviruses, as HHV-6 and HHV-7 also encode β-chemokine receptors (Isegawa et al., 1998; Nakano et al., 2003). In addition, a recent finding indicates that HCMV also encodes a soluble RANTES binding protein (Bresnahan and Shenk, 2000). The transcript for this gene is packaged in the virion, and the protein is expressed immediately following infection.

What is the function of M33 in MCMV pathogenesis? While we have demonstrated a role for M33 in the induction of vascular SMC migration, this finding does not preclude M33 from having other functions. Deletion of either MCMV M33 or RCMV R33 have deleterious effects on virus replication in salivary glands, suggesting that both are important for viral persistence and dissemination in the host (Beisser et al., 1998; Davis-Poynter et al., 1997). Similarly, deletion of the MHV-68 vGPCR does not affect acute phase viral replication but prevents reactivation from latency (Lee et al., 2003). Whether

viral chemokine receptors are utilized as sensors to monitor the host inflammatory response to ensure that the extracellular environment is amenable for replication is unclear. In support of this hypothesis, HCMV reactivates in times of immune stress when chemokine ligands are induced, promoting monocyte differentiation into HCMV-infection competent macrophages.

SMC migration induced by CMV-encoded chemokine receptors has important implications for several inflammatory vascular diseases including restenosis, TVS and atherosclerosis, which involve endothelial cell damage and inflammatory cell infiltration, followed by SMC accumulation, ultimately resulting in stenosis of the vessel. Although HCMV has been linked to these vascular diseases, the pathogenic features of these disease processes are complex and multifactorial. The accumulation of SMC in the intima is hypothesized to involve both migratory and proliferative events. Our studies suggest a novel mechanism for the accumulation of SMC in vascular lesions, whereby viral-encoded chemokine receptors might induce SMC migration to sites of atherogenesis. Further investigation of the signaling pathways involved in CMV-induced SMC migration is warranted in order to develop strategies to prevent and treat CMV-associated vascular diseases.

4.5 Materials/Methods

Cells and Viruses. NIH3T3 fibroblasts, primary rat aortic SMC (rat AoSMC) isolated from rat aortas (Chapter 2), and mouse AoSMC similarly isolated from aortas of p53-/- mice were cultured DMEM supplemented with 10% FCS and PSG (Chapter 2). NIH3T3s were used to prepare and titer stocks of the Smith strain MCMV (ATCC), and MCMV-M33FlagGFP. MCMV-M33FlagGFP was constructed using the λ-derived linear recombination system in combination with the pSM3fr MCMV BAC in the E.coli strain DY380 (provided by Dr. U. Koszinowski) (Wagner et al., 1999). MCMV-M33FlagGFP contains a GFP expression cassette under control of the EF1-α promoter, which was cloned into the IE2 promoter region and a C-terminal Flag epitope was cloned in-frame with M33. NIH3T3 cells were used to prepare and titer MCMV stocks.

M33 Adenovirus Construction. The adenovirus vector expressing M33 containing a C'terminal Flag-epitope tag was constructed as previously described (Hsia et al., 2003; Streblow et al., 1999). Briefly, M33 containing a C'terminal Flag-epitope tag was constructed by subcloning the cDNA fragment into pAdTet7 (Hsia et al., 2003), which contains the tet-responsive enhancer within a minimal CMV promoter followed by the SV40 late poly(A) cassette. Recombinant adenoviruses were produced by co-transfection of pAdTet7-M33C-flag and Ad5-ψ5 DNA into 293-Cre cells (Hsia et al., 2003). Recombinant adenoviruses were expanded on 293-Cre cells and titered by limiting dilution. Expression of M33 was driven by co-infection with Ad-Trans expressing the Tet-off transactivator as previously described (Streblow et al., 1999).

siRNA. The M33-specific siRNAs (M33-345-365, AACCGCAUCUAUCGCAGCUCG and M33-387-409, AACCUGUACUUUGCGAACCUG) and laminA/C-specific siRNA (AACUGGACUUCCAGAAGAACA) were obtained ready to use from Dharmacon (Lafayette, CO) as option A4 (5'-deprotected, annealed and desalted). Selected sequences were searched using BLAST to ensure that the siRNAs were specific for their target sequence.

The methods of siRNA transfection were the same, whether used in assays for monitoring virus replication, protein expression, or SMC migration. Briefly, mouse AoSMCs were cultured on 12-well dishes (or T75 flasks for SMC migration assays). Prior to transfection, the cells were washed twice with Opti-MEM. Various concentrations (1-50nm) of siRNA m33 345, m33-387, or lamin A/C along with 2μl of oligofectamine (GibcoBRL) were diluted in Opti-MEM (200μl). The solution was incubated for 20 minutes, and then added to the cells for 4 hours at which time they were supplemented with 10% FCS-DMEM. Sixteen hours post-transfection, cells were infected with MCMV-M33FlagGFP at a moi=1. To assess the effects of siRNA on MCMV entry, MCMV-M33FlagGFP-infected, GFP-positive cells were visualized using a Nikon TE300 microscope (20X) and enumerated by counting multiple fields. Multi-step growth curves were performed to determine the effects of siRNA on virus replication. Culture supernatants were harvested daily and limiting dilution plaque assays were used to quantitate infectious virus in each sample. For protein expression experiments, the cells were lysed 24 hpi in 2x Laemmli's sample buffer and analyzed by western blotting as described below. For SMC migration experiments, siRNA transfected SMC were

transferred to transwells and infected with MCMV at moi=1. Migration assays were then performed as described below.

SMC Migration Assays. Cellular migration assays were performed as previously described (Chapter 2; Streblow et al., 1999; Streblow et al., 2003c). Briefly, $1x10^5$ cells were added to the upper well of a transwell (3.0μm pore size, Costar Corning, Cambridge, MA). Cells were serum starved and infected with MCMV (moi=1). After infection inserts were washed and transferred to new 12-well tissue culture plates. Cells migrating to the lower chamber were counted at 48-72 hpi using a Nikon TE300 microscope at magnification 10X. Experiments were done in at least triplicate wells and ten random fields were read from each well.

Immunofluorescence Microscopy. For immunofluorescent analysis, samples were washed with PBS and fixed with 2% PFA in PBS. For intracellular staining, the samples were permeablized and blocked in intracellular staining buffer (ISB: 1g BSA, sodium azide, 0.5% Triton-X100, and 500ml PBS) with 10% normal goat serum (NGS) for 20 minutes. The primary antibody (diluted in ISB) was incubated for 2 hrs. Anti-Flag antibodies (M2, Sigma) were used to visualize M33-Flag tagged proteins. Samples were washed with ISB and incubated with fluorescein isothiocyanate (FITC) or L-rhodamine conjugated secondary anti-mouse or anti-rabbit antibodies (BioSource International, Camarillo, CA), diluted in ISB for 1hr. Samples were washed with ISB, mounted and visualized using a Nikon TE300 microscope. Mouse AoSMC were stained with either anti-αSMC actin (1:250) or M2 anti-Flag monoclonal antibody (1:500) diluted according

to manufacturer's instructions. SMC were then incubated with secondary fluorescence conjugated antibodies and photomicrographs were obtained at x60 magnification (Nikon TE300).

Western Blot Analysis. Western blotting of cellular lysates for viral and cellular proteins was accomplished as previously described (Chapter 2; Streblow et al., 2003c). Briefly, mock infected or MCMV-M33FlagGFP-infected NIH3T3s ($5x10^5$) were lysed in 2X Laemmli's sample buffer (Laemmli, 1970). Samples were analyzed by 10% SDS-PAGE and proteins transferred to Immobilon-P membranes (Millipore). The blots were blocked with 3% milk in TBS-T buffer (100mM Tris-Cl, pH 7.5, 150mM NaCl, and 0.1% Tween 20) for 1 hr. The M2 anti-Flag antibody (diluted in 1:2,000 in TBS-Tween 20) was used for primary detection of M33-Flag proteins with a secondary anti-mouse conjugated to Horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). Anti-MCMV antibodies recognizing the MCMV immediate early protein 1 (IE-1) or the early protein pp50 (M44) were used to determine the extent of viral protein expression. Chemiluminescence and autoradiography were used for final protein detection on Kodak MR or Biomax Light Film.

Rac Activation Assay. The CDC42/Rac interactive binding domain (CRIB) of PAK1 (67-150aa) was expressed as a GST-fusion (GST-CRIB, Dr. J. Scott, OHSU) in the E.coli strain BL21 as previously described (Knaus et al., 1998; Manser et al., 1994). Upon obtaining an OD=0.6-0.8, bacteria were induced overnight with IPTG (1mM) and clarified supernatants were bound to Glutathione-linked 4B-CL Sepharose (Amersham).

The beads were washed in PBS followed by 3 washes in Rac wash buffer (50mM Tris pH 7.2, 1% Triton-X100, 150mM NaCl, and 10mM $MgCl_2$) with a final equilibration wash using Rac1 lysis buffer (50mM Tris pH 7.2, 1% Triton-X100, 500mM NaCl, 10mM $MgCl_2$, 0.5% sodium deoxycholate, and 0.1% SDS). For Rac activation assays rat AoSMC were cultured on 15cm dishes and upon achieving 75% confluence were serum starved for 18 hours prior to being infected with Ad-M33C'Flag and/or Ad-trans at moi=1000. The cells were stimulated with mRANTES (10ng/ml) at 16 hours post infection and harvested in Rac lysis buffer at 0 (unstimulated), 5, 10 or 30 minutes. Prior to administering GST-CRIB beads, a sample of each cell lysate was analyzed for total input Rac1 and for ERK-1/2 activation by western blotting for Rac1, phospho-ERK-1/2 (pERK-1/2, Thr202/Tyr204) and total ERK-1/2 (Cell Signaling Technologies). Cell lysates were incubated with GST-CRIB beads (80μl of a 1:1 slurry) for 45 minutes at 4°C, then washed 4 times with 1ml of Rac1 wash buffer. The final pellet was resuspended in 60μl of 2x Laemmli's sample buffer, run on 12% SDS-PAGE, transferred to Immobilon-P membranes and probed for Rac1.

4.6 Acknowledgements

We would like to thank Dr. John Scott from Oregon Health and Sciences University for the GST-CRIB construct. This work was supported by grants from the NIH to J. Nelson (HL65754 and HL71695) and S. Orloff from the Department of Veterans Affairs and NIH (HL 66238-01). D. Streblow was supported by an American Heart Association Scientist Development Award.

Chapter 5

Conclusions and Future Directions

5.1 Conclusions

The presence of chemokine receptors in all β-herpesviruses suggests an important role for these molecules in the pathogenesis of CMVs; however, ascribing a biological function to these receptors has been fraught with difficulties. While disruption of CMV-encoded chemokine receptors does not influence *in vitro* growth characteristics, there are gross deficiencies in the *in vivo* pathogenesis and dissemination of CMVs that lack chemokine receptors compared to wild type viruses, supporting the notion that these molecules have important roles in the biology of CMVs (Beisser et al., 1999; Beisser et al., 1998; Davis-Poynter et al., 1997; Kaptein et al., 2003; Oliveira and Shenk, 2001; Vieira et al., 1998). Expanding upon previous studies demonstrating that HCMV mediates the ligand-dependent migration of vascular SMC, and that the HCMV-encoded chemokine receptor US28 is both necessary and sufficient for promoting these migratory events, the studies described in this dissertation further elucidate the role of US28 in cellular migration, identifying the G-proteins that couple to US28 and critical cellular molecules required for US28-mediated cellular motility. Furthermore, we establish the MCMV-encoded chemokine receptor, M33, as a functional homologue of US28 that is necessary and sufficient for the induction of SMC migration. Through our studies of US28-induced cellular activation and motility, three palpable aspects of US28 signaling have emerged. First of all, in order to signal, US28 requires either endogenously produced ligand (from cultured cells) or a source of exogenous ligand, and is therefore ligand-dependent. Second, US28 signaling varies with respect to the cell-type assayed, displaying differences in cellular signaling that parallel the compliment of endogenously expressed G-proteins, and is therefore cell-type dependent. Finally, signaling events initiated by

US28 are ligand specific, with ligands belonging to different chemokine subfamilies displaying differential abilities to stimulate a number of cellular signaling pathways.

5.1.1 US28 signaling is ligand-dependent.

The most apparent feature of US28 functional activity and cellular signaling is the absolute requirement of ligand. In our original transwell migration assays, US28-induced vascular SMC migration did not require the addition of exogenous ligand since vascular SMC constitutively secrete the US28 ligand MCP-1. Addition of neutralizing MCP-1 antibodies was sufficient to abrogate US28-mediated SMC migration, and upon addition of RANTES, migration was restored (Streblow et al., 1999). Therefore US28-induced SMC migration is ligand-dependent, requiring either endogenously produced MCP-1 or exogenously added RANTES. Similarly, US28 stimulation with either MCP-1 or RANTES promoted the activation of the small G-protein RhoA which itself was required for US28-mediated SMC migration (Chapter 2). In addition to promoting an association between the non-receptor PTKs FAK and Src, RANTES stimulation of US28 expressing cells induced the activation of FAK and Src, and both Src and FAK activity was required for US28-induced SMC motility (Streblow et al., 2003c). Treatment with each of RANTES, MCP-1 or Fractalkine promotes the activation of FAK and formation of FAK-Grb2 complexes (Chapter 3). All three of these ligands also promote actin-cytoskeletal rearrangements in US28 expressing cells. Furthermore, RANTES, Fractalkine or VUF2274 stimulation of US28 resulted in pronounced ERK-1/2 activation (Chapter 3). In all of these assays, US28-induced cellular signaling activity was only observed in the

presence of ligand (exogenous or endogenous). Therefore, US28-induced cellular activation is ligand-dependent.

The ligand-dependent signaling activities that we observed contradict other published accounts of US28 signaling (Casarosa et al., 2001; Fraile-Ramos et al., 2001; Minisini et al., 2003; Mokros et al., 2002; Waldhoer et al., 2003). The majority of these studies suggest that US28 is constitutively active and capable of signaling in the absence of ligand, while contending that US28 acts as a scavenger receptor for chemokines. Unfortunately, the contention that US28 signals in a constitutive manner while functioning as a scavenger receptor is mutually exclusive. How can a surface receptor scavenge extracellular chemokines, yet continue to signal in a constitutive manner? To date, there has been a failure to provide an adequate explanation (let alone any explanation) as to how US28 can both signal in a constitutive manner and bind, and internalize chemokines that have already been demonstrated to potentiate robust cellular signaling activity (Billstrom et al., 1998). Furthermore, these studies have failed to address many critical questions, and before such matters are addressed, US28 cannot be labeled a bonafide constitutively active chemokine receptor. First of all, US28 has only been demonstrated to promote constitutive signaling activity in selected cell types such as COS, HeLa and NIH-3T3 cells, and no experiments have been conducted to address whether constitutive signaling activity is induced in cell types that are important in the biology of HCMV such as SMC, endothelial cells or macrophages. Secondly, there have not yet been any published reports addressing whether constitutive signaling activity is even important for HCMV growth and pathogenesis either *in vitro* or *in vivo*. Does

inhibition of constitutively signaling activity negatively or positively influence HCMV replication, gene expression or pathogenesis? Importantly, all of these studies have failed to demonstrate that the cells in which these experiments were conducted produce ligand(s) for US28.

As described, US28 is capable of binding a diversity of ligands from multiple chemokine subfamilies and *in vitro* cultured cells can produce US28 agonists. Thus, the failure to examine for endogenous ligand production may explain the observed constitutive signaling activities. Another viral-encoded constitutively active chemokine receptor, Orf74 of HHV-8, displays differential constitutive signaling activity in endothelial cells versus COS, HeLa and NIH-3T3 cells (Arvanitakis et al., 1997; Couty et al., 2001; Gershengorn et al., 1998). Interestingly, Orf74-induced constitutive signaling, including PLC-β activation and IP3 production is significantly reduced in endothelial cells compared to other cell types (Couty et al., 2001; Shepard et al., 2001). Since endothelial cells and not COS cells (monkey fibroblasts) are important in the biology of HHV-8, the *iin vitro* and *in vivo* significance of constitutive signaling activity is unclear. Furthermore, cultured endothelial cells constitutively produce of an ORF74 ligand, IL-8, which may account for constitutive signaling activity observed in the absence of exogenous ligand (Pati et al., 2001; Shepard et al., 2001). Alternatively, IL-8 is a potent endothelial cell agonist that can stimulate the production of numerous chemokines, some of which may bind to and activate Orf74. Therefore, an adequate characterization and exploration of both the ligand binding properties of US28 and ligand producing potential

of assayed cells is required prior to asserting that US28 is a constitutively active chemokine receptor.

5.1.2 US28 signaling is cell-type dependent

The second important feature revealed from our studies of US28 is the cell-type dependent nature of US28 signaling. All cell-types express varying compliments of cellular proteins, including differences in the profiles of endogenously expressed G-proteins. Variations in the expression of Gα-proteins can significantly alter the functional response and cellular signaling potential of chemokine receptors. As we have demonstrated PASMC and U373MG cells express similar compliments of Gα-proteins, where as NHDF and COS cells lack Gα12 and Gα13 protein expression (Chapter 2). While US28 promotes the migration of PASMC, and the activation of RhoA in both SMC and U373MG cells, ligand stimulation of US28 expressing NHDF or COS cells fails to stimulate cellular migration or induce the activation of RhoA. Importantly, reconstitution of NHDF with Gα12 restored the US28-induced RhoA pathway of activation. Therefore, introduction of a single protein (Gα12) has a significant impact on cellular signaling, demonstrating that not only does Gα12 have a role in US28-induced cellular migration and RhoA activation, but that US28 signaling is cell-type specific and dependent upon the compliment of endogenously expressed G-proteins. We have also demonstrated that US28 stimulates both Gα12-dependent and Gα-independent signaling events upon binding RANTES. As described above, RhoA activation and SMC migration induced by US28 are enhanced by Gα12. In contrast, FAK and ERK-1/2 activation in fibroblasts are independent of Gαi/o and Gα12 (Chapter 3). Thus,

alternative signaling events intiated by RANTES are cell-type dependent and influenced by the compliment of endogenously expressed G-proteins.

US28 also exhibits differences in the pathways of activation stimulated in other cell types. For example, US28 promotes the activation of PLC-β and NF-κB in COS cells, undergoes constitutive endocytosis and recycling in HeLa and COS cells, and US28 is constitutively phosphorylated in HEK293 cells (Casarosa et al., 2001; Fraile-Ramos et al., 2003; Mokros et al., 2002); yet US28 signaling activity is exclusively ligand modulated in SMC (human or rat AoSMC), U373MG and FAK-/- cells (Chapter 3&4). The aforementioned HHV-8-encoded chemokine receptor Orf74 also displays cell-type specific signaling characteristics. While this receptor promotes the constitutive activation of PLC-β, NF-κB, p38 and c-JunNH_2-kinases/stress activated protein kinases in COS, HeLa and NIH-3T3 fibroblasts (Arvanitakis et al., 1997; Bais et al., 1998; Guo et al., 1997), Orf74 does not demonstrate similar constitutive signaling activities in endothelial cells (Couty et al., 2001; Pati et al., 2001; Shepard et al., 2001). Rather, Orf74 induced endothelial cell signaling is more sensitive to stimulation with exogenous ligand. Interestingly, the only cell types in which viral-encoded chemokine receptors consistently exhibit constitutive signaling activity are COS, HeLa and NIH-3T3 cells. These findings suggest that COS, HeLa and NIH-3T3 cells are either highly sensitive to expression of foreign receptors/proteins and/or that they produce ligand(s) for viral-encoded chemokine receptors and are therefore not suitable cell types to assess chemokine receptor activity.

5.1.3 US28 Signaling is Ligand-Specific

The third characteristic derived from our studies on US28 is the ligand-specific nature of US28 signaling. In other words, different ligands display differential abilities to stimulate the activation of a multitude of signaling pathways. A previous study has revealed that both RANTES and MCP-3 promote Ca^{2+} flux and ERK-2 activation (Billstrom et al., 1998). Prior to our studies, Fractalkine had been exclusively employed as a means of reducing US28-induced constitutive signaling activity, including: reducing PLC-β and NF-κB activation (Casarosa et al., 2001) and modulating US28 endocytosis, recycling and phosphorylation (Fraile-Ramos et al., 2001; Mokros et al., 2002). We have detetmined that while RANTES and MCP-1 promote SMC migration, actin-cytoskeletal rearrangements, the activation of RhoA, FAK, Src and ERK-1/2, as well as the formation of active FAK-Grb2 complexes in US28 expressing cells, Fractalkine only stimulates actin cytoskeletal rearrangements, the activation of FAK and ERK-1/2, and the formation of active Grb2-FAK complexes (Chapter 2&3). Therefore US28 possesses the ability to bind and signal in response to both CC- and CX_3C-chemokines. Thus, not only is Fractalkine a modulator of US28 signaling, but also a potent US28 agonist. Importantly, US28 is the first chemokine receptor known to induce cellular activation upon binding chemokines from multiple chemokine subfamilies.

Of particular interest are the contrasting signaling activities induced by RANTES and Fractalkine. Specifically, US28 stimulation with RANTES but not Fractalkine results in SMC motility, and the activation of the small G-protein RhoA, which is required for US28-induced SMC migration (Chapter 2&3). Comparison of the transcriptional profiles

induced by RANTES and Fractalkine in SMC revealed substantial differences in US28 signaling at the level of gene induction. While RANTES promoted the up-regulation of numerous genes that have roles in actin cytoskeletal rearrangements, cellular motility and signal transduction, Fractalkine down-regulated many of these same genes, demonstrating that these two ligands have very different functional consequences with respect to US28-induced SMC activation and motility. Interestingly, Fractalkine binding to cellular chemokine receptors is known to promote leukocyte migration and signaling acitivty, and in many instances Fractalkine is a more potent agonist for cellular chemokine receptors than are other chemokines. Thus, our findings that Fractalkine failed to promote motility and down-regulated genes upregulated by a chemokine (RANTES) that promotes cellular migration further illustrates the unique nature and signaling potential of US28.

The capability of US28 to signal and elicit functional responses upon binding the CC-chemokines RANTES and MCP-1, but not the CX_3C-chemokine Fractalkine has important implications in the pathogenesis and dissemination of HCMV, as well as in the acceleration of vascular pathologies (Figure 5.1). HCMV persists in endothelial cells, SMC and macrophages, which are also the critical cell types in vascular disease. During times of immune stress, for example inflammatory events such as injury to the vascular endothelium, HCMV lytic cycle gene expression is initiated upon the approapriate stimuli being received by the persistently infected cell. Among the viral genes expressed are the chemokine receptor homologues. These same inflammatory events recruit immune cells such as macrophage/monocytes, B and T cells to the site of vascular injury. Of critical

importance in the development of vascular disease is the migration of SMC from the vessel media to the vessel intima, where they proliferate. Since HCMV has been detected in these cells *in vivo* and US28 mediates SMC migration in response to CC-chemokines, which are highly expressed during vascular lesion formation, this viral-encoded chemokine receptor may promote the migration of HCMV infected cells from the vessel media to the intima. Upon encountering cell-bound Fractalkine, US28-induced SMC migration is arrested, promoting the adherence of HCMV infected and US28 expressing SMC in the vessel intima, where multiple HCMV infection competent cells have accumulated. The infected SMC could then release progeny virus, infecting nearby cells such as macrophage/monocytes, promoting viral dissemination in the host. HCMV infection of macrophages and the accompanying up-regulation of scavenger receptors, lead to enhanced formation of foamy cells, contributing to vascular lesion formation. Ultimately, a fibrous plaque composed of SMC, foamy macrophages, and activated T cells embedded in a collagenous matrix of connective tissue is formed. The plaque protrudes into the lumen, obstructing the flow of blood through the vessel, eventually resulting in vessel occlusion. Therefore, the exacerbation of vascular diseases by HCMV may be a function of virus dissemination in the host and can be explained by the physiological effects of virus infection on the different cell-types involved in vascular lesion formation.

Figure 5.1 The role of US28 in HCMV-induced vascular disease and virus dissemination.

(A) Healthy vessel. Endothelial cells line the lumen of the vessel, and are surrounded by the intima, composed of fibroblasts and extracellular matrix, and the media, composed of SMC. An initial insult or injury to the vascular endothelium (which may be caused by viral pathogens) induces localized inflammatory events. HCMV infected SMC are shown in the vessel media.

(B) The injured endothelial cells secrete chemokines and cytokines, attracting immune cells (macrophage/monocytes, T cells, B cells and platelets) to the site of injury.

(C) During times of immune stress HCMV lytic gene expression initiates. The ensuing expression of US28 and other viral-encoded chemokine receptors on infected cells promotes the migration of HCMV infected SMC to sites of inflammation in response to MCP-1 and RANTES. Upon encountering membrane associated Fractalkine at the site of injury, US28-induced cellular motility is arrested and US28 expressing cells adhere and accumulate in the vessel initma. Subsequent infection of attracted macrophages may promote further exacerbation of the vascular lesion, by upregulating scavenger receptors. Alternatively, infection of macrophage/monocytes or other neighbouring cells can promote dissemination of virus in the infected host. Ultimately, a fibrous plaque that protrudes into the lumen of the vessel is formed.

Figure 5.2 Cellular pathways of activation induced by ligand binding to US28

The pathways of activation induced by US28 binding to RANTES, MCP-1, or Fractalkine. Cellular motility and RhoA activity are induced in response to RANTES and MCP-1. In contrast, FAK, ERK-1/2 activation, formation of FAK-Grb2 complexes and actin cytoskeletal rearrangements are induced by US28 in response to all three ligands. However, US28-mediated SMC migration through FAK is only observed in response to MCP-1 and RANTES.

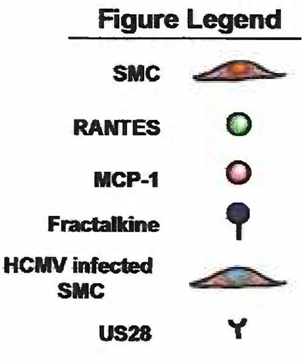
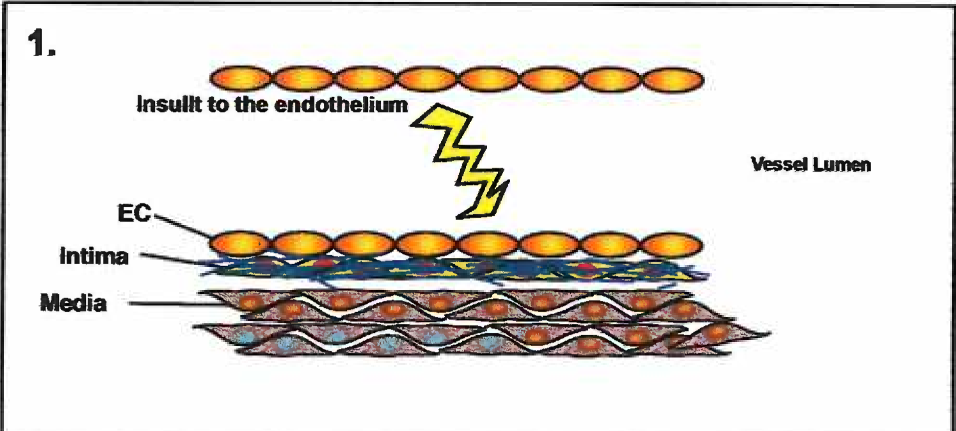
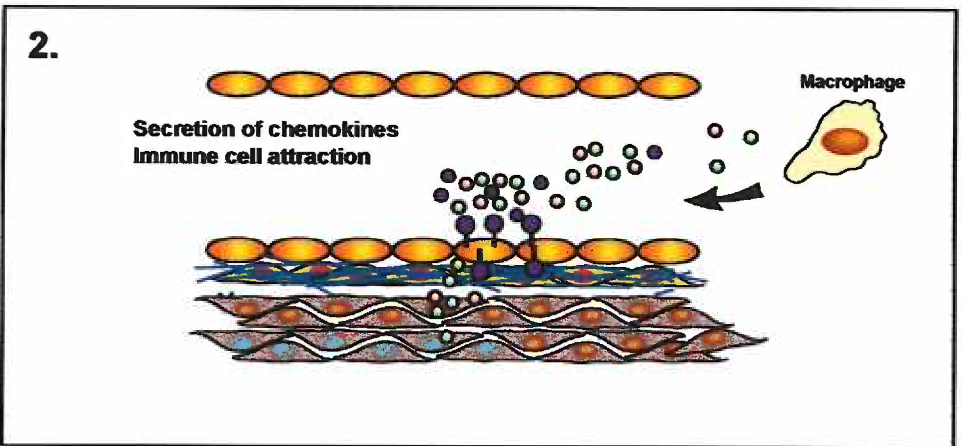
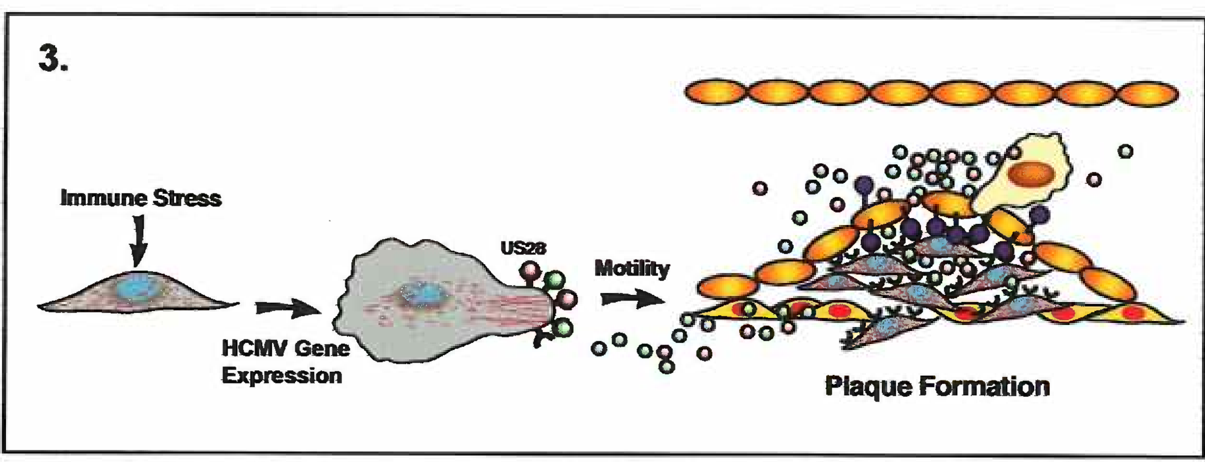


Figure Legend
SMC
RANTES
MCP-1
Fractalkine
HCMV infected SMC
US28

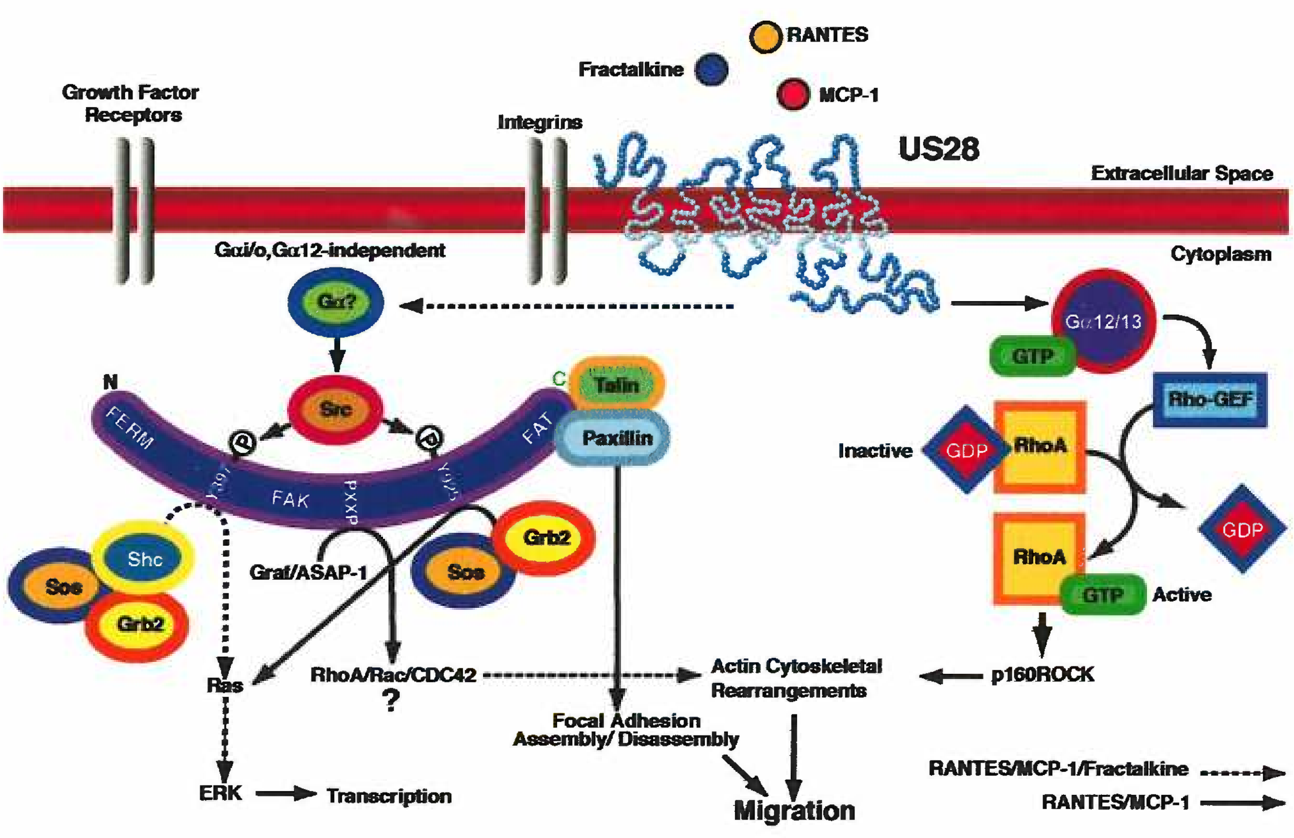

1.
Insult to the endothelium
Vessel Lumen
EC
Intima
Media


2.
Macrophage
Secretion of chemokines
Immune cell attraction


3.
Immune Stress
US28
Motility
HCMV Gene Expression
Plaque Formation

5.1.4 Summary of US28 signaling

US28-induced SMC migration and signaling is ligand-dependent, promoting the Gα12 mediated activation of RhoA and p160ROCK in response to MCP-1 and RANTES. Ultimately, this pathway of activation results in the induction SMC migration. Fractalkine, MCP-1 and RANTES all induce actin cytoskeletal rearrangements, the activation of FAK, ERK-1/2 and formation of FAK-Grb2 complexes; however only RANTES and MCP-1 potentiate cellular motility through FAK. These pathways of activation differ in a cell-type dependent manner, with cell-types that lack Gα12 and Gα13 unable to initiate the US28-induced RhoA activity or cellular motility. However, expression of Gα12 is sufficient to restore RhoA activity. Therefore, US28-induced functional activity and signaling are ligand-dependent, cell-type depedent and ligand-specific (Figure 5.2).



RANTES
Fractalkine
MCP-1
Growth Factor Receptors
Integrins
US28
Extracellular Space
Cytoplasm
Gαi/o,Gα12-independent
Gα?
Gα12/13
GTP
Rho-GEF
N
C
Talin
Src
Paxillin
FERM
FAT
FAK
PXXP
Inactive
GDP
RhoA
GDP
RhoA
GTP
Active
Shc
Sos
Grb2
Grb2
Sos
Graf/ASAP-1
Ras
RhoA/Rac/CDC42
?
Actin Cytoskeletal Rearrangements
p160ROCK
Focal Adhesion Assembly/ Disassembly
ERK
Transcription
Migration
RANTES/MCP-1/Fractalkine
RANTES/MCP-1

5.1.5 The MCMV-encoded chemokine receptor M33 is a functional homologue of US28

Unlike HCMV, all other characterized CMVs except RhCMV contain only two putative GPCRs, specifically the positional homologs of UL33 and UL78 in HCMV. Although US28 is absent from MCMV, this virus contains a functional homologue of US28, as is evidenced by the ability of MCMV to promote vascular SMC migration (Chapter 4). Since M78 is predicted to be a CXC-chemokine receptor, the most likely candidate to be a functional homologue of US28 is the putative CC-chemokine receptor M33. Adenovirus-mediated expression of M33 was sufficient to promote vascular SMC migration, and utilizing siRNA, disruption of M33 protein expression during the context of MCMV infection abrogated SMC migration, indicating that M33 was also necessary. Migratory events stimulated by M33 were enhanced upon addition of mRANTES. Furthermore, M33 promoted the activation of both Rac1 and ERK-1/2 upon stimulation with mRANTES, indicating that mRANTES is a ligand for M33. Thus, like US28, M33 promotes SMC migration and the activation of ERK-1/2 and the small G-protein Rac1, which like RhoA is a critical mediator of cellular motility. Therefore, M33 is a functional homologue of US28 in MCMV and is necessary and sufficient for the induction of SMC migration.

M33 has previously been demonstrated to be important for the *in vivo* growth and pathogenesis of MCMV (Davis-Poynter et al., 1997). Despite these findings, the *in vivo* role of M33 during MCMV infection has not been determined. A clue as to the function of M33 may come from the HHV-7-encoded chemokine receptor U12. While HHV-6

U12 induces Ca^{2+} flux in response to RANTES, MCP-1, MIP-1α and MIP-1β (Isegawa et al., 1998), U12 of HHV-7 has only been demonstrated to bind and induce Ca^{2+} flux in response to MIP-3β/ELC (Nakano et al., 2003). MIP-3β and secondary lymphoid tissue chemokine (SLC) are the only two ligands of CCR-7 and this cellular chemokine receptor was originally referred to as Epstein-Barr Virus (EBV)-induced gene-1 since EBV infection promoted the expression of CCR-7 in lymphocytes (Birkenbach et al., 1993). CCR7 is highly expressed in lymphoid tissues and the two CCR7 ligands, MIP-3β and SLC are constitutively expressed in secondary lympoid organs, regulating lymphocyte and dendritic cell homing and ultimately the development and maintenance of secondary lymphoid tissue architecture (Nagira et al., 1997). M33 is a positional and structural homologue of U12, and since the latter binds and internalizes MIP-3β, M33 deletion mutants that display reduced pathogenesis and defects in virus dissemination *in vivo* may be explained by the inability of virus to traffick within the host. Furthermore, CCR7 only binds MIP-3β and SLC, and these ligands are constitutively expressed in tissues in which CMVs have a tendency to accumulate. Therefore, utilizing MIP-3β to promote virus trafficking *in vivo* would be an ideal mechanism for virus dissemination; however only future experimentation will provide the molecular evidence to support this model.

5.2 Future Directions

Endothelial cells, SMC, and macrophages all support productive CMV infection and have *in vivo* roles in the biology of CMVs. While we have demonstrated a role for US28 in the ligand-dependent migration of SMC, the role of CMV-encoded chemokine receptors in both endothelial cells and macrophages has not been determined. We recently

generated C'-terminal HA-tagged US28 using BAC technologies in the endothelial cell and macrophage tropic clinical isolate TR. This virus along with adenoviruses that express US28 will permit the study of US28 in endothelial cells and macrophages including: the localization and sub-cellular distribution of US28 in both of these cell types; the ability of US28 to induce macrophage migration and/or transendothelial cell migration; and an assessment of cellular activation (as measured by RhoA, Rac1, FAK, Src, and ERK-1/2 activity) in response to ligand stimulation.

Studies in macrophages may be complicated by the plethora on endogenously expressed chemokines and chemokine receptors. These problem are less likely in endothelial cells, as endothelial cells generally express angiogenic ELR and angiostatic non-ELR containing CXC-chemokines that have not been reported to bind US28. Endothelial cells have unique organelles termed Weibel-Palade bodies (Weibel and Palade, 1964). These organelles function as intracellular storage compartments for chemokines, cytokines and adhesion molecules. Weibel-Palade body exocytosis is triggered by thrombin binding to G-protein coupled protease activated receptor (PAR) (de Leeuw et al., 2001), vasopressin binding to vasopressin type 2 receptor (Kaufmann et al., 2000) or β2-adrenergic receptor binding to epinephrine (Vischer and Wollheim, 1997). The release of the contents of Weibel Palade bodies, including cytokines and chemokines that induce immune cell chemotaxis; and P-selectin, which promotes the adhesion of the motile cells, has important implication in both inflammation and vascular disease. Interestingly, while the vasopressin and epinephrine stimulated pathways have not been determined, thrombin induced exocytosis through PAR involves Gαq/11 family G-proteins and the activation

of PLC-β. Subsequent intracellular IP3 accumulation is accompanied by a rise in cytoplasmic Ca^{2+}, and Ca^{2+} along with Calmodulin bind the small G-protein Ral, promoting RalGEF mediated activation of Ral. RLIP76, a down-stream effector of Ral, potentiates the activation of CDC42 and Rac, which may have roles in the actin cytoskeletal rearragements that precede Weibel-Palade body release. US28 has been demonstrated to activate many of the molecules involved in Weibel-Palade body exocytosis; however, the signaling potential of US28 has not been assessed in endothelial cells. Therefore, we will determine the role of US28 in these cells, including the ability of US28 to trigger Weibel Palade body release. These studies may provide new insights into HCMV pathogenesis and vascular disease progression.

MIP-3β and CCR-7 have critical roles in the formation and maintenance of secondary lympoid organs and this chemokine is responsible for the homing of inflammatory cells to these sites. The ligand binding profiles of M33 and UL33 have not been determined, and the only chemokine known to bind to either of these receptors is mRANTES by M33 (Chapter 4). Therefore, we will examine the ability of these two chemokine receptors to bind MIP-3β. Should either of these receptors bind MIP-3β, we will evaluate the signaling characteristics and functional activity induced by this ligand. Examination of the signaling characteristics, for example the activation of small G-proteins such as Rac1 and RhoA, and the migration potential of M33 and/or UL33 expressing cells in response to MIP-3β is important in elucidating the role of CMV-encoded chemokine receptors in viral pathogenesis and dissemination, and will permit subsequent *in vivo* studies utilizing the murine model.

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Appendix A

Additional Data

Figure A.1. Adenovirus mediated expression of Gα-proteins.

SMC were infected with adenoviruses expressing Gαi1, Gαq, Gα11, Gα12, Gα13 and/or Trans. G-protein expression in adenovirus infected was determined by western blotting for Gα-protein expression with antibodies specific for Gαi1, Gαq, Gα11, Gα12 and Gα13.

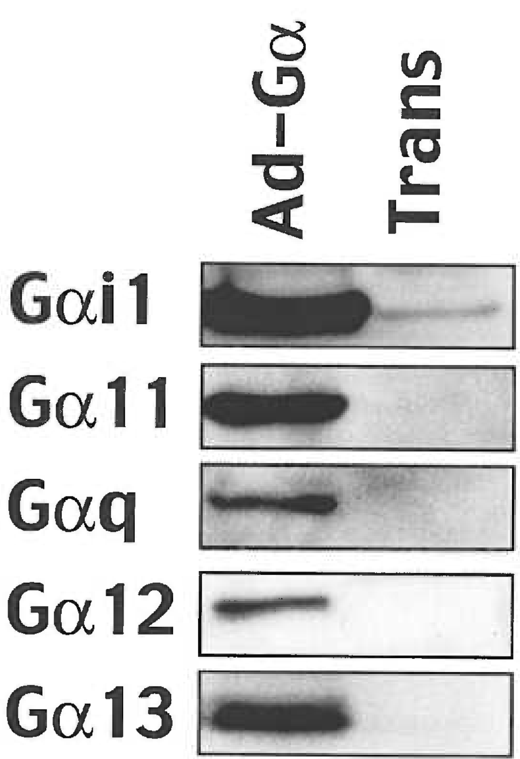

Ad–Gα
Trans
Gαi1
Gα11
Gαq
Gα12
Gα13

Figure A.2. US28 induces morphological changes in U373MG cells.

Serum straved U373MG cells were infected with adenoviruses expressing US28 and/or Trans. Sixteen hours post-infection morphological changes in US28 expressing (right panels) or Trans infected controls (left panels) were assessed by microscopic examination.

Trans **US28**

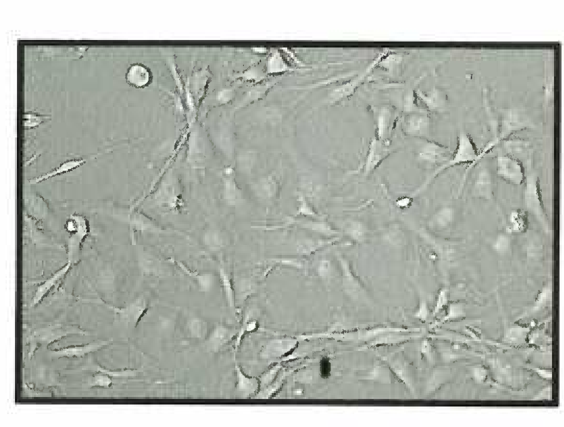


Figure A.3. GST-RhoTekin purification.

The RhoA binding domain of Tekin (Rhotekin) was expressed as a fusion protein with GST. Bacteria were lysed, and clarified supernatants were applied to glutathione sepharose beads. To assess GST-Rhotekin expression and purity, 50μl of GST control (lane 1) and GST-RhoTekin (lane 2) baited beads were analyzed by coomasie blue staining of an SDS-PAGE gel.

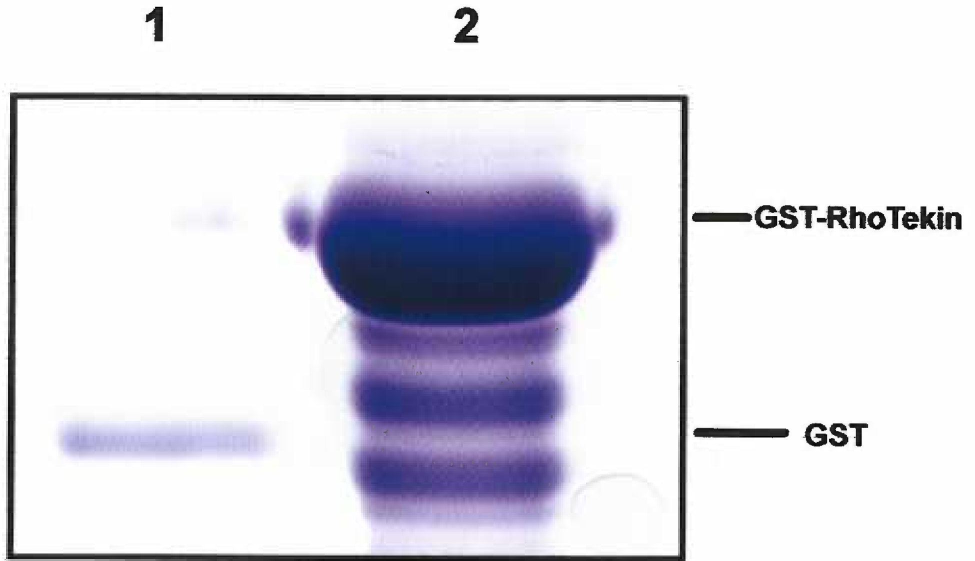

1
2
GST-RhoTekin
GST

Figure A.4. US28 induces RhoA activation in Balb/C-3T3 but not NIH-3T3 cells. Balb/C-3T3 and NIH-3T3 cells were serum starved for 18 hours and then infected with adenoviruses expressing US28 and Trans. Sixteen hpi cells were stimulated with RANTES (10ng/ml) for the indicated times. RhoA and Rac1 one pull down assays were then performed utilizing GST-RhoTekin and GST-CRIB. Active RhoA and Rac1 precipitated with GST-RhoTekin and GST-CRIB respectively was determined by western blotting for RhoA and Rac1. While US28 failed to induce RhoA in NIH-3T3 cells, activation was observed upon ligand stimulation in Balb/C-3T3 cells. In contrast, Rac1 was constitutively active in both cell types.

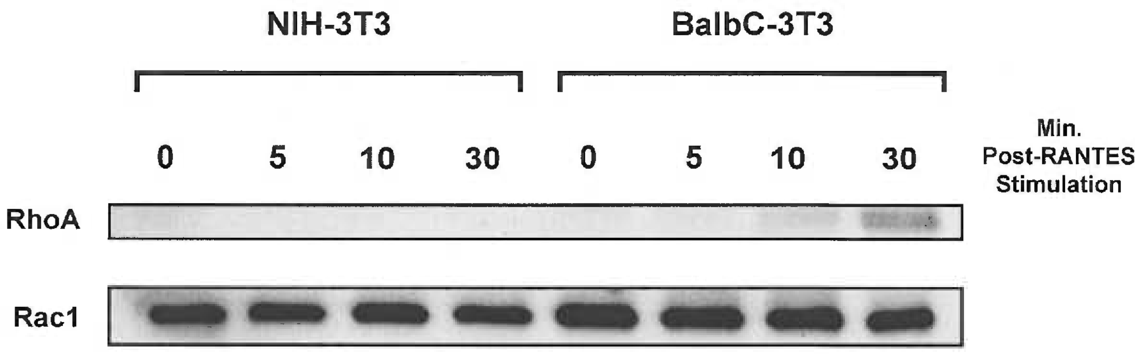
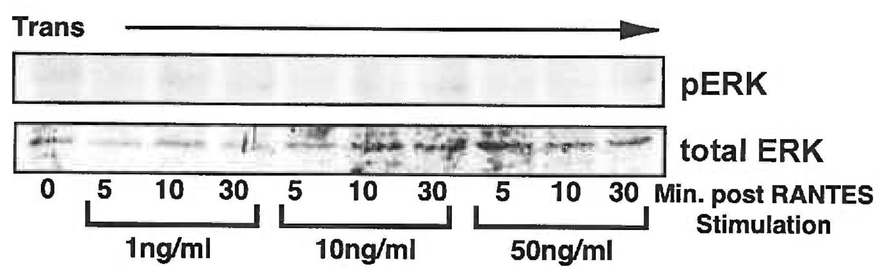

NIH-3T3
BalbC-3T3
0
5
10
30
0
5
10
30
Min.
Post-RANTES
Stimulation
RhoA
Rac1

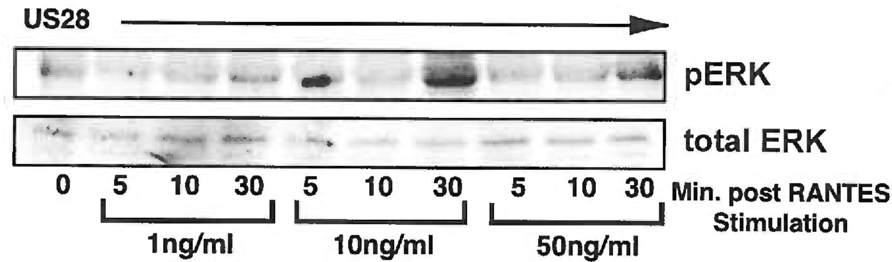
Figure A.5. US28-mediated activation of ERK-1/2 is ligand-dependent.

(A) To determine the ability of US28 to activate ERK-1/2, rat AoSMC were serum starved for 24 hrs. Serum starved cells were then infected with adenoviruses expressing US28 and/or Trans, and 18 hpi infected cells were stimulated with RANTES (1-50ng/ml) for the indicated times. ERK-1/2 activation was determined by probing western blots of cellular lysates with antibodies specific for phospho-ERK-1/2. Blots were then stripped and probed for total ERK-1/2. RANTES stimulation of US28 expressing rat AoSMC potentiated the activation of ERK-1/2.

(B) Fractalkine mediates ERK-1/2 activation in US28 expressing rat AoSMC. Rat AoSMC were serum starved and infected as in (A) and then stimulated with Fractalkine (1-10ng/ml) for the indicated times. Western blots probed for phospho- and total ERK-1/2 were then quantitated using NIH Image. Data is expressed as relative levels of pERK1/2 in relation to total ERK-1/2. Like RANTES, Fractalkine stimulated ERK-1/2 activity upon binding US28.

A.





B.

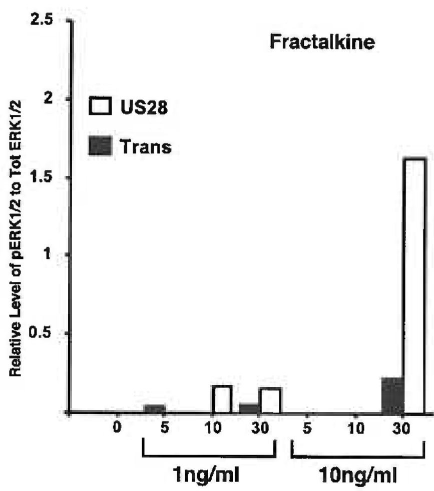


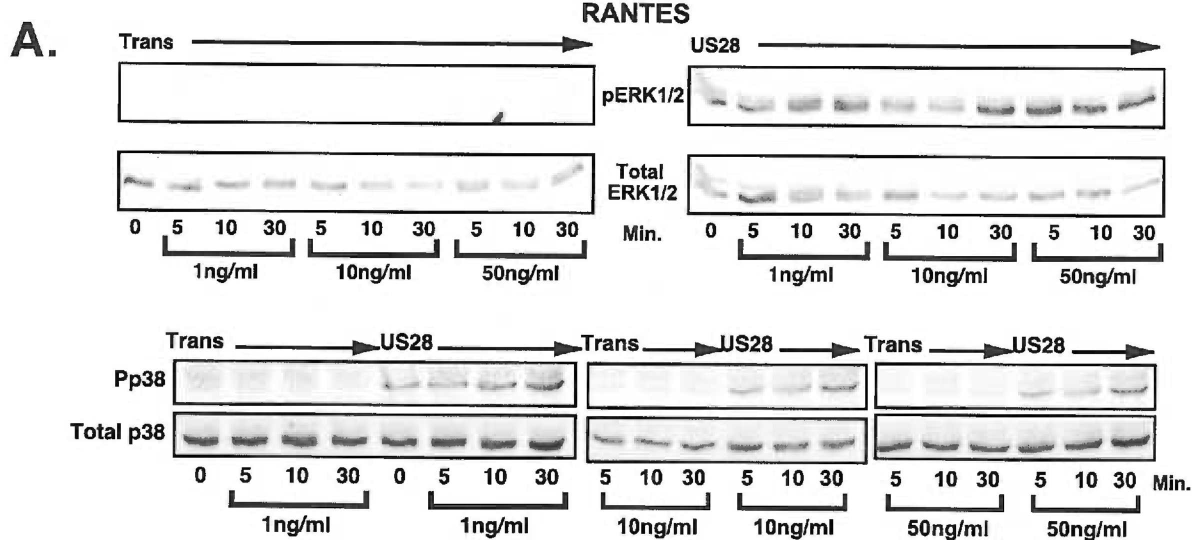
Figure A.6. US28-mediated activation of ERK-1/2 is cell type dependent.

(A) US28-mediated ERK-1/2 activation in response to RANTES was assessed in COS7 cells. COS7 cells were serum starved for 24 hours. Serum starved cells were then infected with adenoviruses expressing US28 and/Trans, and 18 hpi infected cells were stimulated with RANTES (1-50ng/ml) for the indicated times. ERK-1/2 activation was determined by probing western blots of cellular lysates with antibodies specific for phospho-ERK-1/2. Blots were then stripped and probed for total ERK-1/2. Interestingly, addition of ligand had little influence of US28-induced ERK activity in COS7 cells. Instead, ERK-1/2 activation corresponded with ERK-1/2 expression. Thus, either these cells produce ligand for US28 or are highly sensitive to expression of foreign proteins.

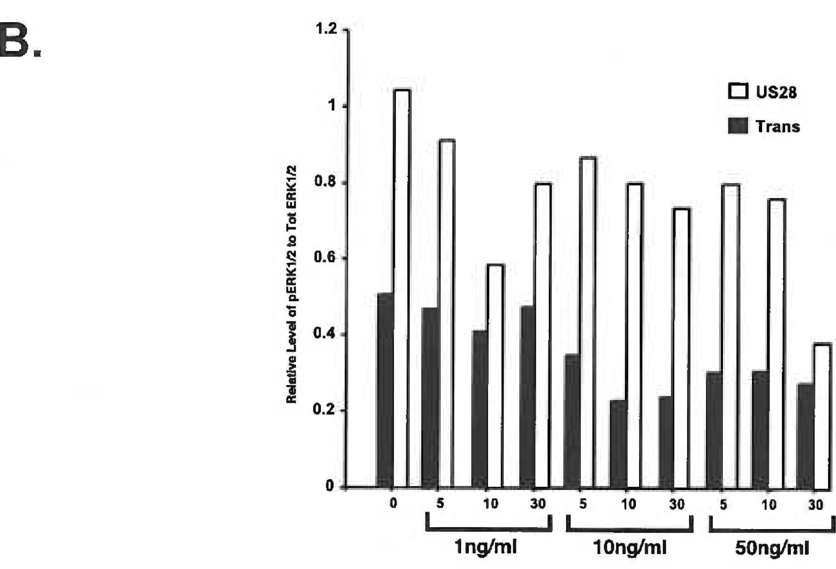
(B) Quantitation of phospho-ERK-1/2 in relation to total-ERK-1/2 as determined using NIH Image. Data is expressed as relative levels of pERK1/2 in relation to total ERK-1/2.

(C) ERK-1/2 activity induced by US28 in RANTES stimulated U373MG cells. US28-induced ERK-1/2 activity was determined in (A). RANTES binding to US28 induced ERK-1/2 activation in U373 cells; however, minimal ERK-1/2 phosphorylation was observed in the absence of ligand. Thus, ERK-1/2 activation is cell type dependent with RANTES stimulating ERK-1/2 activity in rat AoSMC (Figure A.6), ligand-independent ERK-1/2 activation in COS7 cells and an intermediate phenotype in U373MG cells.

A.



B.



C.

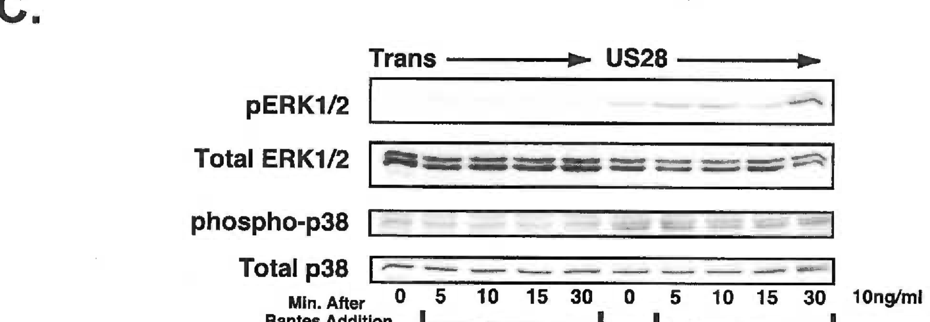
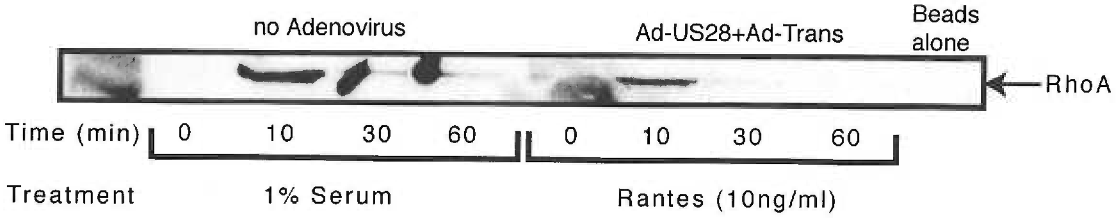


Figure A.7. Gα12 co-expression promotes RhoA activation through US28.

(A) RhoA activation induced by RANTES binding to US28 was compared to serum induced RhoA activation. U373MG cells were serum starved for 18 hours and then infected with adenoviruses expressing US28 and Trans (for RANTES stimulation experiments only) for 16 hours. Infected cells were stimulated with RANTES (10ng/ml) for the indicated times. For serum stimulation experiments, cells were serum starved for 18 hours and then stimulated with 1% serum for the indicated times. RhoA pull-down assays were performed and active RhoA associated with GST-RhoTekin was determined by probing western blots with antibodies specifc for RhoA. RANTES stimulation of US28 induced similar activation of RhoA as 1% serum.

(B) To determine if Gα12 enhances RhoA activity induced by US28, serum staved U373MG cells were infected with adenoviruses expressing Gα12, Gα12CA (consitutively active mutant), US28+Gα12, US28+Gα12CA and or Trans for 16 hours. Infected cells were stimulated with RANTES (10ng/ml) for the indicated times and RhoA activity was determined as in (A). Co-expression of US28 and Gα12 enhanced RhoA activity in response to RANTES.

A.



B.

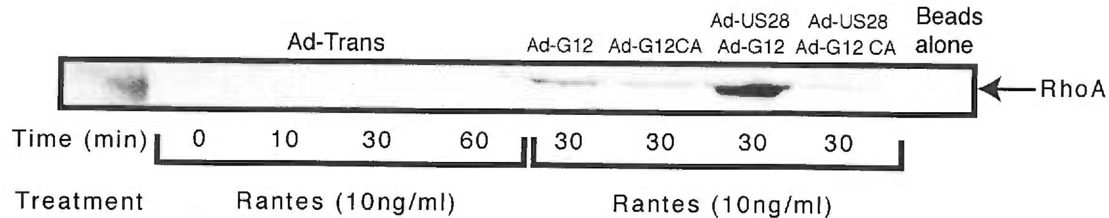
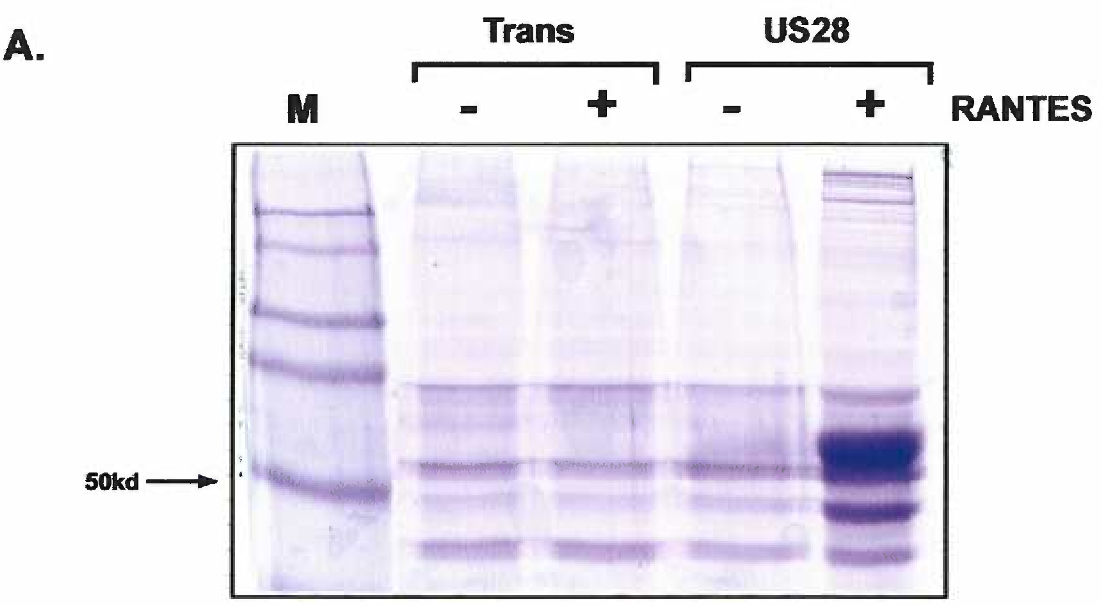


Figure A.8. Identification of 55-kilodalton protein interacting with RhoTekin and stimulated by US28.

RANTES stimulation of US28 consistently induced the co-precipitation of a 55kd protein that cross-reacted with polyclonal RhoA antisera. The appearance of this protein corresponded with US28-mediated activation of RhoA in terms of kinetics and intensity. Therefore, in collaboration with Pacific Northwest National Laboratories (PNNL) we isolated and identified this protein.

(A) Coomasie blue stained gel of RhoA activation assay. U373MG cells were serum starved for 18 hours and then infected with adenoviruses expressing US28 and/or Trans. Infected cells were left untreated or stimulated with RANTES (10ng/ml). RhoA pull-down assays were then performed and precipitating proteins visualized by SDS-PAGE and Coomassie blue staining. The 55kd band was excised and sent to PNNL for Mass-spec proteomic analysis. The protein was identified as the neuronal specific glial fibrillary acid protein GFAP). Interestingly, GFAP has an important role in re-organization of intermediate filaments and is a target of the Rho-associated kinase (Kosako et al., 1997; Matsuzawa et al., 1997). Thus, US28 induces the formation of RhoA signaling complexes in response to RANTES.

(B) Co-expression of Gα12 and US28 enhances GFAP association with RhoTekin. U373MG cells were serum starved, infected with adenoviruses and stimulated with RANTES (10ng/ml for 10minutes) as indicated. RhoA pull-down assays were performed and GFAP associated with GST-RhoTekin visualized by western blotting. Co-expression of Gα12 and US28 but not Gα12, Gαi or Gαi+US28 enhanced GFAP association with GST-RhoTekin upon RANTES stimulation.


A.
Trans
US28
M
-
+
-
+
RANTES
50kd

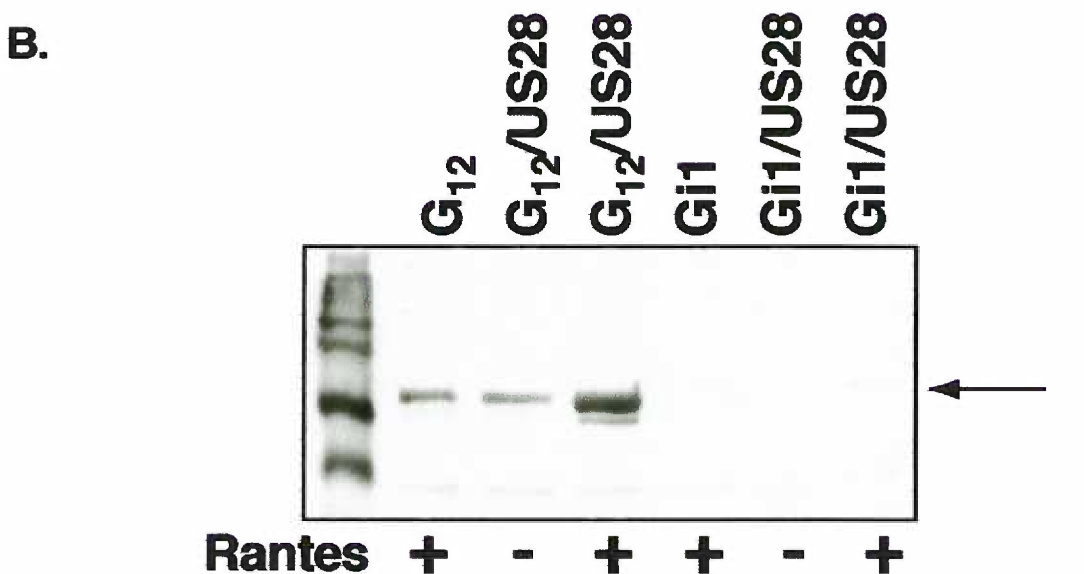

B.
G12
G12/US28
G12/US28
Gi1
Gi1/US28
Gi1/US28
Rantes
+
-
+
+
-
+

Figure A.9. Immunoprecipiation of US28 from human SMC.

Human SMC were serum starved for 16 hours and then infected with adenoviruses expressing C'-Flag-tagged US28 and/or Trans for an additional 16 hours. Infected cells were harvested in modified RIPA buffer, and cellular lysates pre-cleared with protein A beads prior to the addition of M2-flag antibodies (2 hours at 4°C). Protein A/G was added and the lysates incubated for an additional hour at 4°C. Immunoprecipitates were washed and then visualized by SDS-PAGE and silver staining.

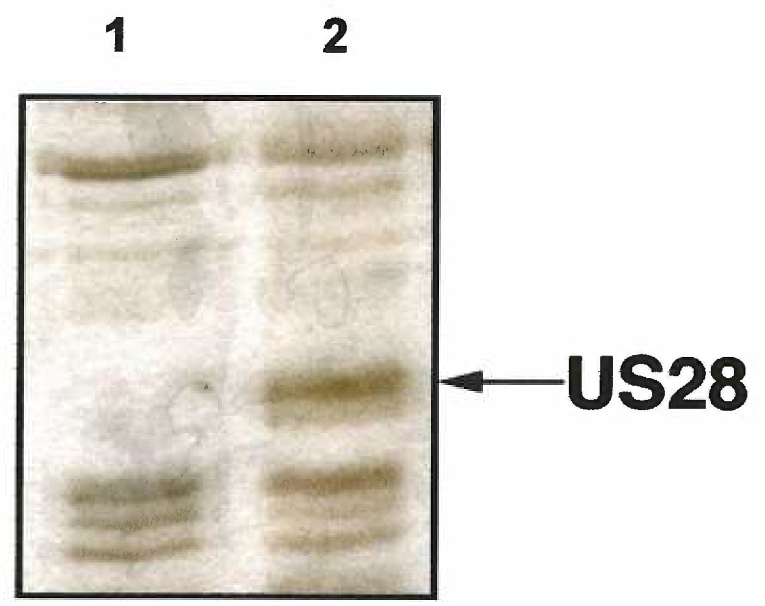

1
2
US28

Figure A.10. Construction of recombinant MCMV.

(A) Schematic of the construction of MCMV-GFP. The EGFP gene under the control of the elongation factor (EF)-α promoter was inserted into the ie2 promoter region (184236-184315) of MCMV.

(B) Insertion of the EGFP gene results in an increase in size of the MCMV BAC genome, which is observed as a shift in the size of the 'L' fragment from 7.2 to 8.7 kb upon HindIII digest of MCMV SM3fr.

(C) NIH3T3 cells were infected with MCMV EGFP and visualized by fluorescence microscopy at day 3 post-infection. The presence of fluorescent viral plaques demonstrates that the EFGP is constitutively expressed in MCMV EGFP infected cells.

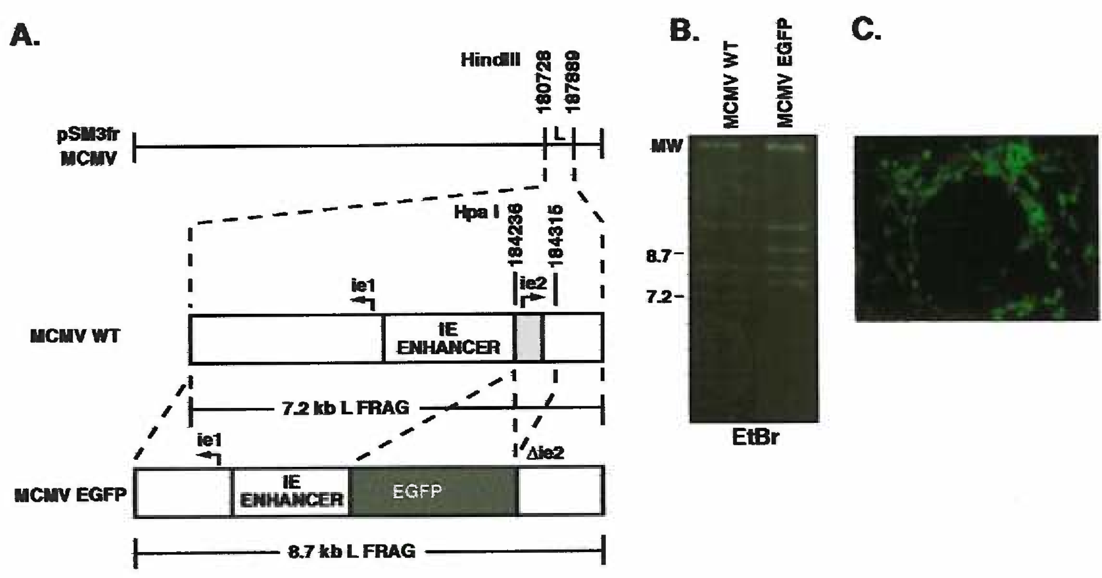
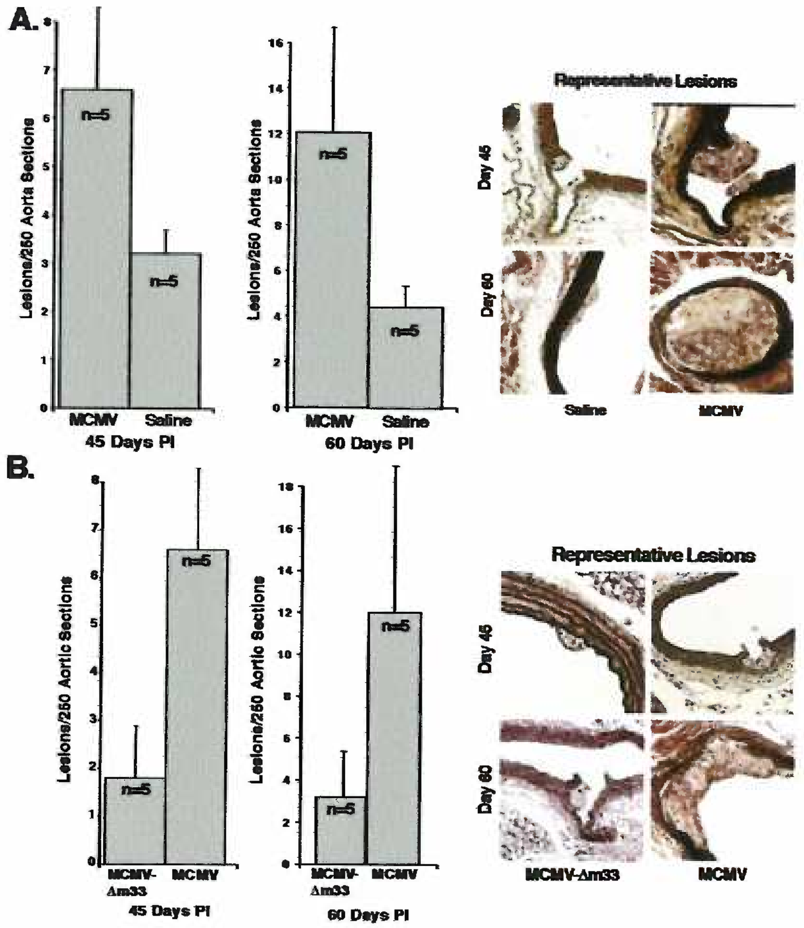

A.
HindIII
180728
187889
pSM3fr
MCMV
L
Hpa I
184236
184315
ie1
ie2
MCMV WT
IE
ENHANCER
7.2 kb L FRAG
ie1
Δie2
MCMV EGFP
IE
ENHANCER
EGFP
8.7 kb L FRAG
B.
MCMV WT
MCMV EGFP
MW
8.7
7.2
EtBr
C.

Figure A.11. MCMV but not MCMVΔM33 infection accelerates atherosclerosis in apoE-/- mice.

(A) At five weeks of age, 5 ApoE-/- mice were injected IP with saline or MCMV Smith strain ($5x10^5$ pfu). At 45 or 60 days pi, mice were perfused with saline followed by perfusion with 4% PFA. The aorta/iliac vessels were cut into five 1cm pieces and fixed in formalin. Paraffin embedded tissues were sectioned (10μm sections, 20μm apart) and stained for elastic fibers with iron hematoxylin stain. Average lesions per mouse aorta were determined by counting the atherosclerotic lesions from 250 aortic sections per mouse (n=5).

(B) MCMV and MCMVΔM33 infected mice at both 45 and 60 days pi.


A.
B.
Representative Lesions
Day 45
Day 60
Saline
MCMV
MCMV-Δm33
Lesions/250 Aorta Sections
Lesions/250 Aortic Sections
n=5
45 Days PI
60 Days PI

Appendix B

JNK-1/2 but not NF-κB or IKK α/β Activity is Essential for Efficient MCMV Replication and Protein Expression in Fibroblasts

Abstract

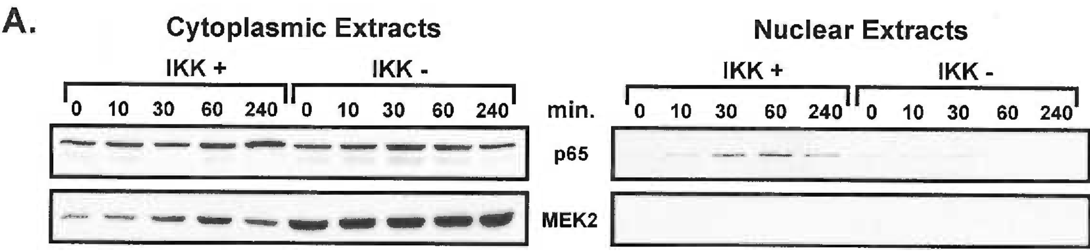
The cellular transcription factors NF-κB and AP-1 are considered to play important roles in the replication cycle of Cytomegaloviruses (CMV). Both of these cellular factors are induced upon CMV infection and several viral promoters contain binding elements that are activated by these proteins. The generation of specific knockout cells in which the induction or activity of transcription factors has been modified, have provided invaluable tools to dissect the importance of these regulatory proteins and the upstream kinases that activate them in MCMV growth and pathogenesis. We have used two knockout cell lines: inhibitor of NF-κB kinase (IKK)-α/β deficient (IKK-/-) and c-Jun NH_2-terminal kinase (JNK)-1/2 deficient (JNK-/-) fibroblasts to determine the influence of these up-stream kinases on the replication of murine CMV (MCMV). We observed that MCMV infection of JNK-/- but not IKK-/- fibroblasts resulted in a significant reduction in the production of progeny virus (1000 fold). Paralleling the lack of virus production, expression of all kinetic classes of MCMV gene products (IE, E and L) was abrogated in JNK-/- cells. Importantly, viral infection was not altered in these cells as viral-encoded GFP under the control of a constitutive cellular promoter was effectively expressed. MCMV synthesis and gene expression was also inhibited in wild-type mouse fibroblasts by a JNK-1/2 peptide inhibitor. Interestingly, stimulation of the PDGF receptor pathway in JNK-/- cells resulted in an increase in virus production that was similar to viral replication observed in untreated wild-type mouse fibroblasts. This increase in viral protein expression and growth was accompanied by the nuclear translocation of the JNK-1/2 substrate and AP-1 transcription factor c-Jun. These observations indicate that JNK-1/2 but not IKK or NF-κB activity is essential for MCMV replication and gene

expression in fibroblasts. These findings also suggest an important role for AP-1 transcription factors in MCMV gene expression and replication.

Figure B.1. MCMV induces NF-κB nuclear translocation and DNA binding in parental but not IκK α/β-/- fibroblasts.

(A) Nuclear and cytoplasmic extracts were prepared from uninfected (0) and MCMV-GFP (MOI=1) infected (10, 30, 60min or 4 hours) parental and IκK α/β-/- (IKK-/-) fibroblasts and examined for RelA (p65) nuclear translocation by western blotting. MCMV infection induces the nuclear translocation of p65 in parental but not IKK-/- fibroblasts.

(B) MCMV-GFP induced NF-κB DNA binding to γ-P^{32}-labeled mutant or consensus NF-κB oligonucleotides using nuclear extracts from uninfected (0) or MCMV-GFP infected (10, 30, 60 min, 4 hrs or 24 hrs) parental or IKK-/- fibroblasts. MCMV infection induces NF-κB DNA binding in parental but not IKK-/- fibroblasts.


A.
Cytoplasmic Extracts
IKK +
IKK -
0 10 30 60 240 0 10 30 60 240
min.
p65
MEK2
Nuclear Extracts
IKK +
IKK -
0 10 30 60 240 0 10 30 60 240

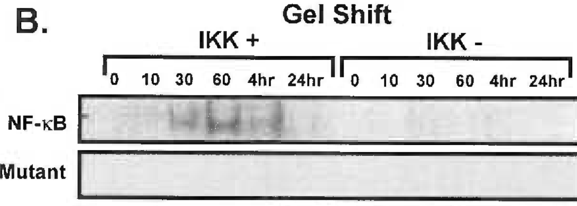
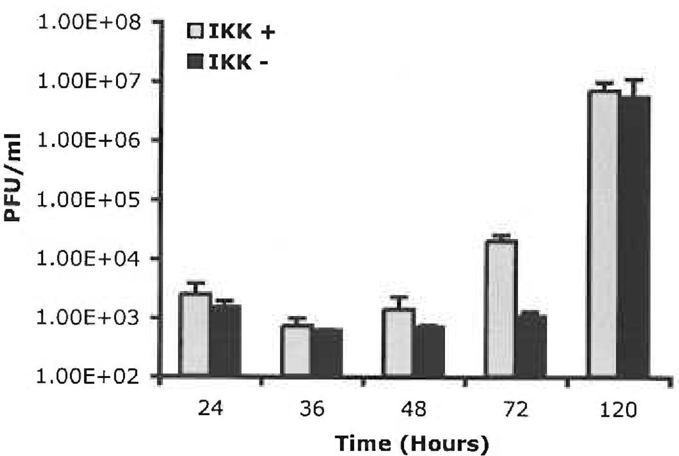

B.
Gel Shift
IKK +
IKK -
0 10 30 60 4hr 24hr 0 10 30 60 4hr 24hr
NF-κB
Mutant

Figure B.2. IκK α/β and NF−κB activity are not required for MCMV replication in fibroblasts.

Parental and IκK α/β-/- fibroblasts were infected with MCMV-GFP at a moi=0.1 (A) or 1 (B) and 24, 36, 48, 72 and 120 hours post-infection, cell supernatants were collected and titers (PFU/ml) determined by limiting dilution plaque assay on WT fibroblasts. MCMV replicated to equivalent levels in both parental and IKK-/- fibroblasts indicating that IKK activity is not required for MCMV replication in fibroblasts. Furthermore, since IKK-/- fibroblasts also lack NF-κB activity, MCMV does not require NF-κB activity for *in vitro* replication in fibroblasts.

A.



B.

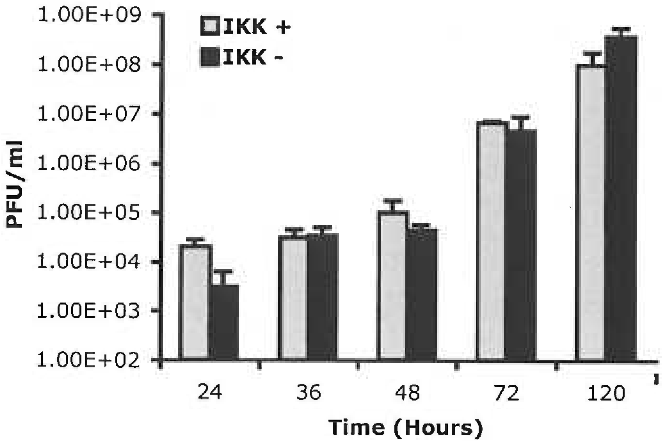


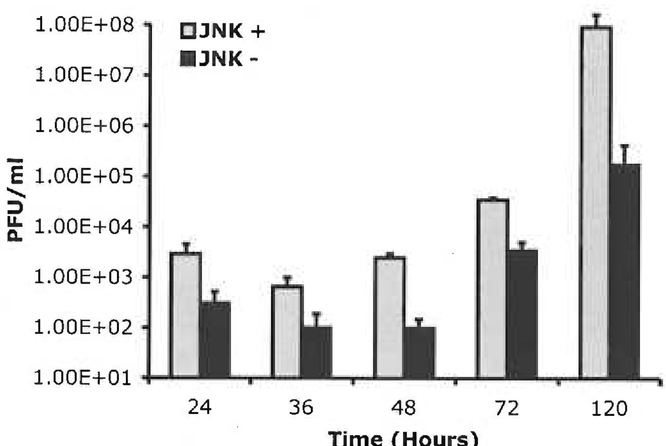
Figure B.3. JNK-1/2 activity is required for efficient MCMV replication in fibroblasts.

WT and JNK-1/2-/- fibroblasts were infected in triplicate with MCMV-GFP at moi=0.1 (A) and 1 (B) and 24, 36, 48, 72 and 120 hours post-infection, cell supernatants were collected and titers (PFU/ml) determined by limiting dilution on WT fibroblasts. MCMV replication was severely compromised in JNK-/- fibroblasts, demonstrating decreases of 3-3.5 logs compared to parental cells. At higher moi, MCMV replication was reduced 1-1.5 logs at 120 hpi in JNK-/- fibroblasts compared to WT/parental cells.

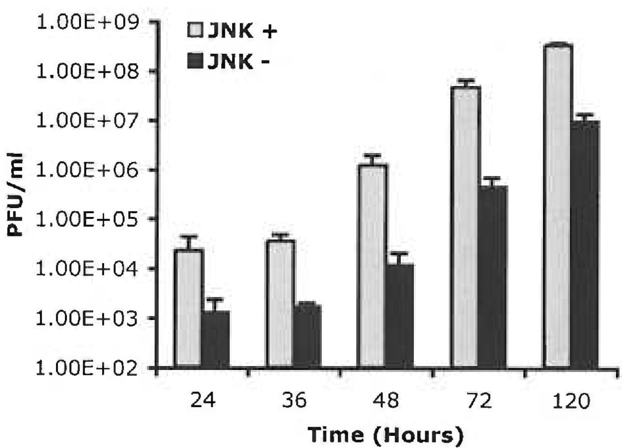
(C) WT and JNK-1/2-/- fibroblasts were infected with MCMV-GFP at moi=1. Total and infected cells were enumerated microscopically 48hpi. Five fields were counted for each cell type. The percentage infection is expressed as the number of GFP-positive (MCMV-infected) cells in the total population of cells (Hoechst positive).

Therefore, efficient *in vitro* replication of MCMV in fibroblasts requires JNK-1/2 activity.

A.



B.



C.

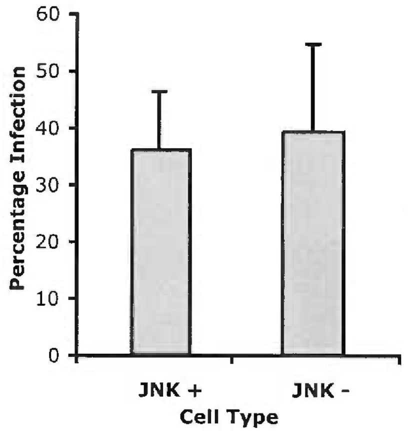


Figure B.4. MCMV IE gene expression is significantly reduced in JNK-1/2-/- fibroblasts.

Lysates of uninfected (time=0) and MCMV-GFP infected WT (left) and JNK-1/2-/- fibroblasts (right) were analyzed by western blotting. Western blots were probed with anti-IE1 (upper most panel), anti-pp50/M44-DNA polymerase accessory factor (2nd from the top), anti-gB (3rd from the top) and Gαi1 as a cellular loading control (lower panel). In parental/WT cells, IE1 expression was readily detectable by 12 hpi and persisted for the duration of the experiment. Expression of the E protein pp50 was observed 24 hpi. The major envelope glycoprotein, gB, was expressed with late kinetics and was first detectable 72 hpi, with peak protein expression occurring 120 hpi. Expression of Gαi1 was not influenced by MCMV infection and was used as a control to ensure equal loading at all time points. In contrast, IE1 expression was significantly reduced in JNK-/- fibroblasts. Similarly, pp50 expression was delayed and reduced in MCMV-infected JNK-/- fibroblasts, while the late protein gB was not detectable. Therefore, expression of all kinetic classes of MCMV gene products is significantly reduced in JNK-/- fibroblasts, indicating that JNK-1/2 activity is critical for MCMV protein expression.

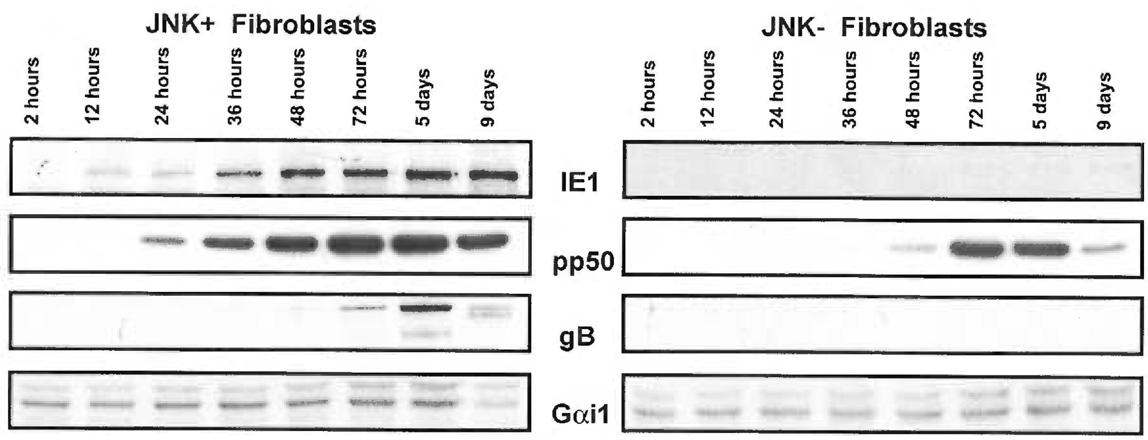
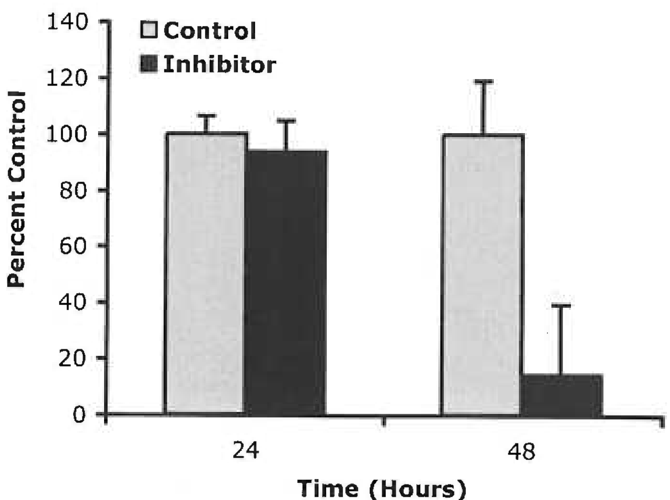

JNK+ Fibroblasts
JNK- Fibroblasts
2 hours
12 hours
24 hours
36 hours
48 hours
72 hours
5 days
9 days
IE1
pp50
gB
Gαi1

Figure B.5. JNK-1/2 peptide inhibitor reduces MCMV IE expression and viral synthesis.

(A) WT fibroblasts were treated in triplicate with a JNK-1/2 tat-conjugated peptide inhibitor or control (tat-alone) at a concentration of 20μM 2 hours prior to infection with MCMV-GFP. At 24 and 48 hours post-infection, cell supernatants were collected and viral titers determined by limiting dilution on WT fibroblasts. MCMV growth rate is percented as a percentage of growth in those cells treated with the JNK-1/2 inhibitor compared to control (tat-alone) treated cells (100%). Confirming that JNK-1/2 activity is critical for efficient MCMV replication in fibroblasts, treatment with the JNK-1/2 inhibitor significantly MCMV replication.

(B) Lysates from WT fibroblasts treated with the JNK-1/2 inhibitor or control (tat-alone) were analyzed by western blotting. Western blots were probed anti-IE1 (upper most panel), anti-pp50/M44-DNA polymerase accessory factor (2nd from the top), anti-gB (3rd from the top) and Gαi1 as a cellular loading control (lower panel). Similar to the MCMV gene expression profiles observed in JNK-/- fibroblasts, expression of IE1, pp50/M44 and gB were greatly reduced in cells treated with the JNK-1/2 peptide inhibitor. Therefore, treatment with the JNK-1/2 peptide inhibitor reduced MCMV gene expression and viral synthesis, confirming that JNK-1/2 activity is required for efficient MCMV gene expression and replication in fibroblasts.

A.



B.

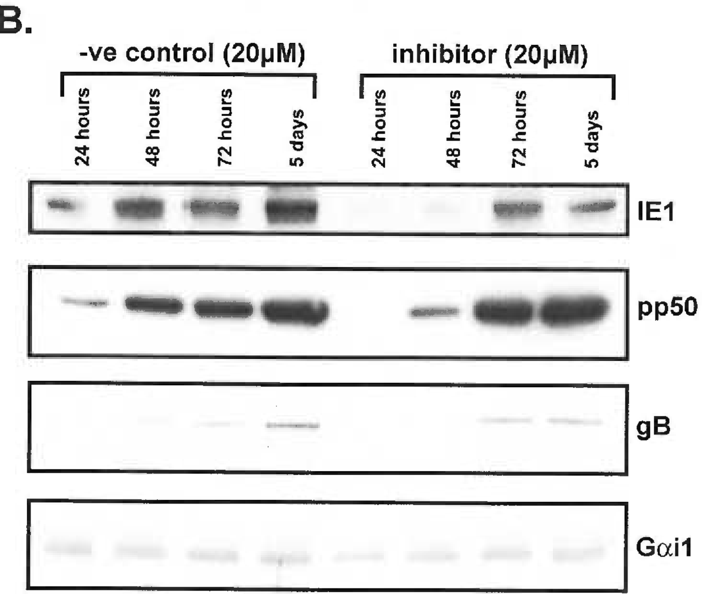


Figure B.6. c-Jun displays cytoplasmic localization in JNK-/- fibroblasts.

Nuclear and cytoplasmic extracts were isolated from uninfected (time=0) and MCMV-GFP infected WT and JNK-1/2 fibroblasts and analyzed by western blotting for c-Jun (upper panel), JunB (2nd from the top), c-Fos (3rd from the top), MEK-2 (2nd from the bottom) and lamin a/c (bottom panel).

MCMV infection induced the phosphorylation of c-Jun by 4 hpi, indicating that MCMV infection promotes JNK-1/2 activation and c-Jun phosphorylation. Surprisingly, MCMV did not promote c-Jun phosphorylation in JNK-/- fibroblasts, moreover, c-Jun was exclusively cytoplasmic in these cells. The subcellular distribution of another Jun family member, JunB, displayed 'normal' nuclear localization. In addition, MCMV infection induced the phosphorylation of the Jun family binding partner and AP-1 transcriptional activator c-Fos in both WT and JNK-/- fibroblasts, indicating that the abnormal localization of c-Jun was specific for this transcription factor. Probing for the cytoplasmic protein MEK-2 revealed that nuclear extracts were not contaminated with exogenous cytoplasmic proteins and equal loading of nuclear extracts was confirmed by probing for Lamin A/C. These results indicate that a lack of JNK-1/2 activity leads to the abherrant localization of c-Jun to the cytoplasm and may account for defects in AP-1 transcriptional activity.

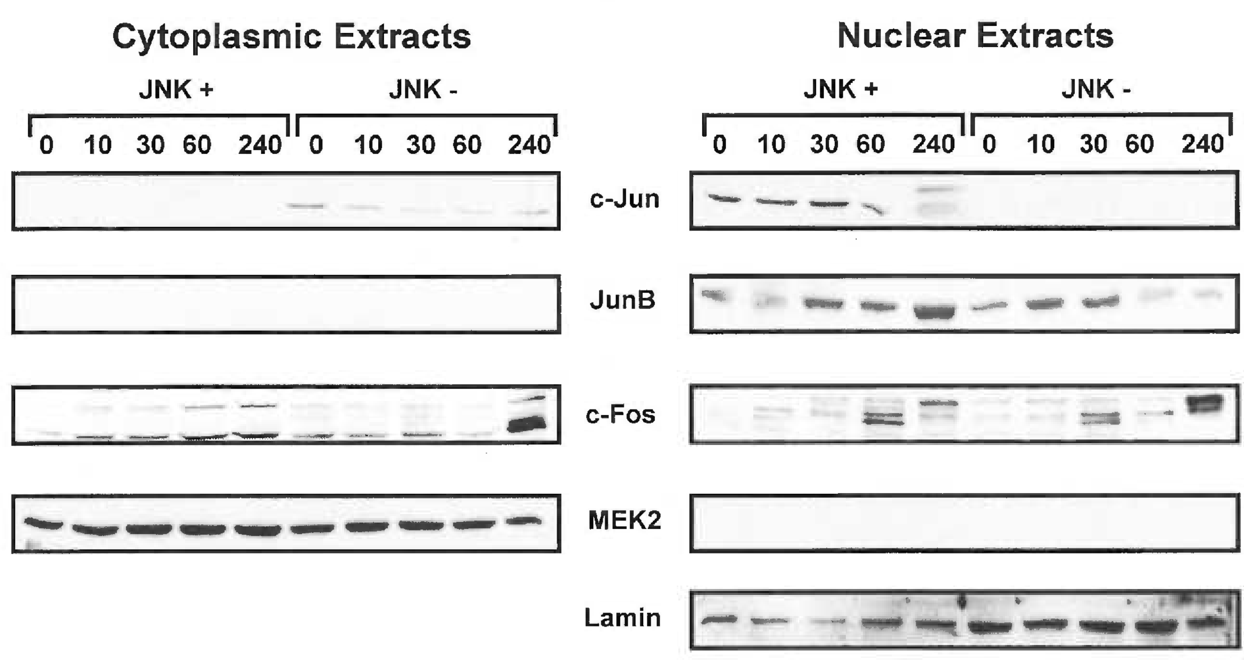
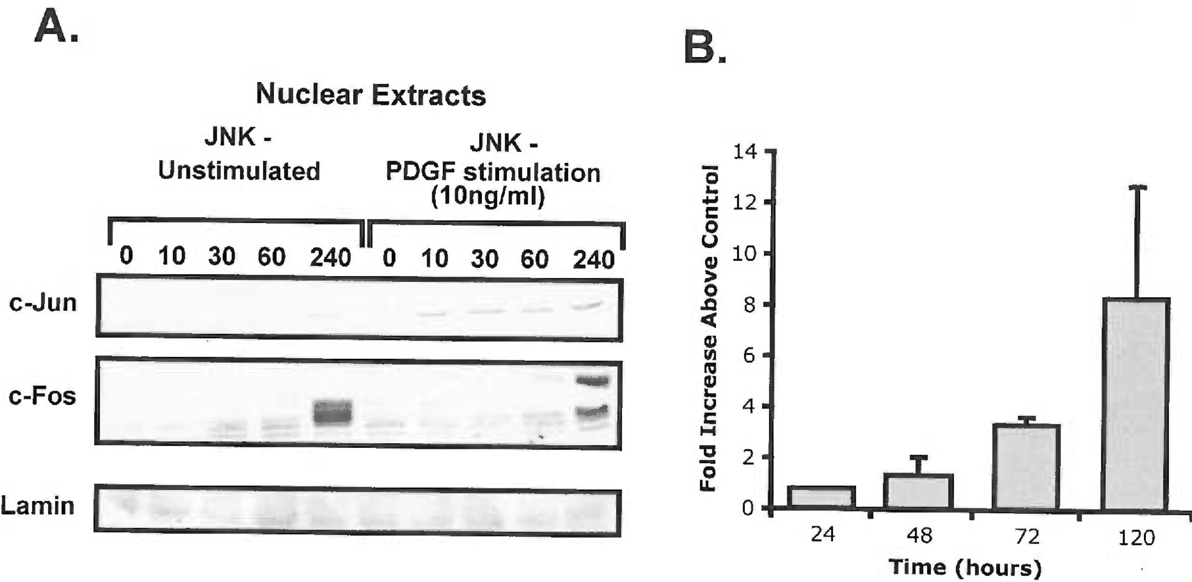

Cytoplasmic Extracts
Nuclear Extracts
JNK +
JNK -
JNK +
JNK -
0 10 30 60 240
0 10 30 60 240
0 10 30 60 240
0 10 30 60 240
c-Jun
JunB
c-Fos
MEK2
Lamin

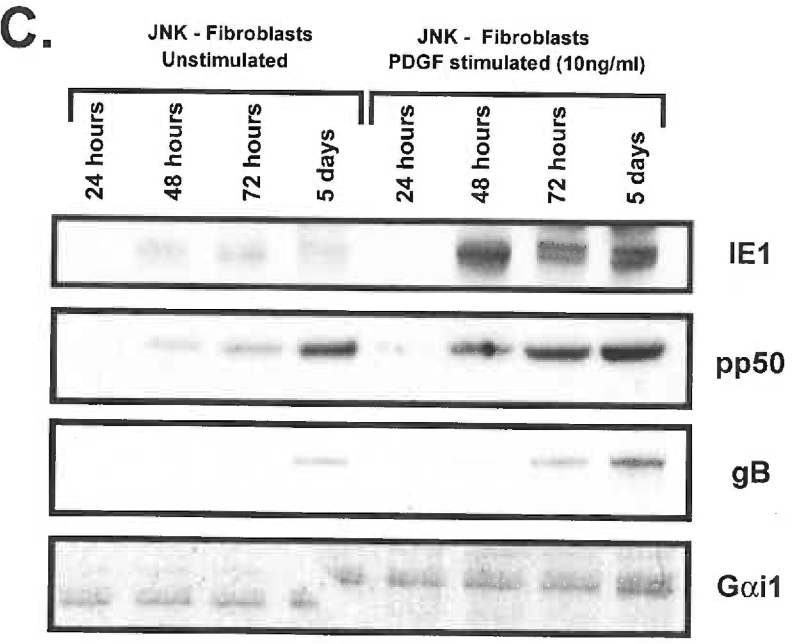
Figure B.7. PDGF stimulates c-Jun nuclear localization with corresponding increases in MCMV gene expression and synthesis.

(A) JNK-1/2 -/- fibroblasts were stimulated with PDGF (10ng/ml) for 5 minutes and then infected with MCMV-GFP (moi=1) for 0 (uninfected, unstimulated control) 24, 36, 48, 72 and 120 hours. Nuclear extracts were prepared and analyzed by western blotting for c-Jun (upper panel), c-Fos (middle panel) and lamin a/c (lower panel). Surprisingly, PDGF pretreatment of JNK-1/2 -/- fibroblasts restored c-Jun nuclear localization without effecting MCMV-induced phosphorylation of c-Fos.

(B) JNK-1/2 -/- fibroblasts were stimulated and infected as in (A) and 24, 36, 48, 72 and 120 hours post-infection, cell supernatants were collected and viral titers determined my limiting dilution on WT fibroblasts. Viral growth is presented as a fold increase in PDGF treated cell as compared to untreated cells for the given time points. PDGF (10ng/ml) pretreatment of JNK-/- fibroblasts resulted in an 8-10 fold increase in MCMV replication at 120 hpi.

(C) Lysates from stimulated and unstimulated MCMV-GFP infected cells were examined by western blotting with anti-IE1 (upper most panel), anti-pp50/M44-DNA polymerase accessory factor (2nd from the top), anti-gB (3rd from the top) and anti-Gαi1 as a cellular loading control (lower panel). PDGF (10ng/ml) pretreatment of JNK-/- fibroblasts resulted in increased expression of all kinetic classes of MCMV proteins.


A.
Nuclear Extracts
JNK -
Unstimulated
JNK -
PDGF stimulation
(10ng/ml)
0 10 30 60 240 0 10 30 60 240
c-Jun
c-Fos
Lamin
B.
Fold Increase Above Control
0
2
4
6
8
10
12
14
24
48
72
120
Time (hours)


C.
JNK - Fibroblasts
Unstimulated
JNK - Fibroblasts
PDGF stimulated (10ng/ml)
24 hours
48 hours
72 hours
5 days
24 hours
48 hours
72 hours
5 days
IE1
pp50
gB
Gαi1