The A5 gene of alcelaphine herpesvirus 1 encodes a constitutively active G-protein-coupled receptor that is non-essential for the induction of malignant catarrhal fever in rabbits

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INTRODUCTION

Malignant catarrhal fever (MCF) is a fatal lymphoproliferative disease of a variety of species in the order Artiodactyla, which includes cattle (reviewed by Reid, 2000). The main causative agents of MCF are two closely related gammaherpesviruses of the genus Rhadinovirus, ovine herpesvirus 2 (OvHV-2) and alcelaphine herpesvirus 1 (AlHV-1). These viruses cause no apparent disease in their natural host species. Wildebeest (Connochaetes taurinus) carry AlHV-1, responsible for the wildebeest-derived form of MCF (WD-MCF) (Plowright et al., 1960). Experimentally, WD-MCF can be induced in rabbits (Buxton & Reid, 1980). The lesions observed are very similar to those described in naturally susceptible host species.

WD-MCF is a disease described as a combination of lymphoproliferation and degenerative lesions caused by unknown mechanisms. It has been suggested that proliferating cytotoxic large granular lymphocytes (LGL) that support a non-permissive infection play a key role in the pathogenesis of MCF (Swa et al., 2001). Despite publication of the entire AlHV-1 genome sequence (Ensser et al., 1997), little is known about the role of individual AlHV-1 genes in WD-MCF pathogenesis. The study of WD-MCF pathogenesis has been impeded by an inability to produce recombinant viruses, mainly due to the fact that AlHV-1...
becomes attenuated during successive passages in culture (Wright et al., 2003). Recently, these difficulties were overcome by cloning the entire AlHV-1 genome as a stable, infectious and pathogenic bacterial artificial chromosome (BAC) (Dewals et al., 2006).

The A5 open reading frame (ORF) of the AlHV-1 genome encodes a putative homologue of cellular 7-transmembrane G-protein-coupled receptors (GPCRs) (Coulter et al., 2001; Ensser et al., 1997). GPCRs form the largest family of membrane proteins. They can be activated by ligands as diverse as hormones, growth factors, neurotransmitters or chemokines. Cell-surface GPCRs transduce external signals through interaction with G-proteins. G-proteins are heterotrimeric proteins named after their α, β, and γ subunits, each responsible for the modulation of different second effectors to give rise to different biological responses in the cell (Marinissen & Gutkind, 2001).

Viral homologues of GPCRs (vGPCR) have been reported in poxviruses and beta- and gammaherpesviruses (Rosenkilde, 2005). The gammaherpesvirus human herpesvirus 8 (HHV-8) ORF74 encodes a vGPCR that functionally binds chemokines to contribute to Kaposi’s sarcoma (KS) pathogenesis (Arvanitakis et al., 1997; Bais et al., 1998). The ORF74 protein is a constitutively active (agonist-independent) receptor that can modulate the transcription of reporter genes under the control of the nuclear factor κB (NFκB) or the cAMP response element-binding protein (CREB) (Cannon et al., 2003). Recently, the human herpesvirus 4 (HHV-4; also called Epstein–Barr virus, another gammaherpesvirus) BILF1 gene has also been described as a constitutively active vGPCR, capable of modulating CREB- and NFκB-mediated transcriptions via Gx_{i}-proteins in a cell-type-dependent manner (Beisser et al., 2005; Paulsen et al., 2005).

The present study was devoted to the A5 ORF of AlHV-1. Here, we show that A5 encodes a constitutively active vGPCR that is not required for viral replication in vitro nor for induction of WD-MCF in rabbits. This study is the first to investigate the role of an individual gene of AlHV-1 in WD-MCF pathogenesis. Knowing that AlHV-1 is extremely well adapted to its natural host, which is an asymptomatic carrier, the present study suggests that, in some viruses, a vGPCR homologue may contribute to the adaptation of the virus to its host rather than always being a virulent factor.

**METHODS**

**Cell lines and virus strain.** Embryonic bovine lung cells (EBL, German collection of micro-organisms and cell culture DSMZ ACC192), EBL cells stably expressing the Cre recombinase (EBL-NLS-Cre; Gillet et al., 2005), and bovine mammary epithelial cells (MAC-T; Huynh et al., 1991) were maintained in Dulbecco’s modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS). Chinese hamster ovary cells (CHO-K1, ATCC CCL-61) were cultured in Ham’s F-12 medium (Invitrogen) supplemented with 10% FCS. Cos-7 cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM, Invitrogen) supplemented with 10% FCS and 2% glutamine. The cell-associated AlHV-1 C500 strain was maintained by limited passage (fewer than five) in EBL cells (Plowright et al., 1975).

**Protein structure prediction.** Structural motifs were predicted based on an A5 published sequence (GenBank accession number NP_065513) using the following software: position of transmembrane domains and molecule orientation in the membrane were predicted using TM Pred (http://www.ch.embnet.org/software/TMPRED_form.htm), prediction of N-glycosylation sites was determined using ProScan (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_proscan.html), and potential phosphorylation sites were predicted using ProScan and NetPhos (http://www.cbs.dtu.dk/services/NetPhos/).

**RT-PCR.** Cytoplasmic RNAs were isolated from cell cultures and from rabbit organs with the RNeasy Mini kit (Qiagen) and then subjected to RT-PCR using the SuperScript III One-Step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen). The pN1-Fwd and pN1-Rev primers described in Table 1 were used to perform RT and PCR reactions.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplicon (bp)</th>
<th>Restriction enzyme</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>pN1-Fwd:</td>
<td>5’-CTCGAGGCCACCATGGGCGACTGACTCTAATCCC-3’</td>
<td>925</td>
<td>XhoI</td>
<td>pEGFP-N1-A5</td>
</tr>
<tr>
<td>pN1-Rev:</td>
<td>5’-GGATCCACTCTAGAAAGGATGAGGTAAGTG-3’</td>
<td>924</td>
<td>BamHI</td>
<td>pCMV-A5-Flag</td>
</tr>
<tr>
<td>pC-Fwd:</td>
<td>5’-GGGCGGCCCCACCATGGGGGCACTGACTCTAACCTCC-3’</td>
<td>926</td>
<td>EcoRI</td>
<td>pEFIN3-A5</td>
</tr>
<tr>
<td>pN1-Rev:</td>
<td>5’-GGATCCCTTACTAAAAGGGAAGCTAGACTGG-3’</td>
<td>3074</td>
<td>Spel</td>
<td>pST-A5WT</td>
</tr>
</tbody>
</table>

Note: The above table includes primers used to generate amplicons (expected sizes are indicated in bp), the latter were cloned into pGEM-T Easy vector (Promega). After digestion with the indicated restriction enzymes (EcoRI and Spel restriction sites are derived from pGEM-T Easy), the inserts were cloned in the vectors listed in the last column.

Table 1. Primers and restriction sites used for plasmid constructions.
Construction of mammalian expression vectors encoding A5. The A5 ORF was amplified from genomic DNA isolated from C500-infected EBL cells using the primers described in Table 1. PCR products were cloned into pGEM-T Easy vector (Promega) and then subcloned into expression vectors using the restriction sites listed in Table 1. For subcellular localization studies A5 was subcloned into pEGFP-N1 (Clontech) and pCMV-Tag1 (Stratagene) expression vectors, to make pEGFP-N1-A5 and pCMV-A5-Flag, respectively. For guanosine 5′-32P-thiphosphate ([32P]GTPS) and S) reporter assays, A5 was subcloned into the bicistronic pEFIN3 expression vector conferring resistance to G418 (final concentration 200 μg ml−1; Invitrogen) (Ghattas et al., 1991). To produce the pEFIN3-DelA5 vector encoding a truncated form of A5 (see below), DelA5 ORF was subcloned from the pST-DelA5 vector (see below) into the pEFIN3 vector using SwaI–NdeI restriction.

Antibodies. The mouse monoclonal antibody (mAb) 15-A (VMRD), specific for the AlHV-1 glycoprotein complex gp115, was used in this study (Li et al., 1994). The mAb M2 (Sigma) that binds to FLAG fusion proteins was also used.

Indirect immunofluorescent staining. Immunofluorescent staining of fixed and permeabilized cells grown on glass coverslips was performed as described elsewhere (Vanderplaschen et al., 2000). The mAb 15-A (0.5 μg ml−1) and mAb M2 (4 μg ml−1) were used as the primary antibodies. Alexa Fluor 488 nm-conjugated goat anti-mouse IgG (Molecular Probe; 2 μg ml−1) was used as the secondary conjugate.

Confocal microscopy analysis. Confocal microscopy analysis was performed with a TCS SP confocal microscope (Leica) as described previously (Gillet et al., 2005).

[32P]GTP-S-binding assay. CHO-K1 cell lines stably expressing pEFIN3 or pEFIN3-A5 were produced as described elsewhere (Gillet et al., 2005). Membrane preparations were produced and quantified from clones resistant to G418, and then submitted to [32P]GTPS-S exchange assays as described in details elsewhere (Blanpain et al., 2001; Minamide & Bamburg, 1990).

Reporter assay. The capacity of A5 to modulate the CREB pathway was investigated using the reporter assay Mercury SEAP Profiling System kit (Clontech). This kit contains the pCRE-SEAP vector in which the expression of the reporter SEAP (Secreted Alkaline Phosphatase) gene is regulated by the cAMP response element (CRE) DNA-binding site. Cos-7 cells (5 × 103) were transiently cotransfected with a mixture of three expression vectors: (i) the pCRE-SEAP vector, (ii) either pEFIN3, pEFIN3-A5 or pEFIN3-DelA5, and (iii) pEGFP-N1. Expression of EGFP (enhanced green fluorescent protein) induced by pEGFP-N1 was used to normalize transfection efficiency (data not shown). To inhibit Gα-proteins, pertussis toxin (PTX, Sigma) was added to the culture medium 24 h after transfection (final concentration 0.4 μg ml−1). To activate adenylyl cyclase (AC), forskolin (Sigma) was added to the culture medium 16 h after transfection (final concentration 20 μM). At 40 h after transfection, culture supernatant was harvested and heated at 65 °C for 30 min to inactivate endogenous alkaline phosphatase. After centrifugation at 10 600 g for 3 min, SEAP activity was quantified in supernatant as follows. Culture supernatant (50 μl) was incubated 1 h at room temperature in the dark with 45 μl reaction buffer [1 mM MgCl2, 20 mM L-homoarginine in 2 M diethanolamine pH 9.8] and 5 μl of a 1 mM solution of the fluorescent phosphatase substrate 4methylumbelliferyl phosphate (4-MUP, Sigma). Fluorescence emission was quantified using a CytoFluor Multi-well plate reader (PerSeptive Biosystems).

Production of AlHV-1 A5 recombinant strains. Mutagenesis of the A5 ORF was amplified with the forward primer 5′-TACGGGAGCCCTGACATTTCATCTCTTTTG-3′ and the reverse primer 5′-ATAACTGTGGTATGTCAGCATGC-ATCTA-3′ were used for this reaction, as described previously (Dewals et al., 2006). Additionally, a PCR reaction allowing the differentiation between strains encoding wild-type A5 (C500 WT Exc and C500 RevA5 Exc strains) or truncated A5 (C500 DelA5 Exc strain and the C500 RevA5 Exc strain, respectively). Infected cells for inoculation were harvested from cultures in which cytopathic effect reached 90% or more. Rabbits were examined daily for clinical signs. According to bioethical rules, rabbits were euthanized when rectal temperature remained higher than 40 °C for two consecutive days. The animal study was accredited by the local ethics committee of the University of Liège (Belgium).

Detection of AlHV-1 genome by PCR. DNA samples were extracted from tissues using a QIAamp DNA Mini kit (Qiagen). PCR amplification specific of AlHV-1 was performed using a set of primers derived from the AlHV-1 ORF50; the forward primer C500-1 5′-TACGGGAGCCCTGACATTTCATCTCTTTTG-3′ and the reverse primer C500-2 5′-ATAACTGTGGTATGTCAGCATGC-ATCTA-3′ were used for this reaction, as described previously (Dewals et al., 2006). Additionally, a PCR reaction allowing the differentiation between strains encoding wild-type A5 (C500 WT Exc and C500 RevA5 Exc strains) or truncated A5 (C500 DelA5 Exc strain) was also performed using the pN1-Fwd and the pN1-Rev primers described in Table 1. Using this reaction, wild-type A5 and DelA5 sequences lead to 909 and 539 bp amplicons, respectively.

Real-time PCR. Popliteal lymph node and spleen DNAs were purified using the QiAamp DNA Mini Kit (Qiagen) before AlHV-1 ORF3 real-time PCR was performed as described elsewhere (Traul et al., 2005), with minor modifications. A 111 bp fragment of the AlHV-1 ORF3 (nt 9098–9121, GenBank accession no. AF005370), consisting of A5 ORF flanked by two regions of 1 kb (named H1 and H2 in Fig. 3), was amplified by PCR and subcloned into pST76Ksr-LacZ to generate the pST-ASWT (see Table 1 for primers and restriction sites used). A deletion of the central 370 bp of A5 was then obtained by a digestion/ligation approach using BfrI and Hpal restriction sites, resulting in pST-DelA5. Mutagenesis of the C500 BAC WT plasmid was performed as described previously (Gillet et al., 2005) to produce the C500 BAC DelA5 plasmid, in which A5 ORF is disrupted, and the revertant C500 BAC RevA5 plasmid with a restored A5 ORF (Fig. 3). Infectious virus from C500 BAC plasmids were reconstructed by transfection in EBL cells. To excise the BAC cassette, reconstructed viruses were propagated in EBL-NLS-Cre cells to generate the corresponding excised (Exc) strain.

Southern blotting. Southern blot analysis was performed as described previously (Markine-Goriaynoff et al., 2003).

Growth curves. Multi-step virus growth experiments were conducted to investigate the effect of A5 deletion on AlHV-1 growth in vitro as described previously (Dewals et al., 2006). Syncytia were revealed by indirect immunofluorescent staining using mAb 15-A as the primary antibody.

Syncytium size. The effect of A5 deletion on AlHV-1 replication in vitro was further investigated by measurement of syncytium size over time. These experiments were performed in EBL cells as described in detail previously (Dewals et al., 2006).

Induction of WD-MCF in rabbits. Specific-pathogen-free New Zealand white rabbits were housed individually throughout this study. Four groups were used, each comprising three rabbits. Animals in the first group were inoculated intravenously with 3 × 108 mock-infected EBL cells, while animals in groups 2, 3 and 4 were inoculated with 3 × 108 EBL cells infected with the C500 WT Exc strain, the C500 DelA5 Exc strain and the C500 RevA5 Exc strain, respectively. Infected cells for inoculation were harvested from cultures in which cytopathic effect reached 90% or more. Rabbits were examined daily for clinical signs. According to bioethical rules, rabbits were euthanized when rectal temperature remained higher than 40 °C for two consecutive days. The animal study was accredited by the local ethics committee of the University of Liège (Belgium).
Purified AlHV-1 BAC plasmid (Dewals et al., 2006) was used to determine standard curves. For cellular gene quantitative amplification, a 178 bp fragment of the rabbit beta-globin gene (nt 372–549, GenBank accession no. V00882) was assayed using the forward primer 5′-GGTATCCTTTTTACACCAAAC-3′ and the reverse primer 5′-CAGGTCCCCAAAAGGACTCG-3′, in the presence of the fluorescent probe 5′-FAM-CTTGAGCTTCTTGATTCCAGG-TAMRA-3′. The respective standard curve employed a 375 bp fragment of the rabbit beta-globin gene (nt 337–711) amplified in the presence of the fluorophores 5′-FAM-CCTGGGCTGTTTTCATTTTCTCAGG-TAMRA-3′. The respective standard curve employed a 375 bp fragment of the rabbit beta-globin gene (nt 337–711), amplified in the presence of the fluorophores 5′-FAM-CCTGGGCTGTTTTCATTTTCTCAGG-TAMRA-3′. The respective standard curve employed a 375 bp fragment of the rabbit beta-globin gene (nt 337–711), amplified in the presence of the fluorophores 5′-FAM-CCTGGGCTGTTTTCATTTTCTCAGG-TAMRA-3′. The respective standard curve employed a 375 bp fragment of the rabbit beta-globin gene (nt 337–711), amplified in the presence of the fluorophores 5′-FAM-CCTGGGCTGTTTTCATTTTCTCAGG-TAMRA-3′.

**Histological analysis.** These experiments were performed as described in detail previously (Dewals et al., 2006).

**Statistical analysis.** Student’s t test was used to test for the significance of the results (P<0.001).

**RESULTS**

**AlHV-1 A5 ORF encodes a putative vGPCR**

To investigate whether the A5 gene expression product possesses the molecular features of GPCR, its amino acid sequence was analyzed with the prediction software listed in Methods. The results of these in silico analysis are presented in Fig. 1(a). They suggest that A5 has several hallmark features of GPCRs: (i) has an extracellular N-terminus, seven-transmembrane helical domain and an intracellular C-terminus; (ii) contains potential N-linked glycosylation sites (positions 6, 10, 154 and 240) and phosphorylation sites (positions 113, 195, 288, 290 and 298); (iii) carries the Asp-Arg-Tyr (DRY) motif (position 105–107) conserved among many GPCRs and involved in coupling with G-proteins; (iv) possesses cysteine residues in each extracellular loop (positions 81, 161 and 244) that might form disulfide bridges; and (v) has a basic third intracellular loop (R and K residues).

**Fig. 1.** A5 ORF encodes a viral GPCR homologue. (a) Bioinformatic analysis of A5 amino acid sequence. The predicted 302 aa sequence of A5 is shown. Grey boxes indicate putative TM domains. Predicted extracellular and cytoplasmic domains are underlined and italicized, respectively. A DRY motif, conserved amongst GPCR homologues, is present at the cytoplasmic end of the third TM domain. Cysteines in the extracellular domains are shown circled. Potential N-linked glycosylation sites are boxed and potential phosphorylation sites are shown in bold. (b and c) Expression of A5 in vitro and in vivo. (b) Cytoplasmic RNA was extracted from EBL cells mock-infected or infected with the AlHV-1 C500 strain for 24 or 48 h. (c) Cytoplasmic RNA was extracted from the spleen (S) and popliteal lymph nodes (LN) of a mock-infected rabbit and a rabbit dying from WD-MCF 19 days after inoculation of the AlHV-1 C500 strain. Cytoplasmic RNAs were then subjected to PCR (left panels) or RT-PCR (right panels) amplifying the A5 ORF (909 bp). C+, positive control using a vector encoding A5. (d) Subcellular localization of A5. MAC-T cells were transiently transfected with vectors expressing (i) A5–EGFP fusion protein, (ii) A5–FLAG fusion protein, (iii) control EGFP protein (pEGFP-N1 vector) and (iv) control luciferase–FLAG fusion protein (pLuc-FLAG, Stratagene). Bar, 5 μm.
A5 ORF is expressed during AlHV-1 lytic replication cycle and in organs of rabbits developing WD-MCF

To provide support for the identification of the A5 ORF as a bona fide gene, before analysing the activity of its gene product, we determined whether the A5 ORF was transcribed during AlHV-1 infection in vitro and in vivo. RT-PCR was performed using first-strand cDNA made from AlHV-1-infected and mock-infected EBL cells. In contrast to mock-infected cells, cDNA from infected cells gave rise to a 0.9 kb PCR product (Fig. 1b, right panel), consistent with the expected size of A5 (909 bp). When RT was omitted from the reactions, no PCR product was detected, indicating that it did not result from amplification of contaminant viral DNA (Fig. 1b, left panel). DNA sequencing confirmed that the 0.9 kb RT-PCR product from infected cells was A5 (data not shown), demonstrating that A5 is expressed during lytic replication of AlHV-1. Similarly, we determined whether the A5 ORF was transcribed in organs of rabbits with WD-MCF. The data presented in Fig. 1(c) demonstrate that A5 is expressed in the spleen and the popliteal lymph nodes of rabbits developing WD-MCF. From a theoretical point of view, the RT-PCR approach could reveal a transcript that crosses the A5 locus on one strand or the other. However, as A5 is encoded by the positive strand of the AlHV-1 genome and as all ORFs located 20.3 kb upstream and 12.6 kb downstream of A5 are all encoded by the same strand, the possibility that the transcripts detected in the present study reflect the expression of an ORF encoded by the negative strand that crosses the A5 locus is very unlikely.

A5 is a membrane protein

The results presented above suggest that A5 could be a functional vGPCR. This hypothesis implies that A5 is expressed as a cell-surface protein. Despite several attempts, we were unable to produce anti-A5 antibodies suitable for A5 detection in AlHV-1-infected or in transiently transfected cells (data not shown). To circumvent this omission, A5 was expressed as EGFP- and FLAG-tagged proteins. Two different constructions were tested to exclude deleterious effects of the tag on cellular localization [Fig. 1d, panels (i) and (ii)]. Immunofluorescent staining of cells transfected with these vectors revealed that A5 is associated mainly with intracellular membranes, but also with the plasma membrane. Abundant A5 expression was observed in the endoplasmic reticulum. This membrane pattern was not observed with control proteins [EGFP and luciferase-FLAG, see Fig. 1d, panels (iii) and (iv), respectively]. Results similar to those presented in Fig. 1(d) were obtained with EBL, CHO-K1 and Cos-7 cells (data not shown).

AlHV-1 A5 ORF encodes a functional vGPCR that constitutively activates endogenous \( \alpha_i \)-type G-proteins

Most of the vGPCRs described to date act as constitutive (agonist-independent) activators of G-proteins (Couty & Gershengorn, 2005; Holst & Rosenkilde, 2003; Rosenkilde, 2005). Here, to test the ability of A5 to signal in an agonist-independent manner, we compared the level of G-protein activation between CHO-K1 cell lines stably expressing pEFIN3 (empty vector) or pEFIN3-A5. G-protein activation was measured on membrane preparations using the \([^{35}S]GTP_\gamma S\)-binding assay described in Methods. Membrane preparations from cells expressing A5 bound significantly more \([^{35}S]GTP_\gamma S\) than membranes from cells transfected with the empty vectors (Fig. 2a) \((P<0.001)\). The data presented in Fig. 2(a) are representative of the results obtained with five independent clones of each cell line (data not shown). Treatment with the G\(_i\) inhibitor PTX abolished the difference observed between the two cell lines (Fig. 2a) \((P<0.001)\). These results demonstrate that A5 displays constitutive vGPCR activity through \( \alpha_i \)-type G-proteins.

A5-mediated activation of G\(_i\)-proteins inhibits gene transactivation by CREB

The G\(_i\) class of G-protein inhibits AC activity. This results in a decrease in cellular cAMP concentration which, in turn, reduces the phosphorylation of CREB transcription factors by cAMP-dependent protein kinases. The whole process leads to a reduced expression of genes regulated by CREB proteins. As the results presented above demonstrated that A5 constitutively activates \( \alpha_i \)-type G-proteins, we investigated whether A5 could inhibit modulation of gene expression by CREB. To test this hypothesis, we used the reporter assay described in Methods based on the pCRE-SEAP vector in which expression of the reporter SEAP gene is regulated by the CRE DNA-binding site (Fig. 2b). As expected, incubation of pEFIN3-transfected cells with forskolin (activator of AC) induced a significant increase of the expression of the reporter gene (Fig. 2b). Interestingly, this forskolin-induced upregulation of the reporter gene was reduced in cell culture expressing A5 (Fig. 2b) \((P<0.001)\) in a dose-dependent manner (Fig. 2c). These results are consistent with the property of A5 described above to activate endogenous \( \alpha_i \)-type G-proteins. This conclusion is further supported by the observation that PTX treatment restored the forskolin-induced upregulation of the reporter gene in pEFIN3-A5-transfected cells to levels comparable to those observed in pEFIN3-transfected control cells (Fig. 2b, grey bars) \((P<0.001)\). Thus, A5 encodes a functional vGPCR that constitutively activates endogenous \( \alpha_i \)-type G-proteins, leading to inhibition of the CREB pathway.

Production and characterization of AlHV-1 A5 recombinant strains

In order to subsequently investigate the importance of A5 in virus replication in vitro and in the pathogenesis of WD-MCF, an AlHV-1 strain deleted for A5 and a revertant strain were produced (Fig. 3). The C500 BAC WT plasmid was used as parental plasmid to generate the C500 BAC
DelA5 plasmid in which the central part of the A5 ORF had been deleted (Fig. 3a, b), restricting the expression of the ORF to the 95 first amino acids. Then, a revertant plasmid was generated. Infectious viruses were efficiently reconstituted by transfection of BAC plasmids into permissive cells. Finally, the infectious AlHV-1 BAC viruses were propagated in EBL-NLS-Cre cells to generate BAC-excised strains. The molecular structures of the recombinant strains produced were confirmed by a combined restriction endonuclease and Southern blot approach (Fig. 4a) and by sequencing the regions used to target recombination (data not shown). All approaches confirmed that the resulting recombinants have the correct molecular structure. Moreover, the absence of polar effect of the A5 deletion on the expression of the ORFs located upstream and downstream of A5 was demonstrated by quantitative real-time RT-PCR (data not shown). Similarly, we demonstrated that the C500 WT and RevA5 Exc strains expressed A5 comparably (data not shown). Finally, to control that the deletion operated in A5 abolishes its GPCR activity, the partially deleted A5 ORF encoded by the C500 DelA5 Exc strain was subcloned in the pEFIN3 plasmid to generate the pEFIN3-DelA5 vector. The latter vector was then tested in the reporter assay described in Fig. 2(b, c). The data obtained demonstrate that the truncated form of A5, in contrast with the full-length form, does not inhibit modulation of gene expression by CREB.

In order to investigate the role of A5 in AlHV-1 growth in vitro, C500 WT Exc, C500 DelA5 Exc and C500 RevA5 Exc strains were compared using the growth and syncytium size assays described in Methods (Fig. 4b, c). All three viruses exhibited similar growth curves and syncytium sizes (P<0.001). Taken together, these results indicate that A5 is not essential for AlHV-1 replication in vitro and suggest that A5 exerts its biological functions in vivo.

**A5 gene is not essential for the induction of WD-MCF in rabbits**

To investigate the importance of A5 in the pathogenesis of WD-MCF, rabbits were inoculated with the C500 WT Exc, C500 DelA5 Exc or C500 RevA5 Exc strains.

All three viral strains induced WD-MCF in rabbits with indistinguishable pathology and similar incubation time. Rabbits infected with the C500 WT Exc, C500 DelA5 Exc and C500 RevA5 Exc strains developed WD-MCF at 19.67±2.08, 21.67±4.62 and 23.33±4.04 days post-infection, respectively (Fig. 5a). Clinical signs were identical in the three groups of infected rabbits and included apathy, anorexia, adipsia, hyperthermia and severe hypertrophy of popliteal lymph nodes. At necropsy, examination of the organs revealed no differences in gross pathology between the three groups of infected rabbits. Characteristic WD-MCF lesions were observed in all infected groups, including severe splenomegaly (the size of the spleens of infected rabbits was at least twice the size of those of mock-infected rabbits) and generalized lymphadenopathy. Congestion and enlargement of the liver with greyish punctiform areas were similarly observed, as well as irregular foci of various sizes (1–5 mm diameter) present

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**Fig. 2.** A5 activity on endogenous \( \alpha_i \)-type G-proteins (a) and on the CREB pathway (b, c). (a) \[^{35}S\]GTP-\( \gamma \)S assay was performed on membranes extracted from CHO-K1 cell lines stably expressing pEFIN3 or pEFIN3-A5 treated or mock-treated with PTX; c.p.m., counts per minute. (b, c) SEAP reporter assays were performed on transiently co-transfected Cos-7 cells. (b) Cells were transiently co-transfected with a mixture of three vectors: (i) pCRE-SEAP (0.3 pM); (ii) either pEFIN3, pEFIN3-A5 or pEFIN3-DelA5 (0.3 pM); and (iii) pEGFP-N1 (0.3 pM). Additionally, cells were treated or mock-treated with forskolin and/or PTX. (c) Cells were transiently co-transfected with a mixture of three vectors: (i) pCRE-SEAP (0.3 pM); (ii) either pEFIN3, pEFIN3-A5 or pEFIN3-DelA5 (increasing concentrations, from 0.03 to 1.5 pM); and (iii) pEGFP-N1 (0.3 pM). Cells were then mock-treated or treated with forskolin. In experiments (b) and (c), EGFP expression was used to normalize the level of transfection (data not shown). Each experiment was repeated three times. The data presented are means±SEM of triplicates.
within the cortical areas of the kidneys. Histological findings were also indistinguishable between the three groups (Fig. 6) and were characteristic of histopathological lesions reported for WD-MCF. Fig. 6 illustrates such WD-MCF lesions in several organs, characterized by infiltration of lymphoblastoid cells. The three mock-infected rabbits survived and remained healthy for the course of the experiment. At necropsy, their organs were normal. To demonstrate that the group of rabbits were infected with the correct virus, to exclude any possibility of virus spread between the different groups and to rule out the hypothesis of contaminant C500 WT Exc or C500 RevA5 strains in the A5 deleted group, PCRs were performed on DNA extracted from popliteal lymph nodes and spleen of infected rabbits. PCRs performed with the C500-1 and C500-2 primers confirmed that the tissues of the infected rabbits contained the AlHV-1 genome (Fig. 5b, upper panel), while reactions performed with the pN1-Rev primers excluded the possibility of virus spread between groups of infected rabbits or contamination of viral inoculums with wild-type virus.

Moreover, in order to investigate the putative effect of A5 deletion on viral replicative fitness in vivo, viral loads were compared in rabbits infected with the C500 WT or DelA5 or RevA5 Exc strains by real-time PCR (Fig. 5c). These data revealed that the various strains tested replicated comparably in vivo.

Fig. 3. Production of AlHV-1 A5 recombinant strains. C500 DelA5 and C500 RevA5 Exc strains were produced by mutagenesis of the C500 BAC WT plasmid. (a) Schematic representation of the 370 bp deletion produced in the A5 gene using a digestion/ligation approach based on BflBI and Hpal restriction enzymes. H1 and H2 represent 1 kb sequences used to target homologous recombinations in bacteria. (b) Flowchart of stages performed to produce C500 WT Exc, C500 DelA5 Exc and C500 RevA5 Exc strains.

Fig. 4. Characterization of AlHV-1 A5 recombinant strains. (a) C500 BAC WT, C500 BAC DelA5 and C500 BAC RevA5 plasmids and corresponding Exc strains were analysed by SacI restriction and further tested by Southern blotting using probes corresponding to the complete C500 genome (left panels) or to the A5 ORF (right panels). Open arrowheads indicate the restriction fragments containing the A5 sequence. MS, molecular size marker (in kb). (b) Replication kinetics of the C500 WT Exc strain (○, dashed line) were compared to those of the C500 DelA5 Exc (●, solid line) and C500 RevA5 Exc (□, solid line) strains. The experiment was repeated three times. The data presented are means ± SEM of triplicate measurements. (c) Effect of A5 deletion on AlHV-1 syncytium size. EBL cells grown on coverslips were infected with C500 WT Exc (white columns), C500 DelA5 Exc (black columns) or C500 RevA5 Exc (hatched columns) strains and then overlaid with DMEM containing 10% FCS and 0.6% carboxymethylcellulose. At successive intervals post-infection, syncytia were revealed by indirect immunofluorescence staining using mAb 15-A. The experiment was repeated three times. The data presented are means ± SEM for the measurement of 15 randomly selected syncytia.
DISCUSSION

Many gammaherpesviruses have been shown to encode vGPCRs with unusual pharmacological and cellular properties and significant biological functions. Several in vivo studies suggested that gammaherpesvirus vGPCRs are important for viral replication, reactivation from latency and for virus-induced pathogenesis in natural hosts. In the present study, in silico analysis showed that AlHV-1 A5 contained several hallmark features of GPCRs. This led us to test whether this ORF encoded a functional vGPCR, and if so, to investigate its role in viral replication and in the pathogenesis of WD-MCF. We found that A5 encodes a functional vGPCR that constitutively activates Gαi-proteins, thereby inhibiting a forskolin-triggered CREB activation. Finally, using an AlHV-1 BAC clone, we produced a strain deleted for A5 and a revertant strain. Interestingly, the strain deleted for A5 replicated comparably to the wild-type parental strain and, when inoculated in rabbits, induced WD-MCF pathology that was indistinguishable from the one induced by the parental strain.

Most of the vGPCRs described to date were found to display constitutive activity through activation of different G-proteins (Rosenkilde, 2005), suggesting that these receptors have fundamentally different properties. Here, we show that A5 constitutively activates Gαi-proteins as suggested by the complete inhibition of the A5-induced
effect by PTX (Fig. 2a, b). Moreover, our data demonstrate that, through activation of G_{s,i}-proteins, A5 inhibits the forskolin-triggered CREB pathway (Fig. 2b, c). Further investigations are required to complete the study of A5 properties to bind to G-proteins and to regulate cellular pathways. The constitutive activity of some vGPCRs can be further increased by specific ligands (Rosenkilde & Schwartz, 2000). Several experiments were performed to determine whether chemokine (n=37) and non-chemo-
kine (n=21) ligands were able to alter intracellular signalling in A5-expressing cells using an aequorin luminescence-based assay (Le Poul et al., 2002). None of the ligands tested led to a positive result (data not shown). Nevertheless, further experiments are required to defi-
tively answer this question.

Many vGPCRs are characterized by an unusual pattern of subcellular localization (Fraile-Ramos et al., 2001, 2002). This is consistent with the observation that A5 was observed mainly in association with intracellular mem-

branes of transfected cells (Fig. 1d). Detailed analysis of the human cytomegalovirus US28 vGPCR showed that it underwent constitutive endocytosis and recycling to the plasma membrane (Fraile-Ramos et al., 2001, 2002). It has been suggested that this endocytosis and recycling could be a mechanism for sequestering host chemokines that bind to this receptor, thereby clearing proinflammatory chem-

kines from the tissue surrounding infected cells (Bodaghi et al., 1998). Furthermore, it has been speculated that the intracellular localization of many vGPCRs could facilitate their incorporation in the envelope of progeny virions (Fraile-Ramos et al., 2002). However, other studies suggested that vGPCRs are not incorporated into the viral envelope (Bechtel et al., 2005; O’Connor & Kedes, 2006). Further studies are required to explain why these studies reached opposite conclusions.

AlHV-1, carried asymptotically by wildebeest, causes WD-MCF following cross-species transmission to a variety of susceptible species. The lesions of WD-MCF are thought to be induced by proliferating cytotoxic infected LGL supporting in vivo a non-replicative infection (Dr Dewals, personal communication). It is attractive to speculate that the cytotoxicity of these infected cells could be the consequence of the expression of a restricted number of viral genes. The present study is apparently the first to investigate the role of an individual gene of AlHV-1 in WD-MCF pathogenesis. The recent BAC cloning of the AlHV-1 genome has opened an area of research that was previously inaccessible due to difficulties in producing recombinant AlHV-1 viruses by homologous recombina-
tion, mainly due to the rapid attenuation of AlHV-1 during passage in culture.

In conclusion, the present study demonstrates that, despite its constitutive activity, AlHV-1 A5 vGPCR is not essential for viral replication in vitro nor for the induction of WD-

MCF in a model organism, the rabbit. Knowing that AlHV-

1 is extremely well adapted to its natural host, which is an asymptomatic carrier, the present study suggests that in some viruses, a vGPCR homologue may contribute to the adaptation of the virus to its host rather than always being a virulence factor.

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