Chemokines and Chemokine Receptors Encoded by Cytomegaloviruses

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Abstract CMVs carry several genes that are homologous to genes of the host organism. These include genes homologous to those encoding chemokines (CKs) and G protein-coupled receptors (GPCRs). It is generally assumed that these CMV genes were hijacked from the host genome during the long co-evolution of virus and host. In light of the important function of the CK and GPCR families in the normal physiology of the host, it has previously been hypothesized that the CMV homologs of these proteins, CMV vCKs and vGPCRs, may also have a significant impact on this physiology, such that lifelong maintenance and/or replication of the virus within the infected host is guaranteed. In addition, several of these homologs were reported to have a major impact in the pathogenesis of infection. In this review, the current state of knowledge on the CMV vCKs and vGPCRs will be discussed.

Abbreviations AC: Adenylyl cyclase; cAMP: Cyclic adenosyl monophosphate; CCMV: Chimpanzee cytomegalovirus; CCR[n] (e.g. CCR5): CC chemokine receptor [n]; CMV: Cytomegalovirus; CRE: Cyclic adenosyl monophosphate responsive

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element; CREB: Cyclic adenosyl monophosphate responsive element-binding factor; ECK-3:England strain rat cytomegalovirus chemokine 3; ELC: Epstein-Barr virus-induced chemokine receptor ligand chemokine; ERK[n] (e.g., ERK1): Extracellular signal-regulated protein kinase [n]; FAK: Focal adhesion kinase; GpCMV: Guinea pig cytomegalovirus; GPCR: G protein-coupled receptor; HCMV: Human cytomegalovirus; HHV-6: Human herpesvirus 6; HHV-7: Human herpesvirus 7; HIV: Human immunodeficiency virus; InsP: Inositol phosphate; KSHV: Kaposi's sarcoma-associated herpesvirus; MCK-2: Murine cytomegalovirus chemokine 2; MCMV: Murine cytomegalovirus; MCP-1: Monocyte chemoattractant protein 1; MDC: Macrophage-derived chemokine; MIP-1α: Macrophage inflammatory protein 1α; NFκ-B: Nuclear factor κB; ORF: Open reading frame; p38/MAPK: 38-kD Mitogen-activated protein kinase; PLC: Phospholipase C; PTK: Protein tyrosine kinase; Rac: Rat sarcoma homolog A-related C3 botulinum toxin substrate; RANTES: Regulated upon activation normal T cell expressed, and secreted; RCK-[n] (e.g. RCK-3): Maastricht strain rat cytomegalovirus chemokine [n]; RCMV: Rat cytomegalovirus; RhCMV: Rhesus macaque cytomegalovirus; RhoA: Rat sarcoma homolog A; SLC: Secondary lymphoid tissue chemokine; SRE: Serum-responsive element; TARC: Thymus and activation-regulated chemokine; vCK: Viral chemokine; vCXC-[n] (e.g. vCXC-1): Human cytomegalovirus CXC chemokine [n]; vGPCR: Viral G protein-coupled receptor; vMIP-II: Kaposi's sarcoma-associated herpesvirus macrophage inflammatory protein II; VSV: Vesicular stomatitis virus

Introduction

Cytomegaloviruses (CMVs) are species-specific betaherpesviruses that establish life-long persistence in their hosts. Their genomes, the largest among herpesviruses, are estimated to contain between 165 (Davison et al. 2003) and 252 (Murphy et al. 2003b; see the chapter by E. Murphy and T. Shenk, this volume) potential open reading frames (ORFs) encompassing up to 241,087 bp of double-stranded DNA (Davison et al. 2003). Approximately 41 human CMV (HCMV) ORFs belong to a core set of genes essential for viral replication in vitro, such as genes encoding DNA polymerase, capsid, matrix and envelope proteins (Yu et al. 2003). Approximately 88 genes were found to be nonessential for efficient CMV replication in vitro (Yu et al. 2003). Some of these nonessential CMV genes are homologous to genes of the host. Among these homologs are genes that share similarities with genes encoding proteins that are associated with the immune system, such as class I MHC proteins (Wills et al. 2005; Prod'homme et al. 2007), a TCR gamma chain (Beck and Barrell 1992), IL-10 (Wagner et al. 2003) and a TNF receptor (Poole et al. 2006). Apparently these gene homologs have been acquired from the host organism and subsequently modified during approximately 180 My of co-evolution (Davison 2002) in order to enable dissemination and maintain life-long persistence. Interestingly, CMVs possess two distinct groups of genes homologous to those of

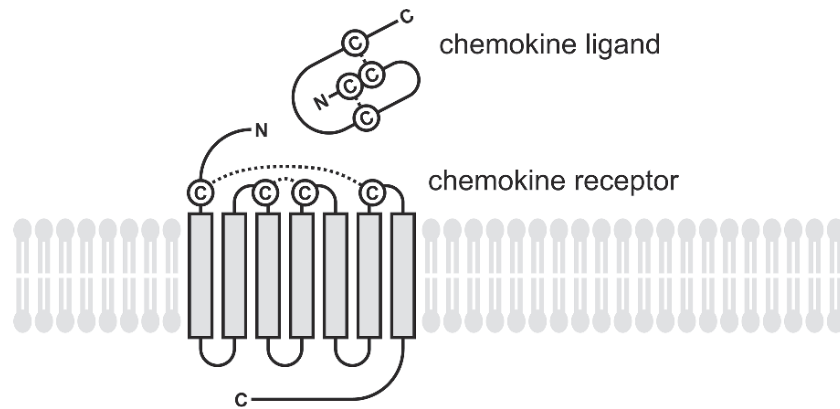

Fig. 1 The 2D peptide structures of a CC chemokine ligand and a chemokine receptor. The *N* and the *C* denote the amino and carboxyl termini, respectively. The encircled *C*s represent conserved cysteine residues. The *dashed lines* indicate conserved disulphide bridges. *Grey boxes* indicate hydrophobic transmembrane alpha helix domains

the host: (a) CC and CXC chemokine genes and (b) G protein-coupled receptors (GPCR) genes, the majority of which resembles chemokine receptor genes. GPCRs form a large family of 7-transmembrane receptors (Fig. 1) that include sensory receptors for sight, smell, and taste as well as receptors for many neurotransmitters, peptide hormones and chemokines. Chemokines comprise a family of immune modulatory cytokine peptides. Currently, four classes of chemokines are known. The classification is based on a conserved structure (Fig. 1) that includes either a single cysteine (C), a CC motif (Fig. 1), a CXC motif or a CX3C motif. Chemokines can be released to initiate inflammatory responses by acting as chemoattractant for infiltrating leukocytes (Glass et al. 2003). They can also stimulate differentiation, maturation and activation of many types of immune-related cells (Glass et al. 2003). Two chemokines, CXCL16 and CX3CL-1, were shown to function as adhesion molecules for leukocytes that are captured from the bloodstream onto the endothelial surface (Haskell et al. 2000; Nakayama et al. 2003). The purpose of this review is to summarize the (putative) functions of the CC and CXC chemokines (vCKs) as well as the chemokine-like GPCRs (vGPCRs) that are encoded by CMV.

Evolution of CMV vCK Genes

To date, three vCK genes have been identified in the HCMV genome (Fig. 2), the CC chemokine-like gene UL128, and the CXC chemokine-like genes UL146 and UL147. To some extent, these genes are conserved among primate CMVs (Table 1). UL128-like genes are also present on the genomes of murine CMV (MCMV) and rat CMV (RCMV), as well as on the genome of human herpesvirus type 6 (HHV-6) (Table 1). The rodent CMV and HHV-6 species lack UL146- and UL147-like

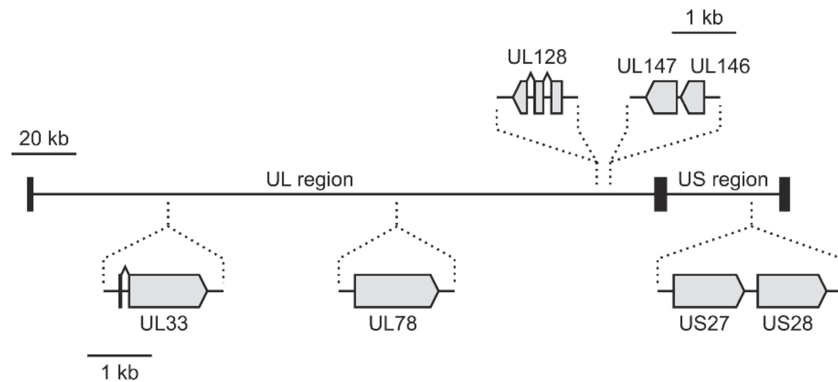

Fig. 2 CMV chemokine and chemokine receptor homolog gene loci. The central line represents the HCMV Merlin strain genome (235,645 kb) derived from GenBank accession NC_006273. The *black boxes* on this line represent repetitive regions. The chemokine homolog gene loci are enlarged above the genomic line, the chemokine receptor homolog gene loci below the genomic line. The *arrowheads* indicate the sizes and directions of the coding content of the genes. The UL128 exon sizes and positions are analogous to those of the HCMV AD169 strain, as indicated in NC_006273. *UL* unique long, *US* unique short

genes. Similar to the UL33- and UL78-like genes, the UL128-like chemokine genes are likely to have been acquired by betaherpesviruses at least 110 My ago. The conservation of this gene family suggests an essential role for these genes in the survival of betaherpesviruses in vivo. This notion is supported by the observation that the UL128 sequences from different clinical HCMV isolates are highly conserved (Baldanti et al. 2006). Paradoxically, the UL128 gene appears to be functional in laboratory strain HCMV AD169, whereas in the clinical, low-passage HCMV strains Toledo and Merlin, as well as in the chimpanzee CMV (CCMV) laboratory strain, the UL128-like genes are disrupted by inversions or frame shift mutations (Table 1). A UL128 counterpart was also identified on the HHV-6 genome, designated U83A. This gene was found to encode a potent CC chemokine (Derwin et al. 2006). Interestingly, the UL130 genes of the primate CMVs also contain chemokine-like sequences, including a CXC domain (Glass et al. 2003). However, the UL130-derived amino acid sequences lack other cysteine residues that are essential for classical chemokine folding (Glass et al. 2003). The MCMV and RCMV genome sequences available to date do not contain UL130-like CXC chemokine sequences. Nevertheless, within the genome of the Maastricht strain of RCMV, a second CC chemokine-like gene, r131, was identified adjacent to r129. These two genes may have originated from duplication of a common ancestor gene (Kaptein et al. 2004; Voigt et al. 2005). In contrast to the UL128-like genes, UL146 and UL147 appear to be restricted to the primate CMVs. Both the HCMV and CCMV UL146 genes encode a CXC vCK (Table 1). Rhesus macaque CMV (RhCMV) does not possess a UL146 homolog (Table 1). Both the HCMV and CCMV genomes contain a UL147 gene, whereas a UL147-like gene is present in

Table 1 Cytomegalovirus chemokine-like genes

CMV Species	Gene (product name)	GenBank accession	References	Comments
HCMV	UL128	NC_006273	Akter et al. 2003, Hahn et al. 2004	Intact in AD169, disrupted in Merlin and Toledo
CCMV	UL128	AF480884	Akter et al. 2003, Hahn et al. 2004	Disrupted
RhCMV	rhUL128	DQ120516	Rivailler et al. 2006	Intact in RhCMV 180.92, not present in RhCMV68.1
RCMV	r129 (RCK–3/ ECK–3)	AF232689	Kaptein et al. 2004	
RCMV	r131 (RCK–2)	AF232689	Kaptein et al. 2004	
MCMV	m131–129 (MCK–1; MCK–2, alternative splice product)	U68299	MacDonald et al. 1999	
HHV–6	U83A	NC_001664	Derwin et al. 2006, Catusse et al. 2007	
GpCMV	GPCMV-MIP	AF500307	Haggerty and Schleiss 2002, Penfold et al. 2003a	Unique, MIP–1-like
HCMV	UL146 (vCXC–1)	NC_006273	Penfold et al. 1999	Intact in Towne, Toledo, Merlin, lost in AD169
CCMV	UL146 (vCXC–1)	AF480884	Miller-Kittrell et al. 2007	
CCMV	UL146A	AF480884	Davison et al. 2003	UL146-like, not present in other CMVs
HCMV	UL147 (vCXC–2)	NC_006273	Penfold et al. 1999	Intact in Towne, Toledo, Merlin, lost in AD169
CCMV	UL147	AF480884	Davison et al. 2003	
RhCMV	rh158	AY186194	Rivailler et al. 2006	Intact in 68.1, not present in 180.92
RhCMV	rh156.2	DQ120516	Rivailler et al. 2006	
CCMV	UL156	AF480884	Davison et al. 2003	Splice variant may encode CXC chemokine, not present in other CMVs
CCMV	UL157	AF480884	Davison et al. 2003	UL146-like, not present in other CMVs

only one of two available genomic RhCMV sequences (Table 1). The sequences of UL146 and UL147 derived from numerous clinical HCMV isolates showed an extensive level of variability. This variability was significant among interindividual strains (Hassan-Walker et al. 2004; Stanton et al. 2004). However, the sequence of

individual strains remained absolutely stable over time in vitro and in vivo, which indicates that sequence drift is not a mechanism for the observed sequence hypervariability (Lurain et al. 2006). UL146 was found to be the more rapidly evolving paralog (Arav-Boger et al. 2005). Despite the observed hypervariability, no specific UL146 or UL147 genotype was associated with disease outcome in newborns with CMV-associated congenital disorders (Arav-Boger et al. 2006; He et al. 2006). Interestingly, a single CXC vCK-like gene (rh156.2) and two CXC vCK-like genes (UL156 and UL157) have been identified in RhCMV and CCMV, respectively (Table 1). While these genes share significant similarity with UL146-like genes, they do not have counterparts in HCMV or rodent CMVs. This indicates that in contrast to UL128-like genes, the UL146-like and UL147-like genes are rapidly evolving in vivo. Finally, a distinct CC vCK was reported to be encoded by guinea pig cytomegalovirus (GpCMV; Haggerty and Schleiss 2002). Both the genomic localization and the DNA sequence of the MIP gene are unique for GpCMV. No counterparts have been found on the genomes of other herpesviruses (Table 1).

The Role of vCKs During CMV Infection

The HCMV UL128 is functionally clustered within the UL128/UL130/UL131A locus. Three mutant HCMV strains were generated in which any of these genes was disrupted. Each of these strains completely lost its ability to replicate in human umbilical cord endothelial cells, as well as its ability to transfer from one cell to another in cultured polymorphonuclear leukocytes (PMNs) and monocytes (Hahn et al. 2004). Thus, these genes appear to determine endothelial cell tropism as well as cell-to-cell passage in vitro (Hahn et al. 2004). The mechanisms by which the UL128, UL130 and UL131A genes govern these processes have recently been addressed. It was suggested that the proteins encoded by the UL128/UL130/UL131A locus might act as ligands for receptors that convey signals into endothelial cells to facilitate intracellular transport or inactivation of innate intracellular antiviral immunity (Patrone et al. 2005). Moreover, these proteins are a component of the attachment/entry machinery, either by acting as a soluble factor or as a virion component, permitting a viral entry pathway that differs from that used in fibroblasts (Patrone et al. 2005). These proteins were also suggested to be involved in the final stages of virus morphogenesis and maturation at membranes (Hahn et al. 2004), as well as in attraction–adhesion of leukocytes to endothelial cells (Hahn et al. 2004). Finally, UL128, UL130 (Wang and Shenk, 2005) and UL131 (Adler et al. 2006) have been shown to be part of a complex with gH/gL in the virion and to play a direct role in entry into epithelial and endothelial cells. The chemotactic activity of each individual gene product of the UL131–128 locus, as well as the potential cooperation with other viral or cellular gene signaling molecules, remains to be elucidated. The HHV-6 counterpart of UL128, U83A, was found to encode a potent CC chemokine capable of inducing Ca^{2+} mobilization and chemotaxis in T lymphocytes (Derwin et al. 2006). However, this provides little insight in the function of

CMV-encoded UL128-like vCKs, since CMVs and HHV-6 occupy different tissue compartments and cell types in the host.

The MCMV counterpart of UL128, m131, was found to encode a potent CC vCK designated MCP-1, which was capable of inducing Ca^{2+} mobilization and chemotaxis of macrophages and cells expressing human CCR3 (Saederup et al. 1999). Similarly, the unique GpCMV MIP chemokine was found to invoke signaling and chemotaxis in cells expressing human CCR1 (Penfold et al. 2003a). These findings indicate a role for the rodent CMV-encoded CC chemokines in leukocyte attraction. It was hypothesized that leukocytes recruited by vCKs can subsequently be subverted by CMV to serve as vehicles to enable viral dissemination. This claim was supported by deletion mutant experiments in vivo. Mutant MCMV and RCMV strains from which the m129/m131 or r131 locus had been deleted, respectively, exhibited reduced virus levels in salivary gland, liver and spleen tissue during acute infection (Saederup et al. 1999; Kaptein et al. 2004). Moreover, leukocyte infiltration in infected foot pad experiments was significantly lower in mice and rats treated with deletion mutant virus (Saederup et al. 1999; Kaptein et al. 2004). Finally, monocyte-associated viremic peak levels in mice infected with the m129/m131 deletion mutant were dramatically lower than those in mice infected with wild-type MCMV (Saederup et al. 1999).

Both HCMV and CCMV UL146 encode potent vCKs, which were designated vCXC-1. Recombinant vCXC-1 peptides derived from both CMV species were found to be capable of inducing calcium mobilization, chemotaxis, and degranulation via stimulation of CXCR2, as well as inducing integrin upregulation and apoptosis in human neutrophils (Penfold et al. 1999; Miller-Kittrell et al. 2007). To study the function of UL146 and UL147 further, mutant HCMV strains were generated in which both genes were disrupted. Viral passage to PMN was reduced in these strains, but not viral passage in monocytes (Hahn et al. 2004). Thus, the chemotactic factors encoded by HCMV UL146 and UL147 appear to be dispensable for viral growth and dissemination in vitro. In order to determine the role of these genes in vivo, the CCMV model may be the most suitable.

Chemokine Receptors Encoded by CMVs

Evolution of CMV vGPCR Genes

Both vGPCR and vCK genes have been identified within the genomes of beta- and gammaherpesviruses, but not within those of alphaherpesviruses. There is no apparent evolutionary relationship for these genes between the beta- and gammaherpesvirus subfamilies. Yet, within the betaherpesvirus subfamily, both vGPCR and vCK genes appear to be conserved among murine and primate CMV species, as well as the HHV-6 and human herpesvirus type 7 (HHV-7). The HCMV genome contains four vGPCR genes, UL33, UL78, US27 and US28 (Fig. 2) (Murphy et al. 2003a). These genes are conserved among all known primate CMVs (Table 2).

Table 2 Cytomegalovirus chemokine receptor-like genes

CMV Species	Gene	GenBank accession	References
HCMV	UL33	NC_006273	Casarosa et al. 2003
CCMV	UL33	AF480884	Davison et al. 2003
RhCMV	rh56	AY186194	Hansen et al. 2003
MCMV	M33	U68299	Davis-Poynter et al. 1997
RCMV	R33	AF232689	Beisser et al. 1998
GpCMV	GP33	AF355272	Liu and Biegalke 2001
HHV–6	U12	NC_001664	Isegawa et al. 1998
HHV–7	U12	U43400	Nanako et al. 2003
HCMV	UL78	NC_006273	Michel et al. 2005
CCMV	UL78	AF480884	Davison et al. 2003
RhCMV	rh107	AY186194	Hansen et al. 2003
MCMV	M78	U68299	Oliveira et al. 2001
RCMV	R78	AF232689	Beisser et al. 1999
GpCMV	GP78	Unavailable	Stropes and Miller 2004
HHV–6	U51	NC_001664	Milne et al. 2000
HHV–7	U51	U43400	Tadagaki et al. 2005
HCMV	US27	NC_006273	Fraile-Ramos et al. 2002
CCMV	US27	AF480884	Sahagun-Ruiz et al. 2004
HCMV	US28	NC_006273	Gao and Murphy 1994
CCMV	US28	AF480884	Davison et al. 2003
RhCMV	rh214, rh215, rh216, rh218, rh220	AY186194	Sahagun-Ruiz et al. 2004

UL33 and UL78 homologs are also present within the genomes of murine, rat and guinea pig CMV (GpCMV), as well as on the genomes of HHV-6 and -7 (Table 2). These species lack US27- and US28-like genes. HCMV and HHV-6 species were estimated to have diverged approximately 110 My ago (Davison 2002). Since the genomic locations of UL33- and UL78-like genes are highly conserved among all known betaherpesvirus species, it is likely that these genes were acquired by a common ancestor of the betaherpesviruses, rather than independently, after divergence of the different betaherpesvirus species. The notion that the UL33 and UL78 gene families have been maintained over such a long period of time suggests an essential role for these genes in the survival of betaherpesviruses in vivo. The US27- and US28-like genes have only been identified in primate CMV species. It is likely that these genes have emerged somewhere after the branching of a common ancestor of rodents and primates, 100 My ago (Li et al. 1990). Interestingly, RhCMV possesses five consecutive US27/US28-like genes (Penfold et al. 2003b) rather than the two genes (US27 and US28) found in CCMV (Davison et al. 2003) and HCMV (Murphy et al. 2003a). Since the sequences of US27- and US28-like genes in primate CMVs are highly similar and consecutively positioned with their respective genomes, it is likely that these genes have emerged from a single hijacked host GCPR gene-by-gene multiplication.

All CMV vGPCRs, with the exception of those encoded by UL78-like genes, contain the hallmarks of chemokine receptors (Ahuja et al. 1993; Davis-Poynter et al. 1997):

1. An N-linked glycosylation site and several negatively charged amino acid residues located in the extracellular N-terminal region
2. Two cysteine residues, which are likely to form a disulfide bridge, thereby joining the N-terminal region with the third extracellular loop (Fig. 1)
3. Several positively charged amino acid residues within the third intracellular loop
4. Invariant amino acids within the transmembrane regions
5. Several serine and threonine residues in the intracellular C-terminal region

The sequences of US27/US28-like genes have the highest similarity with those of chemokine receptors of the host. The sequences of UL33-like genes are also related to those of chemokine receptors, albeit to a lesser extent. The sequences of UL78-like GPCR genes possess none of the chemokine receptor hallmarks. Interestingly, the HCMV genome contains several putative genes encoding 7-transmembrane proteins: UL100 (the putative structural glycoprotein **M**), US12, US13, US14, US15, US16, US17, US18, US19, US20, and US21 (possibly a multiplication of a US12-like ancestor). These genes share some sequence similarity with genes of well-characterized GPCRs (Rigoutsos et al. 2003). Yet, their predicted amino acid sequences lack the cysteine residues that join the second and third extracellular loop of all known GPCRs and are therefore not considered vGPCRs. Nevertheless, it remains tempting to speculate that these genes have arisen from host GPCR genes.

Modulation of Intracellular Signaling by CMV vGPCRs

Various CMV vGPCRs have been investigated for their putative role in the activation of signal transduction pathways. Attempts have been made to identify ligands that bind to these vGPCRs, as well as to identify the downstream intracellular signaling pathways. The most common signaling factors studied were:

1. Ca^{2+} mobilization
2. Increase of inositol phosphate (InsP) by phospholipase C (PLC)
3. Increase of cAMP by adenylyl cyclase (AC) and subsequent CREB-mediated gene transcription
4. NFκ-B-mediated gene transcription

In some cases, other signaling factors were addressed. The results of these studies are summarized in Table 3. The consequences of modulation of these intermediates by vGPCRs may include either activation or inhibition of immune-related responses, such as cell differentiation and maturation, cell proliferation, cytoskeletal remodeling, cell migration, cytokine release, and cytotoxicity. Apparently, viruses benefit from altering these responses by utilizing their own vGPCRs.

For at least one member of each family included in Table 3, one or more ligands were identified. Interestingly, all of these ligands are CC chemokines, of which RANTES appears to be the most common. Some vGPCRs, most notably those encoded by the HHV-6 U51 and CMV US28, interact with more than one CC chemokine species. The combinations of chemokines that interact with each of these vGPCRs are unique and unrelated to those specific for chemokine receptors of the host. The most direct way of determining ligand-dependent vGPCR signaling, as well as subsequent vGPCR desensitization, is measuring intracellular Ca^{2+} mobilization (Table 3). In addition to Ca^{2+} mobilization, cytoplasmic accumulation of inositol phosphates (InsP) was measured in cells that expressed a member of either of the three betaherpesvirus vGPCR families. Interestingly, in all of these studies, vGPCRs were reported to induce a $G_{q/11}$-mediated increase of InsP in a ligand-independent manner (Table 3). Thus, all vGPCRs were found to be constitutively active, a property that is not shared by chemokine receptors of the host. Constitutive signaling was also demonstrated by subjecting betaherpesvirus vGPCRs to reporter gene assays specific for either cAMP/CREB- or NFκ-B-driven modulation of gene transcription. Most notably, the results of these assays were inconsistent when comparing the activities of members of the UL33 vGPCR family: expression of HCMV UL33 and MCMV M33 resulted in an increase in cAMP-mediated gene transcription, whereas expression of RCMV R33 resulted in a decrease (Table 3). The most important factor responsible for this inconsistency is p38/MAPK, which is activated in cells expressing either HCMV UL33 or MCMV M33, but not in cells expressing RCMV R33 (Table 3). Additionally, expression of M33- and R33-encoded vGPCRs resulted in an increase in NFκ-B-mediated gene transcription, whereas expression of HCMV UL33 had no effect (Table 3). Interestingly, NFκ-B-mediated signaling is also increased in cells expressing HCMV US28. These findings support the hypothesis that the primate UL33-like vGPCRs have lost some signaling properties as similar functions might have become redundantly available upon hijacking US28-like genes. Alternatively, the rodent UL33-like vGPCRs may have gained NFκ-B-stimulating activity following the loss of US28-like genes from their corresponding genomes. Gain and loss of genes as well as activities during evolution of the CMVs has become apparent by comparing HCMV US28 with RhCMV US28-like genes. None of the vGPCRs encoded by the five different US28-like genes within the RhCMV genome possess constitutive signaling activities, nor do they signal in the presence of chemokine ligands that are known to modulate HCMV US28-mediated signaling (Penfold et al. 2003b). Moreover, neither of the InsP-, CREB- and NFκ-B-mediated signaling factors were affected by HCMV US27 expression (Table 3).

A recent study has indicated that the HCMV US28-encoded vGPCR not only acts individually by modulating intracellular signaling, but also constitutes a regulatory switch for signal transduction by other Gi/o-coupled receptors (Bakker et al. 2004). In addition, when either HHV-7 U12 or U51 were expressed together with the host chemokine receptors CCR4 and CCR7, they had a broader ligand specificity than when each of these chemokines were expressed individually (Table 3). This suggested that the gene products of U12 and U51 can interact with

Table 3 Signaling factors associated with CMV cGPCRs

Receptor	Ligand(s)	Ca^{2+} mobilization	PLC/InsP	AC/cAMP/CRE	NF-κB	Other signaling effects
HCMV UL33	No binding with RANTES (Casarosa et al. 2003)	Unknown	Constitutively up via $G_{\alpha q/11}$ (Waldhoer et al. 2002, Casarosa et al. 2003), constitutively up, partially via $G_{\beta\gamma}$ from $G_{i/o}$ (Casarosa et al. 2003)	Overall constitutively up (Waldhoer et al. 2002, Casarosa et al.2003), constitutively down via $G_{\alpha i/o}$ (Casarosa et al. 2003), constitutively up via $G_{\alpha s}$ and $G_{\beta\gamma}$/Rho/p38 (Waldhoer et al. 2002, Casarosa et al. 2003)	Unaffected (Waldhoer et al. 2002)	$G_{i/o}$ activation mediated by cytoplasmic tail (Casarosa et al. 2003)
MCMV M33	Mouse RANTES (Melnychuk et al. 2005)	Unknown	Constitutively up via G_{q11} (Waldhoer et al. 2002, Sherrill and Miller 2006), GRK2 scavenges $G_{alpaq/11}$, PLCβ stimulation inhibited (Sherrill and Miller 2006)	Constitutively up (Waldhoer et al. 2002, Casarosa et al. 2003) via p38 (Waldhoer et al. 2002),not ERK 1/2/MAPK(Waldhoer et al. 2002)	Constitutively up (Waldhoer et al. 2002)	Rac1 and ERK1/2 stimulation upon mouse RANTES treatment
RCMV R33	No binding with rat RANTES (Gruijthuijsen et al. 2002)	Unknown	Constitutively up via $G_{q/11}$, constitutively up, partially via $G_{i/o}$ (Gruijthuijsen et al. 2002; Casarosa et al. 2003)	Constitutively down via $G_{i/o}$ (Gruijthuijsen et al. 2002, Casarosa et al. 2003)	Constitutively up via $G_{i/o}$ (Gruijthuijsen et al. 2002)	SRE up via $G_{i/o}$, $G_{i/o}$ activation not mediated by cytoplasmic tail (Casarosa et al. 2003)
HHV–6 U12	RANTES, MIP–1α, MIP–1β, MCP–1 (Isegawa et al. 1998)	By RANTES, MIP–1α, MIP–1β, MCP–1, desensitization by all (Isegawa et al. 1998)	Unknown	Unknown	Unknown	

(continued)

Table 3 (continued)

Receptor	Ligand(s)	Ca^{2+} mobilization	PLC/InsP	AC/cAMP/CRE	NF-κB	Other signaling effects
HHV– 7 U12	ELC, SLC, TARC, MDC (Tadagaki et al. 2005)	By ELC, SLC, TARC & MDC (Tadagaki et al. 2005)	Unknown	Unknown	Unknown	Broadens ligand specificity for CCR4 and CCR7 (Tadagaki et al. 2007)
HCMV UL78	Unknown	Unknown	Unknown	Unknown	Unknown	
MCMV M78	Unknown	Unknown	Unknown	Unknown	Unknown	
RCMV R78	Unknown	Unknown	Unknown	Unknown	Unknown	
HHV–6 U51	RANTES, eotaxin,MCP–1–3, and –4, KSHV vMIP-II (Milne et al. 2000; Fitzsimons et al. 2006)	By RANTES, not by MCP–1 or eotaxin (Fitzsimons et al. 2006)	Constitutively up via Gq/11, more up by RANTES stimulation, not by MCP–1 or eotaxin (Fitzsimons et al. 2006)	Constitutively down via $G_{q/11}$, up by RANTES, MCP–1 and eotaxin via Gi/o (Fitzsimons et al. 2006)	Unknown	Downregulates RANTES transcription (Milne et al. 2000)
HHV–7 U51	ELC, SLC, TARC, MDC (Tadagaki et al. 2005)	By ELC, SLC, TARC & MDC (Tadagaki et al. 2005)	Unknown	Unknown	Unknown	Alters ligand specificity for CCR4 and CCR7 (Tadagaki et al. 2007)
HCMV US27	Unknown	Unknown	Unaffected (Waldhoer et al. 2002)	Unaffected (Waldhoer et al. 2002)	Unaffected (Waldhoer et al. 2002)	
HCMV US28	RANTES, MCP–1, MIP–1α, MIP–1β,	Via $G_{\alpha i}$ and $G_{\alpha 16}$ (Billstrom et al. 1998), by RANTES	Constitutively up via $G_{q/11}$ (Casarosa et al. 2001; Waldhoer et al. 2002), not modulated	Constitutively up (Waldhoer et al. 2002), G_i-independent (Waldhoer et al.	Constitutively up (Casarosa et al. 2001,Waldhoer et al. 2002) via	Various, see text

	fractalkine, MCP–3, KSHV vMIP-II (Billstrom et al. 1998; Vieira et al. 1998; Kledal et al. 1999)	(Billstrom et al. 1998; Vieira et al. 1998), MIP–1α (Billstrom et al. 1998), MIP–1β (Vieira et al. 1998), MCP–3 (Billstrom et al. 1998), fractalkine (US patent 20020127544), desensitized for RANTES by MIP–1β (Vieira et al. 1998), MCP–3 (Billstrom et al. 1998)	by RANTES/MCP–1/MCP–3/MIP–1α/MIP–1β (Casarosa et al. 2001), partially down by fractalkine (Casarosa et al. 2001; Waldhoer et al. 2002), RANTES/MCP–1 antagonize fractalkine (Casarosa et al. 2001)	2002), via p38/MAPK (Waldhoer et al. 2002), down by fractalkine (Waldhoer et al. 2002)	$G_{\beta2\gamma1}$, down by fractalkine (Casarosa et al. 2001)

CCR4 and CCR7, possibly by receptor dimerization. Dimerization and subsequent changes in receptor kinetics have been demonstrated for CCR2 and CCR5 (Mellado et al. 2001). Similarly, other vGPCRs could modulate intracellular signaling by interacting with host receptors. Such a notion may inspire researchers to reevaluate orphan vGPCRs like those encoded by UL78 and US27, as well as by the rhesus US28-like vGPCR genes, which do not seem to be capable of altering signaling by themselves.

The consequences of GPCR signaling (Table 3) for the survival and replication of CMVs in the host remain in most cases unclear. Only a few of these signaling studies have led to the identification of changes in cellular behavior. The US28-encoded vGPCR was shown to trigger smooth muscle cells to undergo chemokinesis and chemotaxis via the $G_{\alpha 12/13}$/PTK/RhoA/FAK/Src pathway upon stimulation with either RANTES or MCP-1 (Streblow et al. 1999, 2003). Thus, US28 could play a role in the spread of CMV through solid tissue by using infected cells as vehicles. HCMV US28-mediated intracellular signaling could cause infected smooth muscle cells to migrate to inflammatory sites such as atherosclerotic plaques. This, together with the finding that a US28-specific antibodies can cross-react with heat-shock protein 60 (Bason et al. 2003), implies that US28-signaling in CMV-infected cells supports the progress of atherosclerosis. Interestingly, in addition to cell migration, US28-mediated signaling was found to trigger two unusual cellular responses: (a) caspase-dependent apoptosis (Pleskoff et al. 2005) and (b) loss of cell contact inhibition and enhanced cell cycle progression, as well as VEGF-mediated enhancement of tumor progression in vivo (Maussang et al. 2007). More investigation is required to determine how these two phenomena benefit CMV infection and how they relate to pathogenesis of CMV infection in humans.

Similar to HCMV US28, the MCMV M33-encoded vGPCR was shown to trigger smooth muscle cell migration, which, in the case of M33 was enhanced upon stimulation with murine RANTES (Melnychuk et al. 2005). Nevertheless, the direct consequences of altered signaling in cells expressing non-RANTES-binding UL33 and R33 (Table 3) still needs to be elucidated. In addition, numerous other questions regarding the function of putative vGPCRs remain. Most important of these is the question regarding the activities of the proteins encoded by UL78, M78, R78 and US27. To date, none of these proteins has been attributed to any signaling activity.

CMV vGPCR Gene Deletion Mutants

Additional roles for CMV vGPCRs in the host have been determined by studying knockout CMV mutants. The HCMV UL33 gene, as well as MCMV M33 and RCMV R33 are dispensable for viral replication in fibroblasts in vitro (Davis-Poynter et al. 1997; Beisser et al. 1998; Casarosa et al. 2003). Yet, the mortality rate of rats infected with RCMV R33 deletion mutant virus was significantly lower

than that of rats infected with wild type virus (Beisser et al. 1998). Additionally, whereas wild type virus can be recovered from salivary glands of both MCMV-infected mice and RCMV-infected rats, their respective M33 and R33 deletion mutant counterparts remained undetectable in these organs throughout infection (Davis-Poynter et al. 1997; Beisser et al. 1998). Other knockout CMVs have been generated that had lower salivary gland replication rate phenotypes (Manning et al. 1992; Xiao et al. 2000; Kaptein et al. 2004), but the M33/R33 deletion mutant studies indicated an absolute requirement of the MCMV M33 and RCMV R33 vGPCR genes for salivary gland tropism in vivo.

Deletion of M78 and R78 from the MCMV or RCMV genome, respectively, resulted in mutant strains that were attenuated both in vitro and in vivo (Beisser et al. 1999; Oliveira and Shenk 2001). In contrast, deletion of UL78 from the HCMV genome resulted in a mutant strain that was neither attenuated in cell culture nor in renal artery explant culture (Michel et al. 2005). Yet, the expression of U51-specific siRNA in HHV-6-infected cells resulted in significantly lower levels of viral replication than the expression of control siRNA in infected cells (Zhen et a. 2005). These results indicate that the vGPCRs encoded by M78, R78 and U51 may fulfill different roles in replication than those encoded by HCMV UL78.

Generation of mutants in which the US28 gene was deleted from the HCMV genome has been instrumental in confirming the US28-specific signaling activities of infected cells. Deletion of US28 resulted in a phenotype in which infected cells were no longer able to bind RANTES and mobilize intracellular Ca^{2+} (Vieira et al. 1998). Infected smooth muscle cells were no longer capable of chemokinesis or chemotaxis (Streblow et al. 1999). This clearly demonstrated a role for US28 in the dissemination of HCMV within the host, by enabling infected cells to navigate through tissue by chemotaxis. Cells infected with US28 deletion mutant virus were no longer capable of sequestering RANTES and MCP-1 (Bodaghi et al. 1998; Randolph-Habecker et al. 2002), allowing more monocytes to be attracted by the medium from cell cultures infected with US28-deleted virus than by medium for cultures infected with wild type HCMV (Randolph-Habecker et al. 2002). Additionally, cells infected with US28 deletion mutant virus showed an increase in IL-8 secretion. These phenotypes indicate that HCMV US28 has an anti-inflammatory effect, which could ensure persistence of the infection.

Localization of CMV vGPCRs

Chemokine receptors are, subsequent to their synthesis, transported to the outer cellular membrane by intrinsic GPCR domains. At the outer membrane, they can interact with both extracellular chemokines and intracellular membrane-bound heterotrimeric G proteins to establish a signaling bridge. Of all CMV vGPCRs detected in either infected or transfected cells, only the vGPCRs encoded by MCMV M33 and RCMV R33 were clearly localized at the outer cellular membrane (Waldhoer et al. 2002; Casarosa et al. 2003). All other CMV vGPCRs were

restricted to the perinucleus, as well as to intracellular, endosomic, multivesicular bodies (Oliveira and Shenk 2001; Fraile-Ramos et al. 2002; Waldhoer et al. 2002; Kaptein et al. 2003; Penfold et al. 2003b; Margulies et al. 2006). The HCMV vGPCRs encoded by UL33, US27 and US28 were shown to be subject to multiple internalization mechanisms, via either the beta-arrestin- or clathrin-dependent endocytosis pathways (Fraile-Ramos et al. 2002, 2003; Waldhoer et al. 2002; Miller et al. 2003; Droese et al. 2004, Margulies et al. 2006). Despite the rapid internalization of the vGPCRs encoded by HCMV UL33 and US28, they both show significant constitutive signaling, and, in the case of US28, ligand binding as well as modulation of signaling. Nevertheless, it was reported that the high rate of endocytosis reduces US28-mediated constitutive signaling as well as the signal transduction modulating effects upon stimulation with fractalkine (Waldhoer et al. 2003). Interestingly, for the HHV-6 U51-encoded vGPCR, it was shown that membrane localization required a T cell-specific factor (Menotti et al. 1999). Most CMV vGPCR signaling and internalization assays have been performed in model cell types such as fibroblasts and immortalized kidney epithelial cell lines. Cell types more relevant to CMV infection in vivo, such as endothelial cells and cells of myeloid origin, might likewise provide the correct factor for stable vGPCR membrane expression.

It was suggested that mere expression of HCMV US28 on the leukocyte cell surface might enable adhesion to endothelial cells expressing membrane-bound fractalkine (Haskell et al. 2000). US28 transcription was shown to occur in latently infected monocytic cells in vitro (Beisser et al. 2001), as well as in PBLs in vivo in lung transplant recipients during primary CMV infection (Boomker et al. 2006b). These findings support the hypothesis that the US28-encoded cGPCR can act as an adhesion molecule aiding infected leukocytes to traffic from the bloodstream to solid tissue compartments. Thus, US28 could be a determinant of viral dissemination in the host. Yet, expression of US28-encoded vGPCRs on the outer cellular membrane of HCMV-infected cells in vivo has not yet been reported.

In addition to cell adhesion, US28 expression was also found to enhance viral entry and cell–cell fusion. The US28-encoded vGPCR was shown to interact with envelope proteins such as those from human immunodeficiency virus (HIV) and vesicular stomatitis virus (VSV) particles, enhancing HIV entry by acting as a (co-)receptor and enhancing membrane fusion upon interaction with HIV and VSV surface glycoproteins (Pleskoff et al. 1997, 1998). A similar role was established for HHV-6 U51 (Zhen et al. 2005). Whether such interactions have an impact on the progression of diseases related to HIV- and VZV-like viruses in vivo is still unclear.

Interestingly, most CMV vGPCRs that localize to intracellular multivesicular bodies, rather than the outer cellular membrane, have also been detected in excreted virus particles. These include vGPCRs encoded by HCMV UL33 (Fraile-Ramos et al. 2002), MCMV M78 (Oliveira and Shenk 2001), HCMV US27 (Fraile-Ramos et al. 2002; Waldhoer et al. 2002; Margulies et al. 2006), HCMV US28 (Fraile-Ramos et al. 2002; Waldhoer et al. 2002) and RhCMV RhUS28.5 (Penfold et al. 2003b). It can be hypothesized that vGPCRs, as

constituents of the viral envelope, are delivered to the cellular membrane immediately after fusion of envelope and membrane at the initial stage of infection. Upon this rapid delivery, modulation of intracellular processes may take place even before the immediate early stage of CMV infection commences. Interestingly, it was shown that HCMV US28-expressing cells were able to enhance the transcriptional activities regulated by the HCMV major immediate early promoter via the p38/MAPK and NFκ-B pathways (Boomker et al. 2006a). This observation supports a role for virion-borne vGPCR delivery for preimmediate early infection events.

Perspectives

The vCKs and vGPCRs described in this chapter are the result of an ever-continuing arms race between cytomegaloviruses and the host organism. The roles of the UL128-, UL146- and UL147-encoded vCKs will likely remain uncertain until the phenotypes of relevant deletion mutant CMVs are examined in vivo. Since UL33- and UL78-like genes are conserved in rodent CMV species, their contribution to viral dissemination and replication have been well established in vivo. Interestingly, a pantheon of phenomena has been attributed to HCMV US28. Similar to the vCKs, more in vivo work needs to be done to determine how these phenomena benefit CMV growth and survival in the host. CCMV is the most appropriate model virus to address these questions.

Most existing antiviral agents against CMV disease target the viral replication cycle. vCKs and vGPCRs are interesting targets in the development of a new class of antiviral agents, such as synthetic small molecule GPCR ligands and chemokine antagonists and inverse agonists. These agents could inhibit viral reactivation or dissemination rather than replication, thereby increasing the range of available drugs and their effectiveness against CMV disease.

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