Alcelaphine herpesvirus 1 glycoprotein B

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Original article

Alcelaphine herpesvirus 1 glycoprotein B: recombinant expression and antibody recognition

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Abstract

The gamma-herpesvirus alcelaphine herpesvirus-1 (AlHV-1) causes fatal malignant catarrhal fever (MCF) in susceptible species including cattle, but infects its reservoir host, wildebeest, without disease. Pathology in cattle may be influenced by virus-host cell interactions mediated by the virus glycoproteins. Cloning and expression of a haemagglutinin-tagged version of the AlHV-1 glycoprotein B (gB) was used to demonstrate that the AlHV-1-specific monoclonal antibody 12B5 recognised gB and that gB was the main component of the gp115 complex of AlHV-1, a glycoprotein complex of five components identified on the surface of AlHV-1 by immunoprecipitation and radiolabelling. Analysis of AlHV-1 virus particles showed that the native form of gB was detected by mAb 12B5 as a band of about 70kDa, whilst recombinant gB expressed by transfected HEK293T cells appeared to be subject to additional cleavage and incomplete post-translational processing. Antibody 12B5 recognised an epitope on the N-terminal furin-cleaved fragment of gB on AlHV-1 virus particles. It could be used to detect recombinant and virus-expressed gB on western blots and on the cell-surface of infected cells by flow cytometry, whilst recombinant gB was detected at the surface of transfected cells by immunofluorescence. Recombinant gB has potential as an antigen for ELISA detection of MCF virus infection and as a candidate vaccine antigen.

Keywords: malignant catarrhal fever; herpesvirus; macavirus; glycoprotein B; fusion tag; mammalian expression; monoclonal antibody

Introduction

Malignant catarrhal fever (MCF) is a fatal lymphoproliferative disease of cattle, deer, bison and other ungulates, caused by ruminant gammaherpesviruses of the genus *Macavirus* [1]. Alcelaphine herpesvirus 1 (AlHV-1), which is among the best studied of the MCF viruses, naturally infects wildebeest, causing MCF among in-contact cattle in sub-Saharan Africa. The other major cause of MCF worldwide is ovine herpesvirus 2 (OvHV-2), which naturally infects domestic sheep [2]. The MCF viruses infect their natural hosts without obvious clinical signs, causing a lifelong, latent infection. In the wildebeest, AlHV-1 cell-free virus appears to be shed in nasal secretions and other body fluids during occasional recrudescence of lytic infection in adults, but the major source of infectious virus appears to be wildebeest calves that shed virus for a prolonged period during their first few months of life. This virus presents a major infectious disease challenge for in-contact cattle, and Massai herdsmen move their cattle to poorer upland grazing to avoid wildebeest during the calving season [3, 4].

MCF in cattle is a dramatic, fatal lymphoproliferative disease characterized by lymphocyte accumulation and necrosis in multiple organs. The disease is characterised by fever, inappetence, ocular and nasal discharge, lesions of the buccal cavity and muzzle, diarrhoea and depression. Cattle may survive for a week or more after the appearance of the first clinical signs, while bison and deer generally die after a few days. Currently there is no treatment or vaccine commercially available for MCF.

AlHV-1, unlike OvHV-2, is the only MCF virus that currently can be propagated in culture and has therefore been used as a model for the sheep-associated form of the disease. The virus genome has been sequenced [5] and is very similar to the genome of OvHV-2 [6]. Small animal models of AlHV-1 infection are available and have been characterised [7, 8]. Extended passage of pathogenic AlHV-1 leads to attenuation [9] and this is associated with rearrangements within the virus genome [10]. Attenuated AlHV-1 has recently been shown to protect cattle from fatal challenge with pathogenic virus and therefore represents a candidate vaccine for MCF [11, 12]. The attenuated and pathogenic AlHV-1 strains have recently been characterised by proteomic methods, demonstrating that there are few major differences between the strains [13].

As part of our studies to understand the basis of MCF pathology in cattle we have been studying the antigenic capsid proteins of AlHV-1[14]. However, interactions between the virus and host are also likely to be influenced by the membrane glycoproteins of the virus, which are responsible for interactions with cell surface receptors on target cell types and fusion of virus and cell membranes. The gp115 glycoprotein complex of AlHV-1 was defined by immunoprecipitation with monoclonal antibody (mAb) 12B5 [15] and mAb 152-A [16] as having components of approximately 115, 110, 105, 78 and 48 kDa. Further analysis demonstrated that this complex appeared to be composed of a single polypeptide that was glycosylated to generate the mature 115 kDa form, which was then cleaved to produce a covalently-linked complex comprising 78 kDa and 48 kDa chains in the virus particle [15].

Recent proteomic analysis of polyacrylamide gel fractionated AlHV-1 virus proteins [13] showed that glycoprotein B (gB) was found in two bands with relative mobility estimated at 80 kDa and 50 kDa, respectively. Examination of the gB peptides identified in the two bands showed that the 80 kDa band contained N-terminal peptides and the 50kDa band contained C-terminal peptides, relative to the position of a furin cleavage site [13]. The existing published data therefore support the hypothesis that mAb 12B5 recognises an epitope on gB and that the 78 kDA and 48 kDa bands identified previously are the furin cleaved N- and C-terminal fragments of gB.

The aims of this work were to confirm the interaction of mAb 12B5 with AlHV-1 gB by expression of a recombinant, epitope tagged polypeptide and to characterise the expression and antigenicity of the recombinant gB polypeptide.

Materials and methods

Cells and virus

AlHV-1 C500 strain was propagated in bovine turbinate (BT) cells grown in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies, Paisley, UK), with 5 % foetal bovine serum (FBS) and 2 % L-glutamine. BT cells were infected with pathogenic AlHV-1 C500 at a multiplicity of infection (MOI) of 3 and virus-infected cells were harvested after 3-5 days. Cell-associated virus particles were isolated after three rounds of freeze-thaw treatment, clarified by centrifugation and stored in batches at -80 °C until required. Representative aliquots of virus were titrated as described previously [11].

Human embryonic kidney 293T (HEK293T) cells were used for transfection studies following previous experiments that had shown that this cell line provided both a high frequency of transfection and a high level of expression of MCF virus genes [17, 18]. For transfection and subsequent protein expression, cells were grown in IMDM with 10 % FBS and 2 % L-glutamine. For fluorescence microscopy, sterile coverslips were first treated with 500 µl of poly-L- lysine for 20 minutes to improve cell adherence. Excess poly-L-lysine was removed and the coverslips were dried in a 37˚C incubator for 1 hour. Cells were seeded onto coverslips at $2x10^5$ per well of a 24 well dish in IMDM before transfection. After 6 hours at 37 °C, 5 % CO_2 the medium was changed to serum-free Opti-MEM (Life Technologies).

Amplification and cloning of the gB gene

The gene encoding gB (ORF8 [5]) from AlHV-1 was expressed in recombinant form as follows: ORF8 was amplified from AlHV-1 strain C500 DNA by PCR using primers ORF8FwdAsc (5'-GGC GCG CCG GCG CAC ACA GGT AGC ACA GTC T-3'), ORF8RevFse (5'-GGC CGG CCC GAC ACC AGT GCT CTC AAA AGA GT-3') and KOD hot-start DNA polymerase (Merck, Nottingham, UK). The 2.6 kbp fragment was inserted into pCR4-blunt-TOPO (Life Technologies) and the DNA sequence of the resulting clones was determined using flanking and internal primers. A verified

ORF8 fragment was excised using restriction enzymes *Asc*I and *Ngo*MIV and inserted into the mammalian expression vector pVR-MCS-HA, derived from pVR1255 (a kind gift from Vical Inc. [19]). To generate pVR-MCS-HA, a synthetic 218 bp double-stranded cassette encoding a ribosome binding site with start codon, a multiple cloning site, a TEV protease cleavage site and a C-terminal triple-HA (haemagglutinin) epitope tag (TOP Gene Technologies, Montreal Canada; accession number FN553440) was inserted between the *Not*I and *Bam*HI sites of pVR1255. The correct structure of the resulting AlHV-1 gB expression vector (pVR-ORF8-HA) was confirmed by restriction enzyme analysis.

Antibodies

Monoclonal antibody 12B5 was raised against whole AlHV-1 virus antigen in BALB/c mice and was shown to be specific for the virus major glycoprotein complex in western blots and immunoprecipitation experiments[15]. For western blotting of HA-tagged proteins, a directly conjugated rat monoclonal anti-HA-peroxidase high affinity (3F10; Roche) was used. Secondary antibodies used for western blotting were HRP-conjugated polyclonal goat-anti-mouse immunoglobulins (Dako , Glostrup, Denmark) and polyclonal rabbit-anti-bovine IgG-HRP (Sigma). For flow cytometry and fluorescence microscopy, the following antibodies were used: mouse anti-HA IgG3 (Abcam); Alexa 488-conjugated goat-anti-mouse IgG3 (Life Technologies); mouse anti-tubulin IgG1 (Abcam); Alexa 555-conjugated goat-anti-mouse IgG1 (Life Technologies).

Transfection

Briefly, HEK293T cells were grown in IMDM containing 5 % FBS and 2 mM L-glutamine, to 70 % confluence before being transfected with plasmid DNA (1µg per 10^6 cells) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. The transfection reagent was replaced with fresh medium after four hours and transfected cells were allowed to grow for up to 72 h before harvesting cells and medium.

Protein extracts for western blotting and ELISA were made 48h post-transfection by lysing single-cell suspensions in 0.8 % CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) in Tris-buffered saline pH 8.3.

Electrophoresis and western blotting

Protein extracts (approx. 10 µg per lane) were fractionated in denaturing, reducing 10 % NuPAGE® Bis-Tris gels (Life Technologies). Protein bands were visualised with SimplyBlue SafeStain (Life Technologies) or transferred to nitrocellulose using the X-cell II semi-wet Blot module in 1× NuPAGE Transfer buffer (Life Technologies) containing 10 % or 20 % methanol, following manufacturer's guidelines. Nitrocellulose membranes were blocked in in PBST [1×PBS, 0.05 % (v/v) TWEEN20 (Sigma)] containing 5 % non-fat milk powder (5 % milk), then washed several times in

PBST. For HA- tag detection, proteins were probed with anti-HA-HRP according to the manufacturer's instructions. Detection of gB with monoclonal antibody 12B5 was done with 1:1000 dilution of affinity-purified 12B5 followed by incubation with HRP-conjugated goat-anti-mouse Ig. For detection by antibodies from MCF vaccinated or infected animals, blood serum or plasma was diluted 1:100 in PBST containing 5 % milk. Bound antibodies were detected with polyclonal rabbit-anti-cow immunoglobulin-HRP. Finally, blots were washed several times with PBST then incubated for 5 min in SuperSignal West Pico Chemiluminescent Substrate (Pierce) before visualisation of bands using the ImageQuant LAS4000 imaging system (GE Healthcare).

Proteomic analysis

The identification of recombinant AlHV-1 gB fractionated by gel electrophoresis was done by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) as described previously [13, 14]. Briefly, protein bands excised from PAGE gels were subjected to standard in-gel destaining, reduction, alkylation and trypsinolysis, followed by liquid chromatography interfaced with a 3-D high capacity ion trap mass spectrometer (Esquire HCTplusTM, Bruker Daltonics). Mass spectral data were analysed using the Mascot V2.2 (Matrix Science) search algorithm against viral and mammalian subsections of the NCBI non-redundant protein sequence databases. Protein identification within a gel band required a minimum of two peptides to be matched, with each peptide containing an unbroken ''b'' or ''y'' ion series of at least four amino acid residues.

ELISA

Extracts of transfected or untransfected HEK293T cells were prepared as described above and serial dilutions (in 0.1M carbonate buffer pH9.6) were used to coat triplicate wells of 96-well microtitre plates (Greiner, high binding). After washing in PBST, detection of the MCF antigen was assayed using a 1:200 dilution of an AlHV-1-positive plasma pool that had previously been used as a control in analysis of vaccinated cattle [12]. Antibody bound in each well was detected using 1:1000 rabbit anti-bovine IgG-Horseradish Peroxidase conjugate (Sigma) and colourimetric detection using Tetramethylbenzidine (TMB; Sigma). Average optical density at 450nm was used as the measure of ELISA response.

Flow cytometry

BT cells were infected with pathogenic AlHV-1 C500 at MOI = 3. After 24h, the infected cells were harvested and stained with mAb 12B5 or isotype-matched control antibody (VPM21 [20]), followed by phycoerythrin-conjugated anti-mouse IgG1 secondary antibody. After staining, the cells were washed twice in FACS buffer and fixed in PBS/0.02 % sodium azide before storing dark and on ice. Samples were acquired using a FACSCalibur instrument (Becton Dickinson, NJ, USA) equipped with 488 nm argon-ion and 635 nm red diode lasers and CellQuest software. Forward (FSC) and side

scatter (SSC) were collected as linear signals, and fluorescent emissions were collected on a four-decade logarithmic scale. A bandpass filter for phycoerythrin was employed to collect the signal in fluorescence channel 2 (FL2, 585 ± 21 nm). A minimum of 10,000 cells were acquired for each sample by gating lymphoid (monocyte/lymphocyte) cells on forward and side scatter.

Fluorescence microscopy

Cells for fluorescence microscopy were incubated for 48 hours after transfection or seeding onto coverslips before fixing with 4 % paraformaldehyde (Sigma) in PBS at room temperature for 1 hour. Cells were washed twice with PBS and once with immunofluoresence buffer (IFF; 2 % FBS, 1 % BSA in PBS). Cells were stored at 4 °C in IFF.

Fixed cells were permeabilised using 0.5 % triton-X-100 in PBS for 10 minutes at room temperature. Cells were then rinsed twice with PBS and once with IFF. Cells were incubated in primary antibody diluted in IFF for 40 minutes at room temperature. Cells were then washed twice with PBS and once with IFF before incubation in the appropriate secondary antibody diluted in IFF for 40 minutes in the dark at room temperature. Cells were washed three times with PBS, refixed with 4 % PFA for 20 minutes, and then rinsed three times with PBS, with the final wash containing DAPI. The cells were rinsed with water and mounted onto slides with Vectashield (Vector Labs) and sealed with clear nail varnish.

Stained cells were visualised using a motorised Zeiss axiovert 200M inverted epifluorescence microscope with apotome and an axiocam MR3 camera. The following Zeiss filter sets were used; set 38H (excitation BP470/40, Beam splitter FT495, Emission BP525/50) set 43 (excitation BP 545/25, Beam splitter FT 570, emission BP 605/70), set 49 (excitation G 365, Beam splitter FT395, Emission BP 445/50) and set 50 (excitation BP640/30, Beam splitter FT 660, emission BP 690/50). Image analysis was undertaken using Zeiss axiovision software.

Results

Expression of AlHV-1 gB

To address the hypothesis that mAb 12B5 recognised an epitope on AlHV-1 glycoprotein B, the AlHV-1 ORF8 gene, encoding gB, was cloned into a haemagglutinin (HA)-tagged expression vector based on pVR1255 [19]. The resulting construct, pVR-ORF8-HA, was transfected into HEK293T cells and protein extracts were made 48h post-transfection. A western blot of whole-cell extracts probed with anti-HA-HRP conjugate showed that AlHV-1 gB was detected as three bands with apparent Mr of approximately 100 kDa, 50 kDa and 20 kDa (Fig. 1A). These bands presumably corresponded to full-length gB and two cleaved forms carrying the C-terminal HA-tag. An equivalent gel, carrying extracts of HEK293T cells expressing gB and BT cells infected with pathogenic AlHV-1 virus, was blotted using mAb 12B5. This showed that whilst a single major band with Mr of about 70 kDa was recognised in the virus extract by 12B5, three bands were recognised within the recombinant gB

extract with approximate Mr of 100 kDa, 70 kDa and 60 kDa (Fig. 1B). This confirmed that mAb 12B5 recognised gB and suggested that the HA-tagged 50 kDa and 20 kDa cleaved products of gB seen in Fig. 1A had lost the 12B5 epitope. The differences in bands recognised by mAb 12B5 between the virus preparation and the gB-transfected cell extract (Fig. 1B) likely reflect differences in post-translational processing between the two cell lines, including both glycosylation and furin cleavage. While gB in the mature AlHV-1 virion appeared to be almost fully cleaved by furin, with only a faint band at the uncleaved ~100 kDa position, the recombinant protein expressed in HEK293T cells was mostly uncleaved, with a smaller proportion of the gB appearing as bands with lower Mr. This is in accord with observations in other gammaherpesviruses that cleavage of gB is closely associated with virion assembly [21]. The detection of two 12B5-detected bands with mobility 60-80 kDa in the recombinant gB lane (Fig. 1B) is in keeping with previous observations that furin cleavage of the 115 kDa and 110 kDa components of the major envelope glycoprotein complex in infected cells gave rise to bands with Mr 78 kDa and 66 kDa respectively [15]. This supports the published evidence that HEK293T cells express furin [22] and suggests that limited furin cleavage of recombinant gB can occur in the absence of virion assembly. Thus, recombinant gB expressed in HEK293T cells appears to be subject to both incomplete post translational modification and additional proteolytic cleavage to produce the lower bands observed in Fig. 1A and 1B. Attempts to express recombinant gB in AlHV-1 infected cells have been unsuccessful to date. This may reflect the poor levels of transfection achievable in the AlHV-1 permissive cell lines tested or the silencing of the recombinant gB construct in AlHV-1 infected cells.

Proteomic analysis of gB fragments

To further confirm the identity of the HA-tagged 50 kDa and 20 kDa bands (Fig. 1A), they were subject to proteomic analysis following excision of the bands from SDS-PAGE of affinity-purified recombinant gB. MS/MS analysis did not provide any evidence of gB-specific peptides in the 20 kDa band but the 50 kDa band yielded multiple gB-specific peptides that were mainly C-terminal of position 300 (Online Resource 1). These observations suggest that recombinant gB may be cleaved at sites on both sides of the furin cleavage site in addition to any furin cleavage that occurs. These data also suggest that the mAb 12B5 epitope is likely to be N-terminal of position 300.

The absence of detectable gB-specific peptides in the 20 kDa HA-tagged band may reflect a lack of suitable peptides for mass spectrometry in the C-terminal portion of the protein. This fragment is likely to include the transmembrane and cytoplasmic regions of gB and the triple-HA tag, which may influence the ionisation or detection of gB peptides. This is supported by the lack of sequenced peptides from the C-terminal region (residues 700-860) of the 50kDa gB-HA fragment and the low abundance of mass-matched peptides (Online Resource 1).

Detection of recombinant gB by sera from infected animals

To further study the antigenic properties of AlHV-1 gB, we analysed the reactivity of affinity-purified recombinant gB in western blots with antibodies from: naturally infected wildebeest, from

cattle MCF cases induced by AlHV-1 or OvHV-2 and from cattle immunised with the attenuated AlHV-1 vaccine [11] (Fig. 2A). This demonstrated that all three recombinant gB bands were recognised by antibodies from both immunised and MCF-affected animals and showed cross-reactivity between AlHV-1 gB and OvHV-2-specific serum. These observations were confirmed by ELISA analysis of extracts of HEK293T cells transfected with pVR-ORF8-HA using an AlHV-1-positive antibody pool. This showed that MCF-specific antibodies could detect gB at dilutions down to at least 1/200 (0.05) of a crude extract of transfected HEK293T cells, whilst an equivalent extract containing a non-antigenic HA-tagged recombinant protein (ORF25 [14]) was not significantly different from an extract of untransfected HEK293T cells (Fig. 2B).

Detection of gB expression by flow cytometry and fluorescence microscopy

Having confirmed that mAb 12B5 recognised recombinant and native glycoprotein B in western blots and ELISA, we then tested whether it could detect expression of gB on the surface of productively-infected cells. BT cells were infected with pathogenic AlHV-1, harvested after 24h and stained with mAb 12B5 for analysis by flow cytometry (FACS). About 10 % of the BT cells expressed the 12B5 epitope (Fig. 3), showing that mAb 12B5 could recognise the gB antigen in its native conformation and that gB was expressed on the surface of infected cells within 24h.

The sub-cellular localisation of recombinant gB in transfected HEK293T cells was also confirmed by fluorescence microscopy (Online Resource 2). Staining with anti-HA showed that gB in transfected cells was detected mainly at the cell surface, and that this localisation could be inhibited by treatment of the cells with Brefeldin A, which inhibits intracellular transport (data not shown).

Discussion

These studies have shown for the first time that the polypeptide which forms the gp115 glycoprotein complex of AlHV-1, identified on infected cells as five bands with Mr 115-48 kDa [15, 16], is glycoprotein B, which is post-translationally modified and cleaved by furin during assembly into virus particles. The observation that recombinant gB, expressed in HEK293T cells, is poorly cleaved by furin and appears to be susceptible to degradation by other proteases, suggests that virus assembly may be an essential part of the correct processing of gB. This is in accord with the known properties of gB in other herpesviruses where association of gB with the gH/gL glycoprotein pair constitutes the core fusion machinery, while additional viral glycoprotein components of the fusion complex may act as cell-type-specific receptors [23]. It is notable that a recombinant OvHV-2 gB fusion polypeptide expressed in transfected cells was also subject to proteolysis, revealing bands of about 110 and 50 kDa [24] when blotted with an epitope tag antibody. Recombinant gB (in addition to other MCF virus antigenic proteins [14, 24]) has been suggested as a good candidate for a recombinant vaccine for MCF and the observation that AlHV-1 gB is recognized by OvHV-2-specific antiserum suggests that cross-protection might occur.

Immunisation of rabbits with OvHV-2 gB induced antibodies that reduced the infectivity of OvHV-2 in an *in vivo* assay [24]. As gB is a known target for herpesvirus neutralization [25], and because mAbs now known to be specific for AlHV-1 gB have been shown to have virus neutralizing activity [15, 16], it will be of interest to determine whether recombinant AlHV-1 gB can induce virus neutralizing antibodies and protect immunized animals from malignant catarrhal fever. This work is currently ongoing.

The data presented here confirm the gB specificity of mAb 12B5 [15] and suggests that other mAbs with specificity for the gp115 complex of AlHV-1 are also likely to recognize epitopes on gB [15,16]. Notably, the mAb 152A described by Li and colleagues [16] recognized a polypeptide with Mr ~ 45 kDa under denaturing conditions. It is now likely that the epitope recognized by this mAb is on the C-terminal furin cleaved fragment of gB.

Previous studies and work here have demonstrated that mAb 12B5 is virus neutralizing and can detect gB by immunoprecipitation, by ELISA, in western blots, flow cytometry and fluorescence microscopy. Attempts to visualize gB expression by immunohistochemistry of fixed tissue sections from MCF affected cattle and rabbits have been unsuccessful to date (data not shown), suggesting that mAb 12B5 is not suitable for this application or that gB is not expressed in MCF-affected tissues. However this versatile reagent has considerable potential for use in diagnostic applications, for analysis of the AlHV-1 fusion machinery and for analysis of glycoprotein expression in AlHV-1 infection and MCF.

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Figure Legends

Fig. 1 mAb 12B5 recognizes AlHV-1 glycoprotein B. **(A)** Western blotting with anti-HA antibody detected three HA-tagged bands in a detergent extract of HEK293T cells transfected with pVR-ORF8-HA (gB). **(B)** Western blotting with mAb 12B5 detected three bands in HEK293T cells transfected with pVR-ORF8-HA (gB), but detected one major band in cell-associated AlHV-1 C500 virus (V). Pre-stained protein size markers (M; SeeBlue® Plus2, Life Technologies) were imaged in parallel and a merged monochrome image is presented. Marker sizes are indicated on the left of each panel

Figure 1

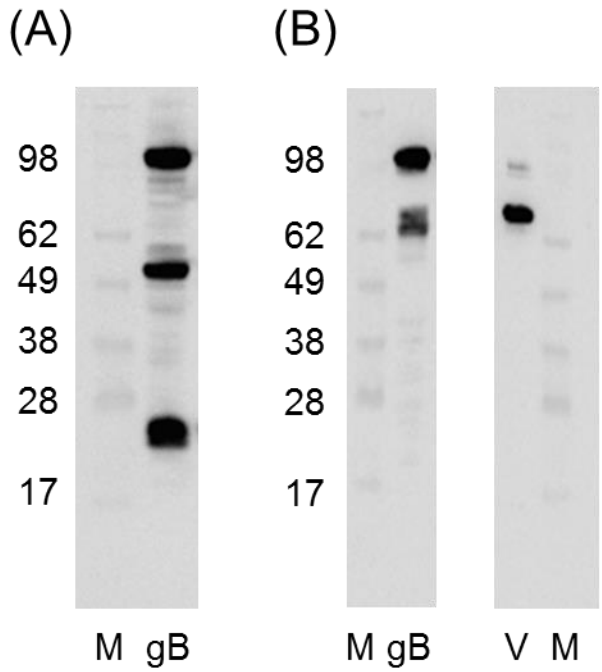


Fig. 2 Detection of recombinant AlHV-1 gB by MCF-positive antibodies using western blotting and ELISA. **(A)** Western blots of recombinant gB extracts probed with serum or plasma from: an AlHV-1 infected wildebeest (lane 1); a cow with AlHV-1-induced MCF (lane 2); a cow vaccinated with attenuated AlHV-1 (lane 3); a cow with OvHV-2-induced MCF (lane 4); and a MCF-negative control cow (lane 5), was used to detect recombinant gB fractionated by denaturing, reducing SDS-PAGE. Antibody binding was detected as described in Fig 1. Pre-stained protein size markers (M; SeeBlue® Plus2, Life Technologies) were imaged in parallel and a merged monochrome image is presented. Marker sizes are indicated on the left of the figure. **(B)** ELISA analysis of recombinant gB. Serial dilutions of detergent extracts of HEK293T cells (filled triangles) and the same cells transfected with pVR-ORF8-HA (filled circles; expressing AlHV-1 gB) or pVR-ORF25-HA (filled squares; expressing HA-tagged AlHV-1 major capsid protein) were coated on ELISA plates and their specific reactivity was tested using 1/200 dilution of pooled MCF-positive plasma Mean optical density (OD) values are plotted against extract dilution

Figure 2

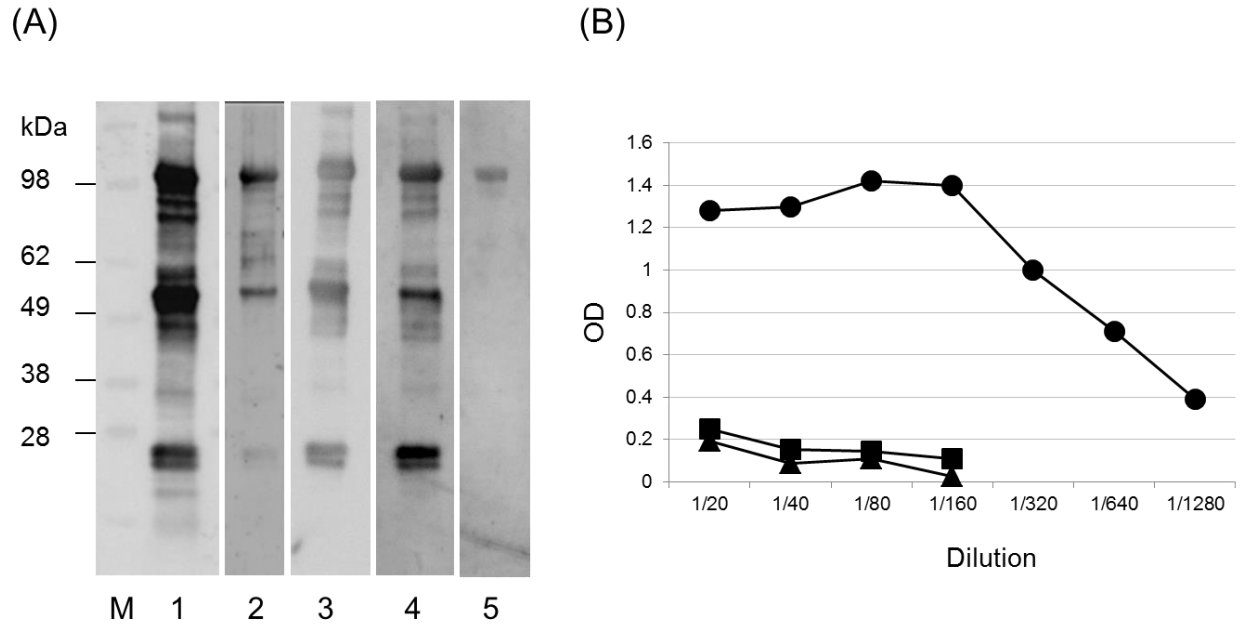
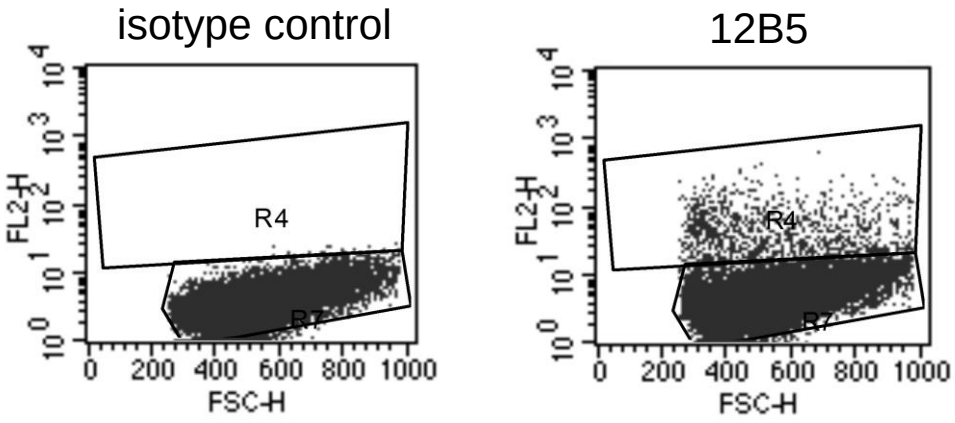


Fig. 3 Flow cytometric analysis of mAb 12B5 binding to infected cells. Bovine turbinate (BT) cells were infected with pathogenic AlHV-1, harvested after 24h, stained with an isotype-matched control antibody (left panel) or mAb 12B5 (right panel) and phycoerythrin-conjugated anti-mouse IgG1 secondary antibody for analysis by flow cytometry (FACS) Phycoerythrin fluorescence (FL2H) was plotted against cell size/complexity (FSC-H) and demonstrated that about 10 % of BT cells expressed gB 24h after infection as detected by 12B5, while less than 1 % of cells were stained by the isotype control antibody (gated region R4 in each panel)

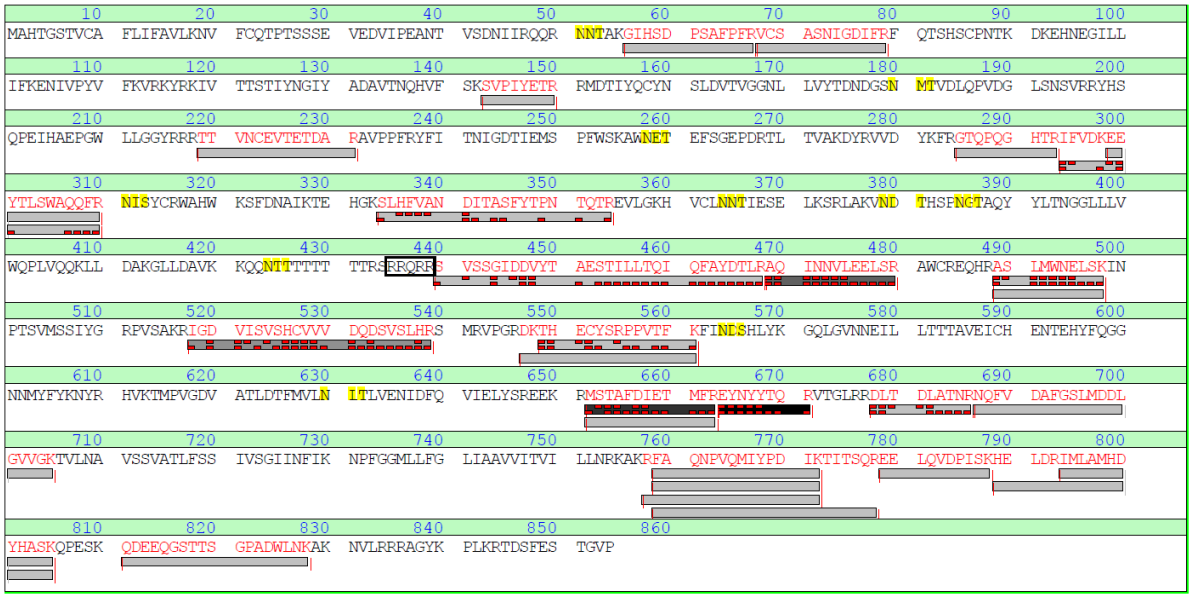
Figure 3



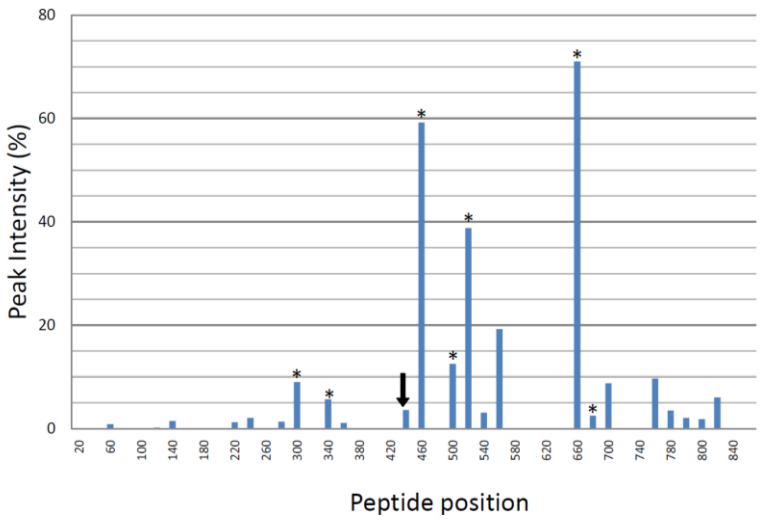
Online Resource 1

Proteomic analysis of 50 kDa HA-tagged band of recombinant expressed AlHV-1 gB

A. Graphical representation of peptide detection by MALDI-MS (grey bars represent peptides with masses that closely match peaks in the MS spectrum; darker bars are more abundant peptides) and amino acid sequences inferred from MALDI-MS/MS secondary fragmentation of abundant peptides (red blocks). Each red block represents a b- or y-ion mass matched to the amino acid residue at that position. An unbroken b or y ion series comprising a minimum of four amino acid residues is considered good evidence for peptide identification. The recognition site for furin cleavage (RRQRR) is boxed and predicted N-linked glycosylation sites are highlighted in yellow.



B. Chart of peptide intensity, derived from MALDI-MS analysis of the 50 kDa band of gB. Peptide peak intensities, derived from the raw mass-spectral data, are plotted as bars at the appropriate positions along the length of the AlHV-1 gB polypeptide sequence. Any peptide with MS/MS evidence of its presence (according to the criteria set out in Figure S1A, above) is indicated by an asterisk (*) above the column and the position of the furin cleavage site is indicated by an arrow.



Online Resource 2.

Immunofluorescence localization of AlHV-1 gB in transiently transfected HEK293T cells.
A: nuclei stained with the DNA–specific stain DAPI (blue);
B: gB-HA stained with mouse anti-HA IgG3 and Alexa 488-conjugated goat-anti-mouse IgG3 (green); and
C: α-tubulin stained with mouse anti-tubulin IgG1 and Alexa 555-conjugated goat-anti-mouse IgG1 (Life Technologies) (red).
D: false-colour image combining the signals from A-C.
Scale bar in panel A represents 20 µm.