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Review

Immune evasion by gammaherpesvirus genome maintenance proteins

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Viruses that establish lifelong latent infections must ensure that the viral genome is maintained within the latently infected cell throughout the life of the host, yet at the same time must also be capable of avoiding elimination by the immune surveillance system. Gammaherpesviruses, which include the human viruses Epstein–Barr virus and Kaposi's sarcoma-associated herpesvirus, establish latent infections in lymphocytes. Infection of this dynamic host-cell population requires that the viruses have appropriate strategies for enabling the viral genome to persist while these cells go through rounds of mitosis, but at the same time must avoid detection by host $CD8^+$ cytotoxic T lymphocytes (CTLs). The majority of gammaherpesviruses studied have been found to encode a specific protein that is critical for maintenance of the viral genome within latently infected cells. This protein is termed the genome maintenance protein (GMP). Due to its vital role in long-term latency, this offers the immune system a crucial target for detection and elimination of virus-infected cells. GMPs from different gammaherpesviruses have evolved related strategies that allow the protein to be present within latently infected cells, but to remain effectively hidden from circulating $CD8^+$ CTLs. In this review, I will summarize the role of the GMPs and highlight the available data describing the immune-evasion properties of these proteins.

Introduction

Herpesviruses represent a group of double-stranded DNA viruses distributed widely within the animal kingdom. The taxonomic classification of herpesviruses has recently been updated with the establishment of the order *Herpesvirales*, which includes the original family *Herpesviridae* and two new families, *Alloherpesviridae* and *Malacoherpesviridae* (Davison *et al.*, 2009). The family *Herpesviridae*, which contains eight viruses that infect humans, is the most extensively studied group within this order and comprises three subfamilies, namely *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. One of the principal characteristics of all herpesviruses is the ability to establish lifelong latent infections of their hosts. Latent infections are established following an initial productive primary infection and are characterized by persistence of the virus within specific host cells, with no detectable infectious virus production. Two fundamental biological features of these latent infections are the maintenance of the viral genome within the latently infected host cell and the ability of the virus to evade detection by the immune system. For the alphaherpesvirus herpes simplex virus, latent infection is established in non-dividing sensory neurons, where the viral genome is maintained in a quiescent state with only a single virus transcriptional unit being transcribed, and no detectable viral antigens expressed (Roizman *et al.*, 2007). Betaherpesviruses, such as human cytomegalovirus, establish latency in cells of the myeloid lineage and, whilst progress has been made in understanding betaherpesvirus latency, the mechanisms involved in maintenance of the viral genome within latently infected cells are poorly understood (Sinclair, 2008). Gammaherpesviruses establish latent infections within either B or T lymphocytes of their hosts and here the mechanisms important for maintenance of latency have been well-characterized. Infection of this dynamic host-cell population requires specific strategies to enable the virus genome to be maintained within actively dividing cells, yet at the same time the virus must avoid alerting the immune system to its presence. Studies of gammaherpesvirus latent infection have highlighted the importance of a single viral protein: termed the genome maintenance protein (GMP), this protein plays a crucial role in ensuring that the viral genome persists within latently infected cells and that it is partitioned evenly into daughter cells as the cell proceeds through cycles of cell division (reviewed by Lindner & Sugden, 2007; Feeney & Parish, 2009). Studies over the last few years have also revealed the strategies that enable the GMP to be expressed within latently infected cells, yet evade efficient detection by host $CD8^+$ cytotoxic T lymphocytes (CTLs). This review will summarize the mechanisms by which gammaherpesvirus GMPs function to ensure that the viral genome is maintained within the lymphocyte population, and focus specifically on the unique properties of these proteins that allow the virus to remain hidden from $CD8^+$ CTLs at crucial times during latent infection.

The subfamily *Gammaherpesvirinae*

Gammaherpesviruses belong to four separate genera: the well-established genera *Lymphocryptovirus* and *Rhadinovirus* and the more recently defined genera *Macavirus* and *Percavirus* (Davison *et al.*, 2009). Lymphocryptoviruses (LCVs) are found mainly in Old World primates, although they have now also been identified in New World primates, and importantly include one of two human gammaherpesviruses, *Human herpesvirus 4*, commonly known as Epstein–Barr virus (EBV) (Wang *et al.*, 2001; Kieff & Rickinson, 2007). Rhadinoviruses are found in both Old and New World primates, as well as in a range of other mammalian species. The rhadinoviruses that infect Old World primates are divided into two distinct lineages, RV1 and RV2 (Damania & Desrosiers, 2001). The RV1 branch includes the second human gammaherpesvirus, *Human herpesvirus 8*, more commonly known as Kaposi's sarcoma-associated herpesvirus (KSHV), and also the direct homologues of this virus that infect other primate species, such as macaque, chimpanzee and gorilla (Greensill *et al.*, 2000; Lacoste *et al.*, 2000; Schultz *et al.*, 2000). The RV2 lineage contains viruses that infect the same Old World primate species as the RV1 lineage, but appear more distantly related to KSHV (Greensill *et al.*, 2000; Lacoste *et al.*, 2000, 2001; Schultz *et al.*, 2000). Rhadinoviruses that infect New World primates include *Saimiriine herpesvirus 2*, commonly known as herpesvirus saimiri (HVS), which infects squirrel monkeys, and *Ateline herpesvirus 3*, which infects spider monkeys (Albrecht *et al.*, 1992; Albrecht, 2000). Another notable rhadinovirus is *Murid herpesvirus 4* (MuHV-4), also known as murine gammaherpesvirus 68, which is a natural pathogen of small rodents (Nash *et al.*, 2001; Blasdell *et al.*, 2003). *Macavirus* and *Percavirus* were recently established as separate genera within the subfamily *Gammaherpesvirinae*, and include species previously belonging to the genus *Rhadinovirus* and additional newly defined species (Davison *et al.*, 2009). Macaviruses include the viruses responsible for malignant catarrhal fever in ruminants. Percaviruses are a lineage of viruses that are hosted by perissodactyl and carnivore species.

Gammaherpesviruses are of primary interest due to the two human viruses, EBV and KSHV, and the diseases with which these viruses are linked. EBV is ubiquitous in the human population; seroconversion normally occurs in early childhood and >90 % of the human population carries the virus. Generally, most EBV-infected individuals carry the virus as an asymptomatic infection; however, a number of clinical diseases are associated with EBV infection. If exposure to the virus is delayed until early adolescence or later, this can lead to infectious mononucleosis (Niederman *et al.*, 1968). Infection with EBV is also associated with a number of human malignancies, including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma and an increasing number of other tumours (Rickinson & Kieff, 2007). KSHV is less ubiquitous than EBV, with geographical and demographic factors influencing its seroprevalence. Most notably, KSHV is present in approximately 5 % of the population in the USA and northern Europe, whereas its seroprevalence is estimated at up to 20 % in certain countries around the Mediterranean and at over 50 % in African countries (Ganem, 2007). KSHV was initially identified in Kaposi's sarcoma lesions isolated from AIDS patients, and is found in all cases of Kaposi's sarcoma (Chang *et al.*, 1994; Chang & Moore, 1996). KSHV is also associated with at least two B-cell malignancies: primary effusion lymphoma (PEL) and a subset of multicentric Castleman's disease (Cesarman *et al.*, 1995; Soulier *et al.*, 1995). The requirement for an animal model of gammaherpesvirus infection has also driven research on many other members of the subfamily *Gammaherpesvirinae*. This has identified a number of related genes that play central roles in the virus life cycle, such as the highly conserved GMP.

Role of the GMP

Upon primary infection, gammaherpesviruses enter the lytic (productive) cycle, but then quickly switch to begin establishment of the latent phase. This is characterized by a reduction in viral gene expression to a small subset of proteins termed the latent antigens, which include the GMP. For all gammaherpesviruses where sequence data are available, in only one case has a predicted GMP not been identified (Table 1). Selected individual GMPs will be discussed in detail later, but first we should consider the role of this protein in genome maintenance and its relevance as a target for the immune system. The model stems mainly from analysis of the GMP of EBV, but is predicted to functionally underpin the mechanism of all gammaherpesvirus GMPs. GMPs are DNA-binding proteins that have two fundamental roles required for effective genome maintenance: (i) they act as a bridge to tether the viral genome to host-cell chromosomes and (ii) they are involved in initiation of genome replication (reviewed by Feeney & Parish, 2009). During latent infection, the virus genome exists as a circular episomal element. By binding to sequence elements within the viral genome and at the same time interacting with a number of host-cell chromosome-associated proteins, the GMP ensures that, as the host cell progresses through mitosis, the viral episomes are partitioned to daughter cells. This provides continuous existence of the viral genome within the host cells. The association of the GMP with specific elements within the viral genome is a key factor in the initiation of replication of the genome, which ensures that there are sufficient copies of the episome to be partitioned evenly into daughter cells. The long-term site of latency is thought to be resting memory lymphocytes, and in these cells it is predicted that there is no viral gene expression (Thorley-Lawson & Gross, 2004). Nevertheless, it is highly likely that the GMP will be present to enable tethering of the viral episome to the host chromosome. Given the capacity of this memory lymphocyte population to divide, the virus will be triggered to express the GMP to ensure that new

Table 1. Details of known or predicted gammaherpesvirus GMPs

Genus/species	Common name	GMP*	Repeat†	Repeat length‡	Content§	GenPept accession no.
Lymphocryptovirus						
Callitrichine herpesvirus 3	Marmoset herpesvirus	ORF39	No	–	–	NP_733892
Human herpesvirus 4	Epstein–Barr virus	EBNA1	Yes	239	GA	YP_401677.1
Macacine herpesvirus 4	Rhesus lymphocryptovirus	rhEBNA1	Yes	47	GSA	YP_067973
Papiine herpesvirus 1	Herpesvirus papio	baEBNA1	Yes	49	GSA	AAA66373
Rhadinovirus						
Ateline herpesvirus 3	Herpesvirus ateles strain 73	ORF73	Yes	173	Acidic	AAC95598
Bovine herpesvirus 4	Movar virus	ORF73	No	–	–	NP_076567
Human herpesvirus 8	Kaposi's sarcoma-associated herpesvirus	LANA1	Yes	585	Acidic	ACC55944.1
Macacine herpesvirus 5‖	Rhesus rhadinovirus	ORF73	Yes/no¶	521	Acidic	ABH07414.1
Murid herpesvirus 4	Murine gammaherpesvirus 68	ORF73	No	–	–	AAB66457.1
Saimirine herpesvirus 2	Herpesvirus saimiri	ORF73	Yes	183	Acidic	NP_040275.1
Macavirus						
Alcelaphine herpesvirus 1	Wildebeest herpesvirus	ORF73	Yes	687	Acidic	ACC58118
Ovine herpesvirus 2	Sheep-associated malignant catarrhal fever virus	ORF73	Yes	328	Acidic	AAX58107
Percavirus						
Equid herpesvirus 2#	Equine herpesvirus 2	–	–	–	–	–

*GMPs have been identified either functionally or by sequence similarity. See text for further details.

†Presence of an internal amino acid repeat domain within the GMPs is indicated.

‡Size of the amino acid repeat is derived from either the prototypic strain or another representative virus isolate. Variation in size is seen in different isolates.

§Content of the amino acid repeat is indicated, either as specific amino acids (A, alanine; G, glycine; S, serine) or as an acidic domain.

‖Macacine herpesvirus 5 represents a group of closely related viruses that infect different Old and New World monkeys, which can be split into two lineages of the genus *Rhadinovirus*, RV1 and RV2 (see text).

¶Where characterized, only viruses of the RV1 lineage contain an internal amino acid repeat. The GenPept accession number given is for southern pigtail macaque herpesvirus strain M78114 ORF73, which contains a repeat domain.

#Sequence data are available for the complete genome of equid herpesvirus 2 (GenBank accession no. U20824); no GMP has been identified.

virus genomes can be tethered to host chromosomes within the new cells, to prevent dilution of the virus as latently infected cells go through rounds of mitosis.

Amongst gammaherpesviruses, there is little sequence similarity between different GMPs, although within each genus there is a higher degree of similarity. The region of highest conservation is within the C terminus, which acts as the DNA-binding domain. The most intriguing sequence feature in the majority of the GMPs studied is the presence of a central domain consisting of repeating amino acids (Figs 1 and 2). There is no overall consensus with respect to size or amino acid content of the repeats (Table 1). The repeat domain is not essential for genome-maintenance functions (Yates *et al.*, 1985; Cotter *et al.*, 2001; Griffiths *et al.*, 2008) and there are examples of GMPs that do not contain a repeat domain (Table 1) (Lomonte *et al.*, 1995; Virgin *et al.*, 1997).

GMPs and immune evasion

In addition to maintaining the viral genome within latently infected cells, at the same time, to achieve long-term latency, there is an absolute requirement to evade detection and elimination by the host immune response. $CD8^+$ CTLs recognize viral antigens expressed endogenously within cells. Viral antigens are degraded by the proteasome, and short peptides are presented on the cell surface in the context of major histocompatibility complex (MHC) class I molecules for recognition by circulating $CD8^+$ CTLs (Pamer & Cresswell, 1998). The actual source of the viral antigens was assumed historically to be the turnover of stable proteins, but whilst in some instances this may be the case, there is now increasing evidence to support a major role for defective ribosomal products (DRiPs) as the main source of antigenic peptides (Yewdell, 2007). DRiPs are derived from aberrant translation products generated from newly synthesized proteins. The use of DRiPs as the main source of antigenic peptides allows the rapid detection of active viral infection.

Gammaherpesviruses have evolved a multitude of approaches to evade immune detection (reviewed by Stevenson, 2004; Hansen & Bouvier, 2009). During latent infection, downregulation of viral gene expression is a key feature of immune evasion. However, due to the central

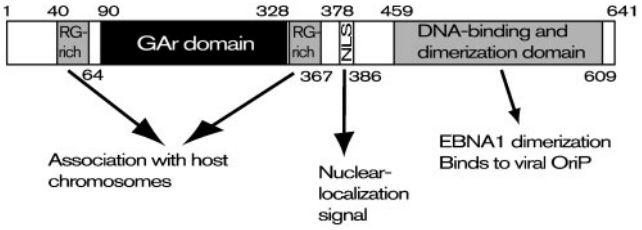

Fig. 1. Schematic representation of the EBV EBNA1 protein, depicting the functional domains essential for genome-maintenance functions. Two arginine/glycine-rich (RG-rich) regions, flanking the internal glycine/alanine repeat (GAr) domain, are involved in binding to host-cell chromosomes. The C-terminal region contains overlapping DNA-binding and dimerization domains, responsible for EBNA1 dimerization and binding to the OriP region of the viral genome. NLS denotes a nuclear-localization signal. For further details regarding the functional domains and interacting proteins, see Lindner & Sugden (2007) and Johannsen *et al.* (2009). The numbers shown refer to the amino acid coordinates for the EBNA1 protein of the B95.8 strain of EBV (GenPept accession no. YP_401677.1).

role of the GMP in long-term latency, any strategy to evade immune detection must per se involve the GMP itself. The overall effect of the immune-evasion properties of gammaherpesvirus GMPs is the same, although there are some important differences in the actual mechanisms employed by the GMPs of different viruses. The first clue came from studies of the EBV GMP.

Lymphocryptovirus GMPs

The prototype LCV is the human virus EBV. Since its identification in 1964 and association with an increasing number of human malignancies, this remains the most extensively studied gammaherpesvirus and is the paradigm for most aspects of gammaherpesvirus infection. The desire for a suitable animal model has driven research into other LCVs (Moghaddam *et al.*, 1997) and allowed the analysis and comparison of many gene homologues, including the GMP.

EBV: Epstein–Barr nuclear antigen 1 (EBNA1). The GMP of EBV is encoded by the latent antigen EBNA1, which is expressed along with seven other viral antigens during EBV latent infection (Kieff & Rickinson, 2007). The initial productive infection with EBV occurs within the oropharynx, involving either epithelial cells or B lymphocytes. EBV subsequently establishes a latent infection within the B-lymphocyte population. Expression of the eight viral latent antigens, six nuclear antigens (EBNA-1, -2, -3A, -3B, -3C and -LP), along with two latent membrane proteins (LMP1 and LMP2), leads to the transformation and proliferation of the infected B cell. This can be mimicked *in vitro* and such transformed B lymphocytes are termed B-lymphoblastoid cell lines (B-LCLs). This pattern of EBV latent infection has been termed 'latency III' (or the growth programme) (Thorley-Lawson & Gross, 2004; Rickinson & Kieff, 2007). As this initial proliferative infection is brought under control by the host immune response, some of the EBV-infected B cells survive to become long-lived peripheral blood memory B cells, carrying the EBV genome as an episomal element. In this form of latency, termed 'latency 0' (or the latency programme), all viral gene expression is switched off (Thorley-Lawson & Gross, 2004; Rickinson & Kieff, 2007). However, it is hypothesized that EBNA1 will be present within these cells to ensure binding of the viral episome to host chromosomes. Indeed, it has been shown that EBNA1 can bind to host chromosomes within cells in interphase (Nayyar *et al.*, 2009). When these EBV-positive memory B cells undergo cell division, EBNA1 transcription is initiated and EBNA1 protein is expressed, so that when the viral episome is replicated and partitioned to daughter cells, sufficient EBNA1 protein is available to ensure that new viral episomes are tethered to the host-cell chromosomes within these cells (Hochberg *et al.*, 2004). This form of latency is termed 'latency I' (or the EBNA1-only programme) (Thorley-Lawson & Gross, 2004; Rickinson & Kieff, 2007). Differential expression of EBNA1 within each form of EBV latency is determined by epigenetic events controlling alternative promoter usage (Schaefer *et al.*, 1997; Hutchings *et al.*, 2006; Day *et al.*, 2007).

The genome-maintenance functions of EBNA1 have been well-documented, and EBNA1 could be considered the prototypic GMP. For the B95.8 strain of EBV, EBNA1 is a 641 aa protein consisting of small N-terminal and larger C-terminal domains, separated by a 239 aa repeat domain consisting of glycine (G) and alanine (A) (GAr domain) (Fig. 1) (Baer *et al.*, 1984). Within different viral isolates, the GAr domain is conserved but varies in size (Falk *et al.*, 1995). Its role in maintaining the viral episome was first deciphered in the early 1980s (Yates *et al.*, 1985). EBNA1 binds via its C-terminal domain to a region in the viral genome termed OriP, the viral origin of replication. This region contains two elements, a family of repeats (FR element) consisting of 20 high-affinity 30 bp EBNA1-binding sites linked to four low-affinity binding sites within a region termed the dyad symmetry (DS) element (Rawlins *et al.*, 1985; Reisman *et al.*, 1985). This interaction is important for initiating replication of the viral episome and is vital for episomal maintenance. EBNA1, while bound to the viral genome, also attaches to host-cell chromosomes. This attachment is mediated by interaction of EBNA1 with host-cell chromosome-associated proteins, including histone H1 and EBNA1-binding protein 2 (Marechal *et al.*, 1999; Kapoor *et al.*, 2005). However, an alternative model where there is direct interaction between the GMP and chromosomal DNA via an AT-hook motif has also been proposed (Sears *et al.*, 2004); see Fig. 1 for a schematic of the structural features of EBNA1 that are important for genome maintenance.

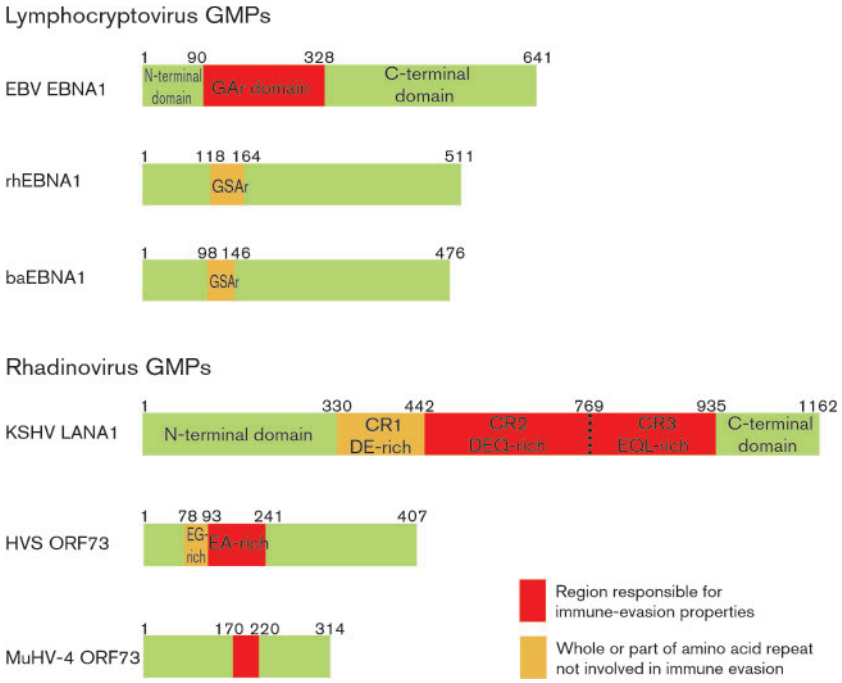

Fig. 2. Schematic representation of the gammaherpesvirus GMPs that have been investigated for immune-evasion properties [lymphocryptoviruses EBV (EBNA1), rhLCV (rhEBNA1) and baLCV (baEBNA1); rhadinoviruses KSHV (LANA1), HVS (ORF73) and MuHV-4 (ORF73)]. EBV EBNA1, rhEBNA1, baEBNA1, KSHV LANA1 and HVS ORF73 all contain N- and C-terminal domains separated by various-sized central amino acid repeat domains, highlighted in orange and red, containing the indicated amino acids. The regions indicated in black correspond to the experimentally defined minimal region required for immune-evasion properties. N.B. the repeat regions present in rhEBNA1 and baEBNA1 are defined as having no inhibitory properties (see text). MuHV-4 ORF73 does not contain a repetitive amino acid sequence, but the region of aa 170–220 (shown in red) has been shown to have immune-evasion properties. The amino acid coordinates shown are for the representative strains of each virus.

The immune response to EBV latent infection has been studied intensively, in particular the determination of how latently infected cells are able to avoid detection and elimination by host $CD8^+$ CTLs. Control of the proliferative latent infection (latency III) is the result of a vigorous CTL response targeting mainly the EBNA3 family of proteins (Rickinson & Moss, 1997). The downregulation of these antigens in other forms of latency allows EBV-infected cells to escape this aspect of the EBV-specific CTL response. However, given the critical role of EBNA1 during latency, recognition of EBNA1 by $CD8^+$ CTLs has been of longstanding interest. In a mouse tumour-rejection model, introduction of EBNA1 into a non-immunogenic carcinoma cell line failed to confer immunogenicity, whereas the equivalent experiment with LMP1-transfected carcinoma cells successfully induced a rejection response (Trivedi *et al.*, 1991), suggesting that EBNA1 may not be a strong immunogen for $CD8^+$ CTLs. These observations were reinforced by studies in humans. Independent reports analysing the fine specificity of the $CD8^+$ T-cell response to EBV latent antigens failed to detect any responses to EBNA1 (Khanna *et al.*, 1992; Murray *et al.*, 1992). However, at the same time, using computer algorithms to predict potential human leukocyte antigen (HLA)-binding peptides and *in vitro* HLA peptide-binding assays, it was shown that EBNA1 contained a number of peptides capable of binding to HLA class I molecules (Stuber *et al.*, 1995). This led to the hypothesis that the EBNA1 protein was protected from recognition by $CD8^+$ CTLs and, amongst the potential mechanisms suggested, it was postulated that the unique GAr domain might in some way prevent the generation of antigenic peptides (Fig. 2). In seminal work from the Masucci group (Levitskaya *et al.*, 1995), this was shown to be the case. Lacking a natural EBNA1 $CD8^+$ T-cell epitope, they introduced a model T-cell epitope into the backbone of EBNA1, with and without the GAr domain, and looked at presentation to epitope-specific CTLs in standard 5 h cytotoxicity assays. The GAr domain was shown to act *in cis* to prevent presentation of antigenic peptide via the MHC class I pathway to $CD8^+$ CTLs (Levitskaya *et al.*, 1995). It was subsequently shown that the GAr domain conferred enhanced stability to the EBNA1 protein by preventing proteasome-mediated degradation (Levitskaya *et al.*, 1997). Thus, it was proposed that, by inhibiting proteasome function, the GAr domain prevented the generation of antigenic peptides (Fig. 3). The ability of the GAr domain to inhibit proteasome-mediated degradation has been well-characterized (Sharipo *et al.*, 1998; Heessen *et al.*, 2002, 2003; Hoyt *et al.*, 2006). However, a more recent report by Daskalogianni *et al.* (2008), comparing the turnover of EBNA1 protein with that of EBNA1 deleted of the GAr domain in cycloheximide pulse–chase experiments, suggested that the stability of EBNA1 was not related to the presence of the GAr domain. However, this requires further experimental support.

Despite the apparent protection of EBNA1 from processing and presentation by the MHC class I pathway, it was subsequently found that EBNA1-specific $CD8^+$ CTLs were present in EBV-seropositive individuals. By conventional *in vitro* stimulation of peripheral blood mononuclear cells with autologous EBV-transformed B-LCLs, EBNA1-specific T-cell clones were identified by screening against EBNA1 deleted of the GAr domain (Blake *et al.*, 1997). The EBNA1 peptide epitopes and HLA-restricting elements

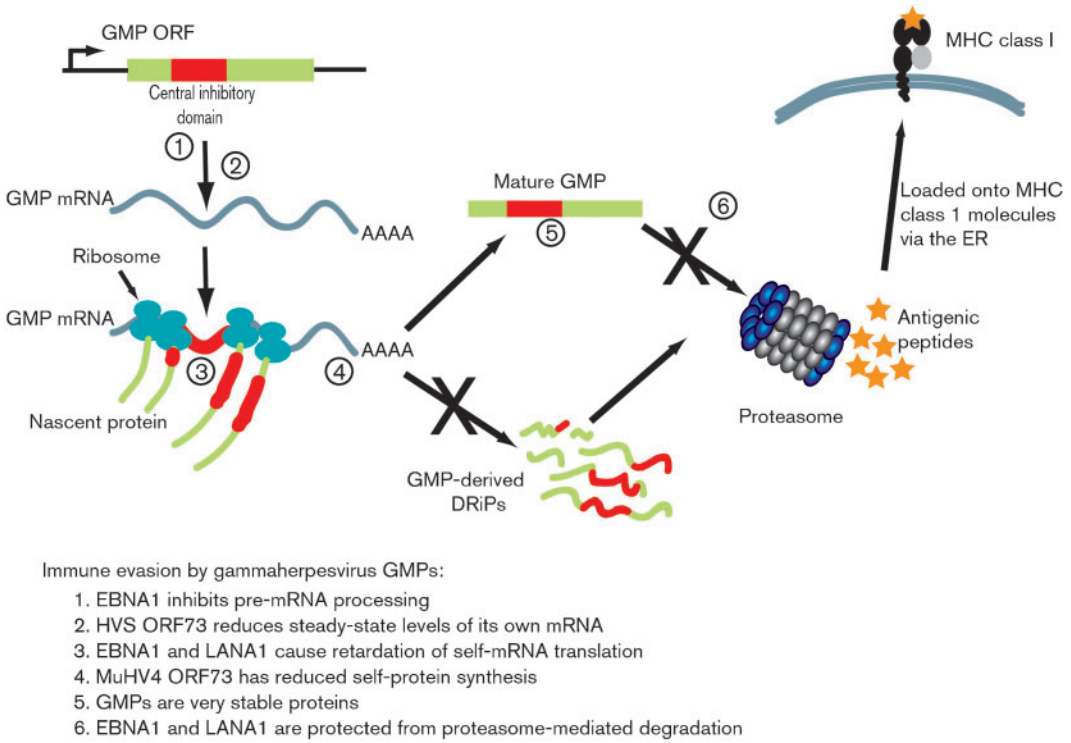

Fig. 3. Properties of gammaherpesvirus GMPs that contribute to their ability to evade processing and presentation by the MHC class I pathway to $CD8^+$ CTLs. EBV EBNA1 inhibits processing of its own pre-mRNA, has a reduced translation rate due to the GAr domain, is a stable protein and is protected from proteasome-mediated degradation by the GAr domain (1, 3, 5 and 6). KSHV LANA1 has a reduced translation rate due to the central acidic domain, is a stable protein and is protected from proteasome-mediated degradation by the central acidic domain (3, 5 and 6). HVS ORF73 reduces the steady-state levels of its own mRNA and is a stable protein (2 and 5). MuHV-4 ORF73 has reduced self-protein synthesis and is a stable protein (4 and 5). The central inhibitory domain as indicated represents the region mapped within the GMPs of EBNA1, KSHV LANA1, HVS ORF73 and MuHV-4 ORF73 that has been shown to have immune-evasion properties. ER, Endoplasmic reticulum.

were mapped, confirming that these were MHC class I-restricted responses. However, in standard cytotoxicity assays, these clones failed to recognize either the autologous B-LCL or full-length EBNA1 when overexpressed in HLA-matched target cells, supporting the role of the GAr domain in protecting EBNA1 from recognition by T cells. It was suggested that these EBNA1-specific clones were primed *in vivo* by the MHC class I cross-priming pathway, which could process and present GAr-positive EBNA1 efficiently (Blake *et al.*, 1997). It was proposed that these cross-primed EBNA1-specific T cells would not recognize endogenously expressed EBNA1, allowing EBNA1-positive cells to remain hidden from immune surveillance. However, although this may indeed be the mechanism of induction of such responses *in vivo*, it turns out that these T cells are able to recognize EBNA1-positive cells, albeit inefficiently.

Based on the premise that EBNA1-specific responses were present in EBV-seropositive individuals, ELISPOT assays were used to re-examine the $CD8^+$ T-cell response to EBNA1. Responses were found in a higher number of individuals than had previously been thought and, in some cases, these responses were found to be the immunodominant response (Subklewe *et al.*, 1999; Blake *et al.*, 2000). The identification of strong EBNA1-specific $CD8^+$ T-cell responses led to the re-evaluation of EBNA1 as a target for CTLs. Three independent studies have subsequently shown that $CD8^+$ T-cell epitopes present in EBNA1 can be presented via the endogenous MHC class I pathway. Using gamma interferon (IFN-γ) release and extended cytotoxicity assays, Voo *et al.* (2004) were the first to report recognition of endogenously expressed EBNA1. Significantly, they reported a small increase in recognition if the GAr domain was removed. They made a second important observation when they showed that new protein synthesis was required for effective recognition of EBNA1. This was the first evidence that $CD8^+$ T-cell epitopes from EBNA1 were derived from DRiPs and was in support of work from the Fahraeus group, which had initially implicated the importance of DRiPS for EBNA1 by biochemical studies (Yin *et al.*, 2003; see later). This was followed by two further reports supporting these observations. In a study re-examining recognition of B-LCLs by EBNA1-specific T cells, Lee *et al.* (2004) used paired cell

lines carrying either EBNA1 or a GAr-deleted EBNA1 as target cells. These experiments measuring IFN-γ release showed that EBNA1 was naturally processed within a B-LCL and presented to $CD8^+$ T cells. Again, recognition was lower than for the matched cell line carrying the GAr-deleted EBNA1. In long-term co-culture assays, this recognition of B-LCLs by EBNA1-specific T cells led to a strong and specific inhibition of B-LCL outgrowth *in vitro*. This did not appear to be influenced by the GAr domain (Lee *et al.*, 2004). The Khanna group have also shown endogenous processing of EBNA1 and, again, GAr-deleted EBNA1 was presented at a slightly higher level (Tellam *et al.*, 2004). They also provided support for DRiPs as the source of EBNA1 antigen.

Taken together, these studies suggested that the GAr-mediated protection was not as robust as first thought and that EBNA1 could be targeted by $CD8^+$ CTLs. However, there was general agreement that deletion of the GAr domain leads to an increase in recognition, implying a protective role for the domain. However, in long-term outgrowth assays, this protection was not sufficient to protect EBNA1-expressing B-LCLs from recognition and destruction by EBNA1-specific $CD8^+$ T cells. These data also suggested that DRiPs derived from the translation of EBNA1 mRNA and not the degradation of stable EBNA1 protein were the source of antigenic peptide. These results sit well with the mechanism of long-term EBV latency. In the resting B cell, EBV gene expression is silent (i.e. latency 0), but EBNA1 protein is present, tethering the viral episome to the host chromosome. As mature EBNA1 protein is not processed for presentation to $CD8^+$ CTLs and is an extremely stable protein, due to the GAr domain preventing proteasome-mediated degradation, no EBNA1 expression is required and this cell will remain hidden from virus-specific CTLs. However, when these cells are stimulated to divide, EBNA1 gene expression is switched on (i.e. latency I) and the latently infected cells will become a target for $CD8^+$ CTLs, due to the production of EBNA1-derived DRiPs. This will prevent uncontrolled expansion of EBNA1-expressing cells, as seen in the outgrowth assay. However, *in vivo* it is likely that this expansion of memory B cells is very brief and that they soon revert back to resting cells, in which EBNA1 expression is turned off. Thus, there is a small period where EBNA1 is being expressed and the cell becomes susceptible to EBNA1-specific CTLs. However, EBV has another trick up its sleeve that allows these cells to evade efficient detection. The Fahraeus group (Yin *et al.*, 2003) showed that the GAr domain also functions to delay the generation of DRiPs by reducing the translation rate of EBNA1 mRNA. Starting from the hypothesis that the GAr-mediated inhibition of proteasomal degradation would mean that, over time, EBNA1 protein would accumulate in cells, they tested this by simply transfecting target cells with appropriate plasmid constructs and analysing steady-state protein levels. The observed result disproved their initial hypothesis, as steady-state EBNA1 protein levels were significantly lower than those of EBNA1 deleted of the GAr domain. This was not an effect at an RNA stage, as mRNA levels for each transcript were equivalent. After further biochemical analysis, they determined that the GAr domain functions *in cis* to inhibit self-synthesis of EBNA1 via a reduction in the mRNA translation rate (Yin *et al.*, 2003). This correlates with a reduction in recognition of EBNA1 by $CD8^+$ CTLs (Fig. 3) (Yin *et al.*, 2003; Tellam *et al.*, 2007a). This evasion strategy will allow EBV to express the GMP as cells divide, without the $CD8^+$ T-cell response being able to eliminate all EBNA1-expressing cells. When some cells revert back to the resting stage, EBNA1 expression will be switched off and the cells will again be invisible to the EBV-specific immune response, thus ensuring a relatively constant population of long-term latently infected cells.

The mechanism by which the GAr domain reduces the rate of translation is unclear. A study by the Khanna group has implicated the EBNA1 mRNA as the mediator of this effect (Tellam *et al.*, 2008). The EBNA1 mRNA sequence has an extensive purine bias within the GAr domain-encoding region, in contrast to the normal mammalian codon usage for these amino acids. Analysis of the secondary structure of the GAr-encoding sequence revealed a lack of any real consensus structure. However, replacing the third base position purines with pyrimidine nucleotides increased the stem–loop structure. This also greatly increased the rate of EBNA1 translation both *in vitro* and *in vivo*, and resulted in enhanced presentation of EBNA1 to $CD8^+$ CTLs. Such an immune-evasion strategy based on codon usage has been proposed previously (Cristillo *et al.*, 2001). Their predicted model is that the lack of any real secondary structure across the GAr domain causes an inhibition of elongation rate. This model has been questioned by a recent study claiming that the nascent GAr peptide delays the assembly of the initiation complex on its own mRNA (Apcher *et al.*, 2009).

The final piece of weaponry that EBV has to protect EBNA1 from the immune response is at the level of transcription. During latency I, EBNA1 autoregulates its own transcription (Sample *et al.*, 1992). In a more recent study, this autorepression of EBNA1 expression is shown to be the result of an inhibition of pre-mRNA processing: EBNA1 acts either post- or co-transcriptionally to prevent processing of the primary EBNA1 transcript (Yoshioka *et al.*, 2008). It is proposed that this autoregulation would control unnecessary new synthesis of EBNA1 protein. In turn, this would subsequently limit the generation of EBNA1-derived DRiPs, so contributing to the overall immune-evasion strategy of EBNA1 (Fig. 3).

$CD4^+$ T-cell recognition of EBNA1 has also been investigated. It has been shown that EBNA1 protein can be processed and presented to $CD4^+$ T lymphocytes (Leen *et al.*, 2001). Importantly, recent studies show that endogenous EBNA1 can be loaded onto MHC class II molecules via an intracellular mechanism, perhaps autophagy (Paludan *et al.*, 2005; Mackay *et al.*, 2009). The GAr domain appears to have little influence on the processing of

$CD4^+$ T-cell peptides from EBNA1. However, the recognition of EBNA1 by $CD4^+$ T cells was found to be an inefficient process, requiring a long period for processing and presentation of EBNA1 protein (in comparison to $CD8^+$ recognition) (Mackay *et al.*, 2009). Nevertheless, these data may have important implications for T-cell recognition of the latently infected cell, which has switched off gene expression but remains GMP-positive. These studies are in their infancy and require further detailed experimental analysis.

Other lymphocryptoviruses. LCVs closely related to EBV have been identified in both Old and New World primates. The establishment of LCV-infected B-LCLs has allowed complete or partial sequencing of the genomes of these viruses, and the LCV genes have been compared with their EBV counterparts (reviewed by Wang *et al.*, 2001). The most extensively studied are the LCVs of baboons and rhesus monkeys: *Papiine herpesvirus 1* (herpesvirus papio; baLCV) and *Macacine herpesvirus 4* (rhesus lymphocryptovirus; rhLCV), respectively. Gene homologues of EBV EBNA1 have been identified in both viruses (Table 1). Baboon EBNA1 (baEBNA1) and rhesus EBNA1 (rhEBNA1), although being slightly smaller than EBV EBNA1 at 511 and 476 aa, respectively, compared with 641 aa for EBV EBNA1, overall each have a high degree of amino acid identity to EBV EBNA1 (Yates *et al.*, 1996; Blake *et al.*, 1999). Both baEBNA1 and rhEBNA1 contain internal amino acid repeats differing slightly from that present in EBV EBNA1, as in addition to having glycine and alanine, they also contain serine (Fig. 2). baEBNA1 has a repeat domain consisting of seven perfect repeats of a GAGAGGS motif. rhEBNA1 contains four perfect repeats of a GAGGS motif preceded by three GAGGS repeats interspersed with 12 additional amino acids, forming seven glycine/alanine-rich repeats within a 47 aa stretch. In this review, these repeats will be termed the GSAr domains to depict the presence of serine amino acids within the repeat. The highest region of conservation is within the C terminus, and the functional properties important for genome maintenance appear well-conserved in both of these predicted LCV GMPs; both proteins have been shown to support EBV OriP-dependent plasmid maintenance and replication (Yates *et al.*, 1996; Blake *et al.*, 1999).

Their predicted role as GMPs and the conservation of GAr-like internal repeats suggested that the ba- and rhEBNA1 proteins would, similarly to EBV EBNA1, be protected from recognition by $CD8^+$ CTLs. This was initially investigated by using a series of chimeric constructs in a manner analogous to that used to first describe the EBV EBNA1 GAr-mediated inhibition of antigen processing (Blake *et al.*, 1999). The ba- and rhGSAr domains were engineered into the EBV EBNA1 sequence in place of the endogenous GAr. These constructs were introduced into both human and simian B-LCLs, and presentation of the endogenous EBNA1 $CD8^+$ T-cell epitopes was investigated by conventional cytotoxicity assay. These experiments suggested that the ba- and rhGSAr domains did not prevent processing and presentation of these chimeric constructs to $CD8^+$ CTLs. Similar data were generated in a parallel experiment using all simian-derived components (Blake *et al.*, 1999), suggesting that, in contrast to the human LCV EBNA1, these simian LCVs did not employ a similar immune-evasion strategy for their GMP. Surprisingly, a follow-up study looking at immune response to rhLCV latent antigens generated contradictory data (Fogg *et al.*, 2005). *Ex vivo* analysis of T lymphocytes from naturally or experimentally infected rhesus macaques at the New England Primate Research Center (Southborough, MA, USA) identified a strong response to rhEBNA1 in 11 of 23 animals. These responses were detected by using an IFN-γ ELISPOT assay after stimulation with full-length rhEBNA1. However, in cytotoxicity assays, polyclonal T cells expanded *in vitro* using autologous rhLCV B-LCL failed to recognize endogenously expressed rhEBNA1. This was despite the fact that putative rhEBNA1 peptide epitopes were mapped and used to confirm the presence of rhEBNA1-specific T cells within the polyclonal populations. Even after the isolation of rhEBNA1-specific T-cell clones by limiting-dilution analysis, there was no recognition of either the rhLCV B-LCL- or recombinant vector-expressed full-length rhEBNA1 in either cytotoxicity or IFN-γ-release assays. However, in the authors' words, there was 'modest' recognition of the GSAr-deleted rhEBNA1, suggesting that, in this system, the internal repeat was functioning to inhibit processing and presentation of rhEBNA1.

A more recent series of experiments has also addressed this issue and provides data in agreement with the first report by Blake *et al.* (1999). Expanding on work probing the effect of the rate of translation on T-cell recognition of EBNA1, Tellam *et al.* (2007b) used a biochemical approach to compare translation of simian and EBV EBNA1 proteins. Both rh- and baEBNA1 were translated at a higher rate than EBV EBNA1 and, unlike EBV EBNA1, deletion of the GSAr domains had no effect on translation rates. The authors inferred from these data that the rh- and baGSAr domains had no effect on self-protein synthesis and were unlikely to affect the generation of DRiPs. In support of this, the rhGSAr domain had no significant effect on the presentation of a model epitope from a chimeric rhEBNA1 in *in vitro* antigen-presentation assays and *ex vivo* T-cell stimulation assays (Tellam *et al.*, 2007b). Studies with cycloheximide suggested that the source of antigenic peptide in these assays was newly synthesized protein.

It is difficult to reconcile the different results from these studies. It may simply be that the rhEBNA1-specific clones isolated were of low affinity such that, in both IFN-γ-release and cytotoxicity assays, they failed to recognize endogenously expressed rhEBNA1 and only marginally recognized the GSAr-deleted form. This contrasts directly with the EBV EBNA1 studies (see earlier). In the study by Fogg *et al.* (2005), no mechanism was put forward for the

proposed inhibitory action of the rhGSAr domain and, based on the work of Tellam *et al.* (2007b), any mechanism is unlikely to be similar to that of the EBV GAr. The mechanism(s) for controlling transcription of simian EBNA1 genes appear analogous to those of EBV EBNA1 (Ruf *et al.*, 1999). Thus, it would appear surprising that the simian EBNA1 proteins are not protected from CTL recognition, but this remains an open question that can only be resolved by further studies.

The first LCV from a New World monkey was identified when an EBV-related virus was isolated from spontaneous B-cell lymphomas of common marmosets; it was termed marmoset lymphocryptovirus or, more formally, *Callitrichine herpesvirus 3* (CvHV3; Cho *et al.*, 2001). Sequencing of the viral genome revealed a high degree of similarity to EBV and other Old World primate LCVs, with a large number of gene homologues (Cho *et al.*, 2001; Rivailler *et al.*, 2002). Open reading frame (ORF) 39 was found to have 39 % overall amino acid identity with EBV EBNA1, and 42 % identity within the C-terminal regions. Despite this high degree of sequence similarity, no significant GAr-like internal repeat was found in this protein. No functional studies have yet been carried out on ORF39, so it remains to be determined whether this is the CvHV3 GMP (Table 1). However, based on the sequence similarity, particularly within the C terminus, this would seem likely. It also remains to be determined whether ORF39 has immune-evasion properties.

Rhadinovirus GMPs

The prototypic rhadinovirus is HVS; however, since identification of KSHV, this human virus has become the focus of attention within this genus and has driven research into related viruses as animal models of KSHV. GMPs have been identified through functional studies or predicted based on sequence analysis for a number of rhadinoviruses (Table 1). The genomes of rhadinoviruses are collinear and the GMPs are encoded by ORF73. In the case of KSHV, the ORF73 GMP is termed latency-associated nuclear antigen 1 (LANA1); for all other rhadinoviruses, the GMPs will be termed ORF73. The best-characterized GMPs are those of KSHV and HVS, although analysis of the MuHV-4 GMP has also allowed valuable *in vivo* studies to be carried out in an animal model of gammaherpesvirus infection.

KSHV: LANA1. In asymptomatic KSHV carriers, the virus establishes a latent infection in B lymphocytes and the viral genome persists within cells as a nuclear episome (Mesri *et al.*, 1996; Renne *et al.*, 1996). Understanding of KSHV latency is less advanced than that of EBV latency, although it is assumed that there will be a number of parallels between the two. KSHV gene expression is restricted within latently infected cells. *In vitro* studies using KSHV-infected PEL cells show that five genes are expressed in this model of latent infection. The major latent transcript is a tricistronic message encoding three genes: ORFs 71, 72 and 73 (Dittmer *et al.*, 1998; Talbot *et al.*, 1999). Two other transcripts have been identified, corresponding to the kaposin locus and ORF K10.5 (Sadler *et al.*, 1999; Rivas *et al.*, 2001). On the major latency transcript, ORFs 71 and 72 encode a viral homologue of cellular cyclin D (v-cyclin) and a viral inhibitor of FLICE (v-FLIP), respectively (Chang *et al.*, 1996; Thome *et al.*, 1997), whilst ORF73 encodes a large nuclear protein termed LANA1 (Kedes *et al.*, 1997; Kellam *et al.*, 1997; Rainbow *et al.*, 1997). LANA1 derived from the PEL line BC-1 comprises 1162 aa (Russo *et al.*, 1996) and consists of three distinct regions: N- and C-terminal domains of 337 and 240 aa, respectively, are separated by a large (585 aa) central repeat (CR) domain consisting mainly of the acidic amino acids glutamine (Q), glutamic acid (E) and aspartic acid (D). The CR domain comprises imperfect repeats of these amino acids and is subdivided further into three subdomains based on the amino acid content: CR1 (aa 30–442) consists of a DE-rich region, CR2 (aa 442–768) consists of a DEQ-rich region and CR3 (aa 769–935) consists of an EQL-rich region (Fig. 2). Variation in the size of the central acidic domain contributes to the considerable variation in the sizes of LANA1 seen in different KSHV isolates (Gao *et al.*, 1999).

Although LANA1 has little similarity to EBNA1, it has been identified as the GMP. Its role in episomal maintenance has been well-characterized. LANA1 associates with the viral genome in infected cells and co-localizes with the viral genome in interphase nuclei and on mitotic chromosomes (Ballestas *et al.*, 1999; Cotter & Robertson, 1999). Studies using both KSHV-derived cosmids and a recombinant KSHV cloned in a bacterial artificial chromosome (BAC) have shown that LANA1 is essential for long-term maintenance (Ballestas *et al.*, 1999; Cotter & Robertson, 1999; Ye *et al.*, 2004). LANA1 binds preferentially to the terminal repeat (TR) region of the KSHV episome via its C-terminal domain (Ballestas & Kaye, 2001; Cotter *et al.*, 2001) and, via its N-terminal domain, interacts with the host chromosome by association with histone H2A and H2B (Barbera *et al.*, 2004, 2006). Although not yet formally proven, by analogy with EBV, it is predicted that similar forms of KSHV latency occur in asymptomatic individuals, where LANA1 is likely to be present within resting memory lymphocytes but not expressed, and where LANA1 alone is expressed as the resting latently infected cells divide. Thus, it was predicted that LANA1 would be protected from recognition by $CD8^+$ CTLs and that the acidic CR domain would play a functional role. A number of studies using ELISPOT analysis and T-cell proliferation studies have identified KSHV-specific $CD8^+$ T-cell responses targeting LANA1 (Brander *et al.*, 2002; Woodberry *et al.*, 2005; Bihl *et al.*, 2007). So, like EBNA1, LANA1 is recognized by $CD8^+$ T cells *in vivo*. However, no-one has yet isolated these LANA1-specific T cells *in vitro* and tested them for recognition of endogenously expressed LANA1. Presentation of endogenously expressed LANA1 to $CD8^+$ CTLs, and the effect of the acidic CR domain, have been investigated by using chimeric LANA1 constructs contain-

ing a model T-cell epitope (Zaldumbide *et al.*, 2007). This revealed that the acidic repeat domain of LANA1 did block presentation of $CD8^+$ T-cell epitopes. The LANA1 repeat functioned *in cis* and also contributed to the stability of LANA1 (Fig. 3) (Zaldumbide *et al.*, 2007). A more biochemical approach to investigate the properties of the acidic CR domain was used by the Moore group (Kwun *et al.*, 2007). As with the EBNA1 GAr domain, the acidic repeat was shown to reduce the translation rate of LANA1. The effector region was mapped to a peptide sequence spanning the junction of the CR2 and CR3 subdomains (Fig. 2). The CR2CR3 region was also shown to stabilize proteins by inhibiting proteasomal processing (Kwun *et al.*, 2007). Thus, it was proposed that the acidic CR domain has similar immune-evasion functions to the GAr domain of EBNA1 (Fig. 3). Retardation of translation will interfere with production of DRiPs, and stabilization of LANA1 will maintain the presence of the protein in resting latently infected cells.

Although there were clear functional similarities to the EBNA1 GAr domain, intriguingly there were also a number of areas in which the LANA1 acidic domain behaved differently. When looking at the ability of the domain to act *in cis* or *in trans*, surprisingly Kwun *et al.* (2007) found that it could also block translation *in trans* and, in their hands, this was also the case for the EBNA1 GAr domain. However, this required high levels of expression for both LANA1 and EBNA1 in these *in vitro* experiments and so may not reflect the *in vivo* mechanisms accurately. The CR2CR3 domain was also found to retard translation when fused to a heterologous protein at both the N- and C-terminal regions, contrasting with the EBNA1 GAr domain, which was shown to retard translation only when located towards the N terminus (Yin *et al.*, 2003; Kwun *et al.*, 2007). The mechanism by which the central acidic domain influences the translation rate remains to be determined. However, the effect is mediated by the amino acid sequence rather than at the nucleotide level, as introduction of a stop codon between the CR2 and CR3 subdomains negates the inhibitory effect (Kwun *et al.*, 2007). This is a significant observation, as although there is little amino acid similarity between the repeat domains of LANA1 and EBNA1, it is interesting to note that the mRNA sequences are very similar. Interestingly, a frame-shift in the EBNA1-encoding sequence switches from a repeat domain consisting of the amino acids G and A to a repeat of amino acids G, Q and E that shares approximately 65 % similarity with the LANA1 repeat, and can inhibit the presentation of $CD8^+$ T-cell peptides (Ossevoort *et al.*, 2007). This is suggestive of a common origin for the individual nucleotide repeat sequences, although they may have diverged translationally and mechanistically.

HVS: ORF73. HVS naturally infects squirrel monkeys and establishes an asymptomatic latent infection in T lymphocytes, although infection of other New World primates can lead to acute T-cell lymphomas (reviewed by Fickenscher & Fleckenstein, 2001). HVS is also capable of transforming T lymphocytes of humans and rabbits (Medveczky *et al.*, 1989; Biesinger *et al.*, 1992). HVS strains are classified based on their pathogenic potential and sequence divergence within the left-hand TR region of the genome into three subgroups: A, B and C (Medveczky *et al.*, 1984). The transforming properties of HVS subgroup A are due to a single protein, the saimiri transforming protein A (stp-A), whereas subgroup C viruses encode a homologue of this protein termed stp-C and a second transforming protein termed TIP (Biesinger *et al.*, 1990; Jung & Desrosiers, 1991; Jung *et al.*, 1991). Immortalized T cells contain the viral genome as a multi-copy episome, without production of virus particles (Werner *et al.*, 1977; Kaschka-Dierich *et al.*, 1982). Initially latently infected T cells were shown to express the transforming genes, although subsequent analysis revealed that they were dispensable for long-term episomal maintenance (Fickenscher *et al.*, 1996, 1997; Duboise *et al.*, 1998). Using a lung carcinoma cell line stably maintaining HVS DNA as a non-integrating episome as an *in vitro* model of HVS latency, gene expression was shown to be limited to ORFs 71, 72 and 73 (Hall *et al.*, 2000). These genes are expressed as a polycistronic message and, as with KSHV, ORF71 encodes a v-cyclin D (Chang *et al.*, 1996), ORF72 encodes a vFLIP (Thome *et al.*, 1997) and ORF73 encodes the GMP (Collins *et al.*, 2002; Verma & Robertson, 2003; Calderwood *et al.*, 2004). The GMP from the prototype HVS strain A11 encodes a 407 aa protein. Like most GMPs, it comprises three distinct domains, with a large repeat region flanked by small N-terminal and larger C-terminal domains (Fig. 2). With the exception of the C-terminal domain, there is little similarity to KSHV LANA1 or other GMPs. However, the HVS ORF73 internal repeat domain is analogous to that of KSHV LANA1, being composed of acidic amino acids. In this case, it consists of two distinct elements, a glutamic acid- and glycine-rich (EG-rich) region linked to a glutamic acid- and alanine-rich (EA-rich) region (Fig. 2). In the A11 and C488 strains, respectively, the EG-rich region is 15 and 111 aa, whilst the EA-rich region is 147 and 132 aa (Albrecht *et al.*, 1992; Ensser *et al.*, 2003; Verma & Robertson, 2003). HVS ORF73 has been shown to have similar genome-maintenance properties to KSHV LANA1 and EBV EBNA1. A recombinant HVS BAC lacking ORFs 71–73 failed to persist within dividing cell populations, and episomal maintenance could be rescued by reintroduction of ORF73 (Calderwood *et al.*, 2005). HVS ORF73 binds to *cis*-acting DNA elements in the TR region of the virus genome via its C-terminal domain and this association is essential for episomal persistence (Collins *et al.*, 2002; Verma & Robertson, 2003; Calderwood *et al.*, 2004). HVS ORF73 also interacts with host-cell mitotic chromosomes, mediated by interaction with the host-cell proteins methyl-CpG-binding protein 2 and histone H1 (Griffiths & Whitehouse, 2007; Griffiths *et al.*, 2008).

The important role played by HVS ORF73 suggested that it too would be protected from recognition by $CD8^+$ CTLs.

Perhaps not unexpectedly, the large acidic repeat of HVS ORF73 was shown to have immune-evasion properties. By using chimeric HVS ORF73 constructs containing a model T-cell epitope, it was shown that the presence of the acidic repeat reduced presentation of peptides to $CD8^+$ CTLs (Gao *et al.*, 2009). Steady-state levels of ORF73 protein are reduced due to the presence of the acidic repeat; however, analysis of the mechanisms responsible revealed some critical differences with respect to the mechanism of action of the HVS acidic repeat, compared with both KSHV LANA1 and EBV EBNA1 repeats. The stability of HVS ORF73 is not influenced by the acidic repeat and, importantly, neither does the acidic repeat have any inhibitory effect on the rate of ORF73 mRNA translation. In contrast, it was shown that steady-state levels of ORF73 mRNA were reduced due to the presence of the repeat (Fig. 3). Comparison of the ORF73 sequences from the A11 and C488 strains of HVS highlighted the EA region as the most conserved element of the repeat. A single copy of the motif EEAEEAEEE, present multiple times in the EA-rich region of both strains, was sufficient to inhibit antigen presentation and to reduce ORF73 mRNA levels, and an additive effect was seen as the number of motifs was increased to six (Gao *et al.*, 2009). It is proposed that the reduction in mRNA levels will reduce the total amount of ORF73 translated, rather than translation rates per se, and thus will also influence the generation of ORF73-derived DRiPs. It was predicted that the HVS ORF73 acidic repeat functions either by destabilizing ORF73 mRNA or by reducing the transcription rate of the ORF73 gene. Surprisingly, we found that the presence of the acidic domain actually acts to stabilize the mRNA (Gao *et al.*, 2009). To maintain steady-state levels, it must also act to control the rate of accumulation, i.e. transcription rate, although this has yet to be formally proven. The inhibitory action of the acidic domain is mediated via the nucleotide sequence rather than the amino acid sequence (E. Callery & N. Blake, unpublished data). However, it is likely to be distinct from that proposed for the EBNA1 GAr domain, which was shown to have no effect on mRNA levels and not to influence the stability of EBNA1 mRNA (Yin *et al.*, 2003; Tellam *et al.*, 2008). It is postulated that the HVS ORF73 nucleotide domain functions at two levels. Firstly, it is proposed that the nucleotide domain acts as a target of an RNA-binding protein that has mRNA-stabilization properties (Garneau *et al.*, 2007). Secondly, it is predicted that the domain acts to repress self-transcription. Clues as to how the repeat-encoding domain may control transcription rates come from work on the inherited disease Friedreich's ataxia (Cossee *et al.*, 1997). This disorder is due to expansion of the trinucleotide repeat GAA within the frataxin gene, which causes low levels of transcription of the gene (Pandolfo, 2002). Mechanistically, this is thought to be due to either transcriptional pausing or induction of heterochromatin regions within the gene (Krasilnikova *et al.*, 2007; Soragni *et al.*, 2008). The long stretches of glutamic acid within the repeat domain of HVS ORF73 are encoded exclusively by the GAA codon and, thus, this sequence may mimic the expanded repeat present in the disease-associated frataxin gene.

MuHV-4: ORF73. The rhadinovirus MuHV-4 is related more closely to KSHV than to EBV, but has been used widely as a small-animal model of general gammaherpesvirus infection and pathogenesis. In a mouse model, intranasal inoculation with MuHV-4 leads to an acute, self-limiting infection in epithelial cells of the lung, followed by establishment of latent infection in the lungs and lymphoid organs. Long-term latency is established in B cells, although macrophages, dendritic cells and epithelial cells are also recorded as sites of latent infection. Similarly to EBV, latent infection is characterized by expansion of latently infected germinal centre B cells, followed by persistence in the resting memory B-cell population (Flano *et al.*, 2002; Willer & Speck, 2003). As with other gammaherpesviruses, there is a reduction in viral gene transcription during latent infection (Virgin *et al.*, 1999). For MuHV-4, the number of genes expressed varies depending on the cell analysed (Marques *et al.*, 2003). Within the latently infected B-cell population, the profile of gene expression also varies, reminiscent of EBV latency. Amongst the predicted latent genes is MuHV-4 ORF73. Based on co-localization of ORF73 within the MuHV-4, KSHV and HVS genomes, this was predicted to be the GMP of MuHV-4. Within the latently infected B-cell populations, ORF73 is expressed in the germinal centre and other B-cell subsets (Marques *et al.*, 2003). It has also been shown that there is different promoter usage for ORF73 during latent infection (Coleman *et al.*, 2005; Allen *et al.*, 2006). This all suggests that there is tight control of MuHV-4 ORF73 expression during latent infection. The *in vitro* functional properties of MuHV-4 ORF73 have not yet been investigated; however, *in vivo* models have shown it to be essential for the establishment and maintenance of latency. A MuHV-4 mutant virus containing a translation-termination codon within the coding region was found to have a severe defect in its ability to establish latency in the spleen of C57Bl/6 mice after intranasal infection (Moorman *et al.*, 2003). A second study using two separate ORF73-deficient viruses, an ORF73-deletion mutant and a frameshift mutant, also demonstrated a severe latency defect after both intranasal and intraperitoneal inoculation of BALB/c mice (Fowler *et al.*, 2003).

The MuHV-4 ORF73 protein encodes a 314 aa protein (Fig. 2) with little overall similarity to KSHV LANA1 or HVS ORF73 (or EBNA1). There is a greater degree of similarity if the C-terminal regions alone are compared. However, significantly, MuHV-4 ORF73 does not encode an internal amino acid repeat sequence. Despite the lack of an internal repeat domain, in light of the importance of ORF73 in establishment and maintenance of latency in the mouse model, Bennett *et al.* (2005) predicted that there would be similar pressure on ORF73 to evade recognition by $CD8^+$ CTLs. Again by using chimeric constructs, this

group showed that, when a model epitope was fused to MuHV-4 ORF73, there was limited presentation of the peptide to $CD8^+$ CTLs (Bennett *et al.*, 2005). The region responsible for this inhibition was mapped to aa 170–220 (Fig. 2). This sequence lies within the C-terminal half of MuHV-4 ORF73 that has similarity to the C-terminal domains of both KSHV LANA1 and HVS ORF73. These regions in the KSHV and HVS GMPs are important for genome maintenance and have not been implicated in immune-evasion properties. Bennett *et al.* (2005) showed that, in MuHV-4 ORF73, this amino acid sequence causes a reduction in steady-state levels of ORF73 protein and contributes to the stability of ORF73. Labelling cells at various time points after transfection, these authors showed that aa 170–220 cause a reduction in self-protein synthesis, although the effect on the rate of translation was not analysed (Bennett *et al.*, 2005). However, it is predicted that this region causes a reduction in ORF73-derived DRiPs and thus acts as a *cis* immune-evasion element (Fig. 3). The mechanism of action has not been deciphered. The authors speculated that it may act at the RNA stage, but this has yet to be investigated.

Perhaps the most important observation from this study came from an *in vivo* experiment analysing the effect of bypassing the *cis*-inhibitory domain and expressing an immunogenic peptide from the ORF73 mRNA. A mutant virus was constructed with three tandem $CD8^+$ T-cell epitopes linked by an internal ribosome entry site to the C terminus of the ORF73 mRNA. These immunogenic peptides would be translated efficiently from this message, which would be transcribed as for a wild-type virus. Crucially, this led to a severe reduction in virus latency, mediated via an MHC class I-restricted and CTL-dependent mechanism. This was the first clear evidence for the importance of immune evasion by the GMP for establishment of gammaherpesvirus latency *in vivo* (Bennett *et al.*, 2005).

Other rhadinoviruses. The genomes of a number of other species of the genus *Rhadinovirus* have been sequenced completely or in part. ORF73 positional and sequence homologues have been identified in *Ateline herpesvirus 3* (herpesvirus ateles strain 73), *Bovine herpesvirus 4* (Movar virus) and *Macacine herpesvirus 5* (rhesus rhadinovirus) (Table 1). Ateline herpesvirus 3 ORF73 is a 447 aa protein that contains a 169 aa internal repeat consisting of the amino acids glycine, aspartic acid and glutamate (Albrecht, 2000). The ORF73 gene of bovine herpesvirus 4 has been sequenced and found to encode a small protein of 253 aa; no internal repeat motif was identified (Lomonte *et al.*, 1995). The species *Macacine herpesvirus 5* represents a number of rhadinovirus strains first found in rhesus macaques, but closely related viruses have also been identified in other macaque species (Damania & Desrosiers, 2001). ORF73 sequence data are available for a number of these viruses: rhesus macaque herpesvirus strains H26-95 and 17577 (Alexander *et al.*, 2000; Searles *et al.*, 1999), Japanese macaque herpesvirus strain JM153 (GenBank accession no. AY528864) and southern pigtail macaque herpesvirus strains M78114 and J97167 (Burnside *et al.*, 2006). As discussed earlier, the rhadinoviruses of Old World primates have been split into two lineages (RV1 and RV2). The southern pigtail macaque herpesvirus strain M78114 is within the RV1 lineage, which includes KSHV. The ORF73 gene from this virus encodes a 1071 aa protein that contains a 521 aa repeat (consisting of glutamate and proline) (Table 1). The other sequenced strains all belong to the RV2 lineage. The ORF73 genes encode proteins of 436–448 aa that do not contain internal amino acid repeat domains. There have been few functional studies on these ORF73 proteins. Rhesus macaque herpesvirus strain H26-95 ORF73 has been shown to bind the viral episome during latency and to be essential for establishment of latency within B cells (DeWire & Damania, 2005; Wen *et al.*, 2009). There have been no studies investigating any immune-evasion properties of these proteins.

Genera *Macavirus* and *Percavirus*

The genera *Macavirus* and *Percavirus* have recently been proposed by the International Committee on Taxonomy of Viruses and contain viruses that were previously within the genus *Rhadinovirus*, or otherwise newly defined species (Davison *et al.*, 2009). Where data are available, positional and sequence homologues of the rhadinovirus ORF73 gene have been identified in two species members of the genus *Macavirus*. Internal amino acid repeat domains have been identified in both viruses (Table 1). *Alcelaphine herpesvirus 1* (also known as wildebeest herpesvirus or malignant catarrhal fever virus) ORF73 encodes a 1300 aa protein containing a large acidic internal repeat region. The repeat is composed of three distinct regions: a 365 aa section rich in glycine, proline and glutamate, linked to a short run of glutamate amino acids and, finally, a 118 aa region rich in glycine and glutamate (Ensser *et al.*, 1997). Recently, an ovine herpesvirus 2 (OvHV-2) or sheep-associated malignant catarrhal fever virus isolate derived from a bovine T-cell lymphoma line was sequenced and the ORF73 homologue identified (Hart *et al.*, 2007). OvHV-2 ORF73 is 495 aa in length and contains an internal 329 aa acidic repeat domain. Again, the internal repeat domain consists of three regions: a 234 aa stretch rich in glycine, glutamate and proline, followed by a run of 57 glutamates and a short 38 aa region rich in glycine, glutamate and aspartic acid. No functional data are available for macavirus ORF73 proteins. Sequence similarity within the C-terminal domains is indicative of conserved genome-maintenance functions and the presence of the internal acidic repeat domains is suggestive of conserved immune-evasion properties. However, this remains to be demonstrated mechanistically.

Within the genus *Percavirus*, sequence data are available for one species, *Equid herpesvirus 2* (Telford *et al.*, 1995). Analysis of the coding potential of this virus genome did

not identify a positional or sequence homologue of ORF73. It remains to be determined which gene, if any, within this virus functions as the GMP, and whether this has immune-evasion properties.

Concluding remarks

The establishment of latent infection in a cell that undergoes periodic rounds of cell division has presented a unique challenge to gammaherpesviruses. To establish and maintain long-term latency within this active cell population, gammaherpesviruses have developed an approach that relies on the long-term presence and active expression of the virus-encoded GMP. The presence of this viral antigen within the latently infected cell provides an opportunity for the host immune response to act. Gammaherpesviruses have addressed this conundrum by evolving an immune-evasion strategy specific to the GMP itself. The proposed model, derived mainly from studies on EBV, but thought to be relevant to most gammaherpesviruses, is shown in Fig. 4. Long-term latency is established in a resting memory lymphocyte. The GMP is present as a stable protein that acts to tether the viral episome to the host-cell chromosome. The stable GMP protein is protected from proteasome-mediated degradation. No active GMP transcription or translation occurs, so no GMP-derived DRiPs are generated and the cell remains hidden from virus-specific $CD8^+$ CTLs. When the resting cell is stimulated to divide, the viral genome is replicated and GMP is expressed, so that new genomes can be tethered to host chromosomes to prevent dilution of viral genomes. GMP-derived DRiPs will be generated and thus make these cells susceptible to detection by $CD8^+$ CTLs. However, a reduction in expression of the GMP reduces the rate at which DRiPs, and antigenic peptides, are generated. The expansion of this memory lymphocyte will be a brief event, quickly reverting back to a resting stage. It would be expected that sufficient GMP-expressing cells evade elimination by the immune system. Once the cell ceases dividing, there will no longer be the need to generate new GMP, hence turning off the source of viral antigenic peptide.

As outlined in this review, the GMPs of EBV, KSHV, HVS and MuHV-4 have all been shown to have immune-evasion properties. In the case of EBV EBNA1, KSHV LANA1 and HVS ORF73, this has been shown to be due to the presence of amino acid repeat domains within these GMPs. Of specific interest is the fact that these repeat domains have no significant sequence similarity and appear to function via different effector mechanisms. The EBNA1 and LANA1 repeats are most closely related functionally in that they both cause a reduction in the rate of self-mRNA translation, although, mechanistically, it is speculated that EBNA1 acts via its mRNA, whereas LANA1 acts via a peptide sequence. The situation for HVS ORF73 is again different, although the outcome is the same. Here the repeat, although highly acidic (similar to LANA1), targets the levels of ORF73 mRNA rather than the protein directly.

Regardless of the detailed mechanism, the outcome of these immune-evasion strategies is the same: a reduction of the presentation of antigenic peptides from the GMPs. It is intriguing that these proteins with similar functions in the virus life cycle, which require immune-evasion properties, have utilized the presence of amino acid repeat domains with different amino acid content to target the same immunological pathway. It is not clear whether this is a recurring feature in the subfamily *Gammaherpesvirinae*. Where sequence data are available, GMPs have been identified or predicted in all gammaherpesvirus species except *Equid herpesvirus 2*. Whether that is reflective of this virus species or an anomaly of the particular strain sequenced is not clear. Of particular interest is the genus *Lymphocryptovirus*, which contains EBV. The closely

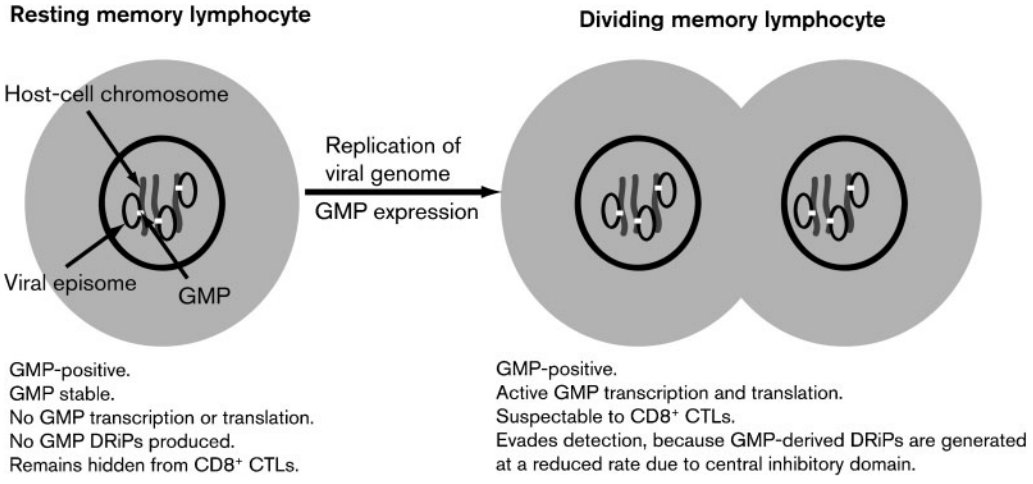

Fig. 4. Model for the role of the immune-evasion properties of GMPs that allow gammaherpesviruses to maintain a latent infection of their host.

related viruses rhLCV and baLCV both contain GMP with internal repeat domains. Whether they also function to inhibit antigen presentation is open to question, although the strongest data imply that they do not have this function, perhaps suggesting that the immune-evasion properties of the EBV EBNA1 GAr repeat are a more recent acquisition, indicative of an adaptation to human cells. Within the genus *Rhadinovirus* and the related, but recently defined, genus *Macavirus*, more GMP sequence data are available. The majority of GMPs contain acidic repeat domains that have yet to be characterized. Based on the KSHV and HVS data, one might predict that the immune-evasion properties will be conserved. However, things are never so straightforward. For MuHV-4 ORF73, which does not contain a repeat domain, immune-evasion properties were identified and an effector domain was mapped to a short sequence with no distinguishing features. Importantly, for this virus it was shown that this immune-evasion property was essential for establishment of a latent infection *in vivo*. An amino acid repeat domain is also missing from bovine herpesvirus 4 ORF73, the ORF73 of members of the RV1 lineage of the species *Macacine herpesvirus 4* and the predicted GMP of the LCV of the New World marmoset monkey. Whether another sequence element in these proteins has immune-evasion properties is not known.

In conclusion, the immune-evasion properties of GMPs are likely to be well-conserved within the subfamily *Gammaherpesvirinae*. A complete and thorough understanding of the different mechanisms used by each virus will not only generate information on the basic pathways of antigen presentation, but may ultimately reveal therapeutic approaches that will allow disruption of the site of long-term latency.

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