Glycoprotein B of equine herpesvirus type 1 has two recognition sites for subtilisin-like proteases that are cleaved by furin

Bart Spiesschaert,1 Heike Stephanowitz,2 Eberhard Krause,2 Nikolaus Osterrieder1 and Walid Azab1,3

Correspondence
Walid Azab
wfazab@zedat.fu-berlin.de

1Institut für Virologie, Robert von Ostertag-Haus, Zentrum für Infektionsmedizin, Freie Universität Berlin, Robert-von-Ostertag-Str. 7–13, 14163 Berlin, Germany
2Leibniz-Institut für Molekulare Pharmakologie, Robert-Rössle-Strasse 10, D-13125 Berlin, Germany
3Department of Virology, Faculty of Veterinary Medicine, Zagazig University, Egypt

Glycoprotein B (gB) of equine herpesvirus type 1 (EHV-1) is predicted to be cleaved by furin in a fashion similar to that of related herpesviruses. To investigate the contribution of furin-mediated gB cleavage to EHV-1 growth, canonical furin cleavage sites were mutated. Western blot analysis of mutated EHV-1 gB showed that it was cleaved at two positions, $518 \text{RRRR} 521$ and $544 \text{RLHK} 547$, and that the 28 aa between the two sites were removed after cleavage. Treating infected cells with either convertase or furin inhibitors reduced gB cleavage efficiency. Further, removal of the first furin recognition motif did not affect in vitro growth of EHV-1, while mutation of the second motif greatly affected virus growth. In addition, a second possible signal peptide cleavage site was identified for EHV-1 gB between residues 98 and 99, which was 13 aa downstream of that previously identified.

INTRODUCTION

Proprotein convertases, which belong to the conserved subtilisin-like family (subtilases), are calcium-dependent serine proteinases, which are responsible for generating a large diversity of bioactive proteins and peptides through cleavage within secretory pathway compartments, more specifically, the trans-Golgi network and endosomal vesicles (Bosshart et al., 1994; Gu et al., 2001). Numerous mammalian precursors have been identified to date, including PC1, PC2, furin, PC4, PC5, PACE4 and PC7. Each of these enzymes, either alone or in combination with others, is responsible for specific processing of multiple protein precursors at single and/or pairs of basic residues. Precursors are usually cleaved at the consensus motif Lys/Arg-X$_n$-Lys/Arg, where $n=0, 2, 4$ or 6 and X can be any amino acid but usually not Cys (Seidah et al., 1999).

Furin, formerly known as paired basic amino acid cleaving enzyme (PACE), is an endoprotease that plays an important role in homeostasis, cancer, dementia, bacterial and viral diseases and many more pathologies (Thomas, 2002; Wise et al., 1990). Furin activates cellular, but also viral and bacterial proteins, by cleaving at a conserved arginine-rich consensus site (Arg–X–Lys/Arg–Arg; where X is any amino acid); however, cleavage at the Lys/Arg-X$_n$-Lys/Arg motif can also take place (Hallenberger et al., 1992; Molloy et al., 1992; Seidah et al., 1999; Stieneke-Grober et al., 1992; Volchkov et al., 1998). The function of the furin cleavage motif of glycoprotein B (gB) was evaluated for several herpesviruses. Abolishing furin cleavage of MuHV-4 gB had no effect on cell entry of fibroblasts and epithelial cells, but showed a significant entry deficit in myeloid cells such as

One supplementary figure is available with the online Supplementary Material.
macrophages and bone marrow-derived dendritic cells (Glauser et al., 2013). gB of herpes simplex virus type 1 (HSV-1), on the other hand, is not cleaved (Claesson-Welsh & Spear, 1986). It was clear from these studies that the role of furin cleavage of gB differs significantly from one virus to the other, but, when present, furin cleavage seemed to be critical for full gB functionality.

Equine herpesvirus (EHV)-1 is a member of the genus Variceliovirus within the subfamily Alphaherpesvirinae. The virus induces several severe clinical syndromes with infection of PBMCs being a key aspect in the pathogenesis, as viraemia contributes to the systemic distribution of virus. The subsequent infection of different organs can result in abortion and neurological syndromes as well as respiratory disease (Borchers et al., 2004; Meindl & Osterrieder, 1999; Neuabauer et al., 1997; Stokes et al., 1996; Wellington et al., 1996b). Information about EHV-1 gB is limited, but it appears to be a 138 kDa protein that, when fully glycosylated, is proteolytically cleaved into two subunits (77 and 55 kDa). These subunits are linked by a disulfide bond(s) to form a 145 kDa complex (Sullivan et al., 1989). Although the site responsible for cleavage of EHV-1 gB into two subunits was believed to be a conserved furin cleavage motif (544RLHK547), an alternate endoproteolytic cleavage site (544RLHK547) was identified for EHV-1 gB. However, cleavage at 518RRRR521 was not excluded, suggesting that the 28 aa between both cleavage sites are removed (Wellington et al., 1996a). As there are no previous studies addressing the role of furin cleavage during EHV-1 replication, we addressed the question of whether abolishing individual or all possible furin cleavage sites of EHV-1 gB would have attenuating effects on virus infection. Whether effects on PBMC infection, similar to those seen for MuHV-4 (Glauser et al., 2013), were also identifiable in the case of EHV-1 was of particular interest due to the important role these cells play during EHV-1 pathogenesis. Here, we report on the importance of furin-mediated cleavage of EHV-1 gB by analysing gB expression in equine dermal (ED) cells. Furthermore, virus growth in vitro, after mitigating furin cleavage motifs, was also investigated.

RESULTS

Mutation of the furin cleavage site

To study the effect of gB cleavage on EHV-1 replication, two-step Red-mediated recombination was used to either delete or replace putative furin cleavage sites (Fig. 1). The newly generated viruses comprised EHV-1Δ518RRRR521, EHV-1Δ518AAAA521, EHV-1Δ544AAAA547, EHV-1Δ495AAAA498/518AAAA521 and EHV-1Δ495AAAA498/518AAAA521Δ544AAAA547. The respective revertant viruses, in which the original sequences were restored, were also generated. The genotypes of all the mutants were confirmed by PCR, RFLP and Sanger sequencing (data not shown).

gB expression and cleavage

To determine the effect on gB expression of mutating the conserved arginine-rich consensus furin cleavage site (518RRRR521), ED cells were infected with either parental or mutant (EHV-1Δ518RRRR521 and EHV-1Δ518AAAA521) viruses or transfected with pCDNA-gB wt, pCDNA-gBΔ518RRRR521 or pCDNA-gBΔ518AAAA521. Cell lysates were then collected and subjected to Western blot analysis. Using the anti-gB 3F6 mAb, we detected the uncleaved gB precursor of approximately 138 kDa (parental and mutant EHV-1) and the 75 kDa large subunit (parental EHV-1). In the case of EHV-1Δ518RRRR521 and EHV-1Δ518AAAA521 an extra band was identified with apparent molecular masses of approximately 145 kDa and approximately 90 kDa (EHV-1Δ518AAAA521) and 85 kDa (EHV-1Δ518RRRR521; Fig. 2a). A haemagglutinin (HA)-specific antibody used in Western blots containing lysates of cells transfected with C-terminally HA-tagged gB reacted with two bands exhibiting molecular masses of approximately 138 and 60 kDa (parental and mutant EHV-1; Fig. 2b).

In order to explain the increase of apparent weight from 75 to 90 kDa, the possibility of existence of alternative furin cleavage sites was explored. Two motifs (518RRRR521; 544RLHK547) fulfilled the requirements of the general motif Lys/Arg-Xn-Lys/Arg (Seidah et al., 1999; Thomas, 2002). To investigate the importance of these motifs for gB cleavage, ED cells were either infected with parental or mutant (EHV-1Δ495AAAA498/518AAAA521) viruses or transfected with pCDNA-gB wt, pCDNA-gBΔ544AAAA547, pCDNA-gBΔ495AAAA498/518AAAA521 or pCDNA-gBΔ495AAAA498/518AAAA521Δ544AAAA547. Expression of gB was evaluated using Western blot analysis. Probing blots with either anti-gB 3F6 or anti-HA antibodies (Fig. 3a, b, respectively) did not reveal any difference in molecular mass between EHV-1Δ518AAAA521 and its parental EHV-1Δ518AAAA521 (Fig. 3a, b, lanes 1, 2 and 4). Interestingly, mutating 544RLHK547 resulted in the prevention of proteolytic cleavage of gB as evidenced by the presence of the precursor molecule (138 kDa) and the absence of the large (75–80 kDa) and small (60 kDa) subunits (Fig. 3a, b, lanes 1, 3 and 5). This indicates that either furin or another subtilisin-like proprotein convertase was responsible for the cleavage of gB at this site.

To further confirm these results, ED cells were incubated with either furin or convertase inhibitors before infection with EHV-1. Western blot analysis showed that both inhibitors did indeed inhibit gB cleavage, indicated by
a reduced signal obtained for the large gB subunit (75–80 kDa; Fig. 3c).

Next, we performed deglycosylation analyses. Peptide-N-glycosidase (PNGase) treatment resulted in proteins of approximately 110 kDa (parental and mutant EHV-1), 55 kDa (parental EHV-1), 65 kDa (EHV-1_518AAAA521) and 60 kDa (EHV-1Δ518RRRR521; Fig. 4a). The molecular mass of the 145 kDa band observed in lysates of cells infected with EHV-1_518AAAA521 and EHV-1Δ518RRRR521 was reduced to 120 kDa after PNGase treatment (Fig. 4a). Deglycosylation with EndoH resulted in less pronounced decreases of molecular size for the proteins resulting in a molecular mass of approximately 120 kDa (parental and mutant EHV-1), 70 kDa (parental EHV-1), 80 kDa (EHV-1_518AAAA521), 80 kDa (EHV-1Δ518RRRR521) and 130 kDa for the extra band (mutant viruses; Fig. 4b).

**Virus growth in vitro**

No significant differences (n=3; Friedman test/Dunn’s multiple comparison test; P>0.05) were observed during single-step growth kinetics in ED cells for either EHV-1_518AAAA521 or EHV-1Δ518RRRR521 when compared with parental virus (Fig. 5a, b). We did not observe any significant differences either when evaluating the plaque sizes (n=100; one-way ANOVA; P>0.05; Fig. 5c). We, therefore, concluded that the growth properties of mutant EHV-1 in vitro were not significantly affected by the mitigation of the conserved furin cleavage site (518RRRR521). However, mutating the 544RLHK547 cleavage site resulted in a significant reduction of virus growth. While we were able to reconstitute virus from cloned DNA, and some plaques were visible after transfection, infection could not be maintained or propagated (data not shown). Finally, we were unable to reconstitute the virus where all furin motifs are mutated (EHV-1_495AAAA498/518AAAA521/544AAAA547).

**Infection rates of different PBMC subpopulations by parental and mutant EHV-1**

Monocytes were the most highly infected subpopulation by both parental [69.02±6.62% (EHV-1)] and mutant EHV-1 [70.17±5.98% (EHV-1Δ518AAAA521)];
61.72 ± 16.07 % (EHV-1 \( \Delta^{518}\)AAAA\( ^{521} \)), followed by the B-lymphocytes [24.28 ± 15.27 % (EHV-1); 33.75 ± 13.33 % (EHV-1 \( \Delta^{518}\)AAAA\( ^{521} \)); 12.67 ± 2.53 % (EHV-1 \( \Delta^{518}\)RRR\( ^{521} \)) and T-lymphocytes [4.98 ± 4.92 % (EHV-1); 7.48 ± 3.65 % (EHV-1 \( \Delta^{518}\)AAAA\( ^{521} \)); 4.12 ± 1.15 % (EHV-1 \( \Delta^{518}\)RRR\( ^{521} \))]. Contrary to what was reported for MuHV-4, no significant differences in infection rate between parental and mutant EHV-1 were seen (\( n = 3; \) Kruskal–Wallis one-way ANOVA; \( P > 0.05 \)) for monocytes, B-lymphocytes or T-lymphocytes (Fig. 6).

**Fig. 3.** Mutation of furin cleavage motifs and expression of gB in ED cells. (a, b) Western blot analysis was performed under reducing conditions using either anti-gB (3F6) mAb (a) or anti-HA antibodies (b). (c) Effects of convertase (Con) and furin (Fu) inhibitors on cleavage of gB. ED cells were pre-treated with either convertase or furin inhibitors (400 \( \mu M \)) before infection with viruses and subjected to Western blotting. gB expression was detected using an anti-gB (3F6) mAb under reducing conditions. As controls, cells were infected with viruses in the absence of inhibitors or treated only with the inhibitors (mock-Con and mock-Fu) without infection.

Mass spectrometry of EHV-1 gB after mutation of the furin cleavage site (\( ^{518}\)RRR\( ^{521} \))

In order to determine the apparent upward shift of the lower gB band from 75 kDa to approximately 90 kDa, this subunit was immunoprecipitated from cell lysates of EHV-1 or EHV-1 \( \Delta^{518}\)AAAA\( ^{521} \)-infected ED cells, separated by SDS-PAGE and stained with Coomassie blue. The band, corresponding to approximately 75 kDa (EHV-1) or 90 kDa (EHV-1 \( \Delta^{518}\)AAAA\( ^{521} \)), previously identified by Western blot (Fig. 2a), was precisely excised from the gel (Fig. 7a). The protein corresponding to 75 kDa was digested with trypsin, while the protein corresponding to 90 kDa was digested with either trypsin, elastase, AspN or chymotrypsin. The resulting peptides were eluted from the gel slices and subsequently subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS). EHV-1 gB was unambiguously identified with high confidence in all samples. In the samples subjected to the trypsin digest, two predicted fragments [residues 28–66 and residues 67–84; predicted by ExPASy PeptideCutter (http://web.expasy.org/peptide_cutter/)] at the N-terminal end of gB were missing. The absence of
this sequence indicated that this sequence is the signal peptide sequence, which was removed in an earlier stage of gB processing (Fig. 7b). Because MS analysis using unspecific enzymes for protein digestion may generate higher resolution due to overlapping peptides, we performed elastase digestions. LC-MS analysis clearly identified peptides with N-terminal Ser99. Furthermore, peptides covering aa 1–98 could not be observed indicating a signal cleavage site between Thr98 and Ser99 (98TS99).

By LC-MS analysis of the band corresponding to the approximately 90 kDa gB of EHV-1_518AAAA521, it was possible to detect peptides of the C-terminal end of gB. A good coverage of the gB sequence between the respective putative N-terminal signal cleavage sites and the gB C-terminal end was obtained, especially after trypsin and elastase digestion (Fig. S1a, available in the online Supplementary Material). The C-terminal end of gB was never identified in the EHV-1 parental 75 kDa band (Fig. S1b).

**DISCUSSION**

Herpesvirus gB homologues are highly conserved and are essential for replication (Cai et al., 1988; Herrold et al., 1996; Neubauer et al., 1997; Peeters et al., 1992; Rauh & Mettenleiter, 1991; Spiesschaert et al., 2015). Across most of the members of the herpesvirus subfamilies, gB seems to possess a conserved cleavage site Arg–X–Lys/Arg–Arg targeted by the cellular protease furin (Backovic et al., 2007; Glauser et al., 2013; Okazaki, 2007; Oliver et al., 2009; Sorem & Longnecker, 2009). EHV-1 gB was predicted (Fig. 1) to have at least three motifs recognized by substilisin-like proteases in the region where gB is expected to be cleaved: one fulfils the requirement of the conserved motif (518RRRR521), while the other two follow the general motif Lys/Arg–Xn–Lys/Arg (595RSNR498 and 544RLHK547). In order to investigate the role of each motif in gB cleavage, we created different mutant gB molecules where only one and/or more motifs were targeted.
We first confirmed that proprotein convertases, particularly furin, are responsible for gB cleavage. In the presence of convertase or furin inhibitors, the majority of EHV-1 gB remained uncleaved in ED cells. Comparative analyses of gB expression (Figs 2 and 3) revealed that gB is cleaved at both the $518\text{RRRR}521$ and $544\text{RLHK}547$ motifs, resulting in the removal of the 28 aa between the two sites. This may explain the difference in apparent molecular mass between the large subunits of the wt (around 75–80 kDa) and the mutant viruses (EHV-1$\Delta 518\text{RRRR}521$ and EHV-1$\Delta 518\AAA5471$, 85–90 kDa), where around 28 aa are missing. Furthermore, probing gB with anti-HA antibodies (which reacts with the small subunit) did not show any differences between mutant (gB$\Delta 518\text{RRRR}521$ and gB$\Delta 518\AAA5471$, but not gB$\Delta 544\AAA547$) and wt viruses, indicating that $544\text{RLHK}547$ is the main cleavage site of EHV-1 gB. Since there was no difference in gB expression between EHV-1$\Delta 518\AAA521\AAA498$ and parental EHV-1$\Delta 518\AAA521$, we concluded that the $495\text{RSNR}498$ site is not cleaved by furin. Of note, there was no difference between parental and mutant (EHV-1$\Delta 518\text{RRRR}521$, EHV-1$\Delta 518\AAA521$ and EHV-1$\Delta 518\AAA521\AAA495$ AAA498) viruses with regard to the reduction of the molecular masses of gB after deglycosylation. This indicates that both mutant and parental gB are glycosylated to the same extent with a mixture of high-mannose and complex carbohydrate chains.

It seems likely that double cleavage results in the excision of a stretch of amino acids that are not important for gB function. It is interesting to note that these amino acids are absent in other gB homologues (for example, the prototype Varicellovirus VZV; Fig. 1). Our data are in agreement with a previously proposed model in which gB was predicted to be cleaved at the $544\text{RLHK}548$ motif and possibly also at the conserved furin cleavage site $518\text{RRRR}521$ (Wellington et al., 1996a). One similarity with VZV is what appears to be partial cleavage of gB when the cognate furin cleavage recognition sites is mutated. Previously, two alternative cleavage sites in proximity to the furin recognition site were proposed to be responsible for partial cleavage of VZV gB, but the functionality of these motifs was not tested (Oliver et al., 2009). For other members of the genus Varicellovirus, such as PRV and BHV-1, no conclusion with regard to additional cleavage sites can be drawn, since they were evaluated by deleting large portions of gB (BHV-1) or only shown using chemical inhibitors (PRV) (Kopp et al., 1994; Okazaki, 2007).

Double cleavage by furin has also been confirmed for other viral fusion proteins. For example, the F protein of the respiratory syncytial virus (RSV) needs to be cleaved in two distinct sites by furin in order to have full fusion activity in vivo (González-Reyes et al., 2001; Zimmer et al., 2001). In the case of RSV, however, the two events appear to occur independently, which is in contrast to the furin-mediated cleavage seen for EHV-1 gB. Here, the cleavage events seem to occur sequentially since mitigating cleavage at the $544\text{RLHK}547$ motif also blocks cleavage at the $518\text{RRRR}521$ motif, but not vice versa. A similar sequential proteolytic cleavage of the fusion protein of the SARS (severe acute respiratory syndrome) coronavirus has been reported (Belouzard et al., 2009). It is postulated that cleavage at the first site allows subsequent cleavage at the second site, which in turn results in full fusion activity of the protein (Belouzard et al., 2012). We propose a similar course of events for gB of EHV-1 where the $518\text{RRRR}521$ motif is inaccessible until the $544\text{RLHK}547$ site has been cleaved.

Previous studies done on furin cleavage of gB homologues of other herpesviruses have made evident differences in mutant viruses when evaluated for their growth properties in vitro (Glauser et al., 2013; Kopp et al., 1994; Okazaki, 2007; Oliver et al., 2009; Sorem & Longnecker, 2009; Strive et al., 2002). Cleavage of gB has been shown to be important for syncytium formation in the case of PRV (Okazaki, 2007) and for cell-to-cell spread in the case of BHV-1 (Kopp et al., 1994). On the other hand, as previously reported for VZV, EBV and MuHV-4 (Glauser et al., 2013; Oliver et al., 2009; Strive et al., 2002), cleavage of gB has no effect on growth in vitro. Similarly, we show here that mitigating the conserved furin cleavage motif (EHV-1$\Delta 518\text{RRRR}521$ and EHV-1$\Delta 518\AAA521$) was dispensable for EHV-1 replication at least in ED cells. However, mutating the $544\text{RLHK}547$ furin cleavage site to $544\AAA547$ significantly affected viral growth. Moreover, when cleavage at both the $518\text{RRRR}521$ and $544\text{RLHK}547$ motifs was mitigated, no more viral replication occurred, indicating that cleavage of gB is essential for in vitro replication. In addition, EHV-1 also differed from MuHV-4, since no reduction in EHV-1-infection rates could be detected in myeloid cells (Glauser et al., 2013). This was
of particular interest for EHV-1, since the cell-associated viraemic phase allows EHV-1 to spread throughout the host without being exposed to the immune system, thereby facilitating more severe pathologies such as abortion and neurological symptoms (Ma et al., 2013).

Mass spectrometry analyses were conducted in order to further confirm the existence of two furin cleavage sites. Peptides derived from the approximately 90 kDa and 90 kDa gB protein of parental EHV-1 and EHV-1_518AAAA521, respectively, demonstrated that the signal peptide is cleaved from the gB protein, since no peptides corresponding to the signal peptide region were identified. Most N-terminal peptides identified after AspN and chemotrypsin digestion were located more downstream compared with the N-terminal peptides found after elastase digestion. This may result from difficulties in the identification of AspN or chymotryptic peptides in the region of residues 99–117. Due to the unspecific nature of elastase digestion, which may generate higher resolution due to overlapping peptides, more detailed results could be obtained from the subsequent MS results. Taken together, the LC-MS data point to another possible putative signal cleavage site between residues 98TS99, which is downstream of the previously reported signal cleavage site (85AV86) (Wellington et al., 1996a).

After evaluating the approximately 90 kDa gB protein with mass spectrometry using different enzymic digestions as preparation, the entire protein was covered ranging from the newly identified signal cleavage sites to the C-terminal end of gB. However, these results are in contrast to what was observed during the Western blot analysis of gB with mutated 518RRRR521 and/or 544RLHK547 motifs (Figs 2 and 3). When considering these mass spectrometry results in combination with the Western blot data, it seems likely that the C-terminal peptides detected during mass spectrometry originate from either misfolded full-length protein or C-terminal gB that co-migrated to the region of the gel that was excised.

Taken together, we conclude that furin is responsible for the cleavage of EHV-1 gB and that the 544RLHK547 motif is the main cleavage site of gB and altering this motif greatly affected virus growth. However, mutating the canonical furin cleavage motif (518RRRR521) does not have an effect on in vitro replication of EHV-1. Finally, it would be interesting to examine if furin cleavage has an impact on the pathogenesis of EHV-1 in the natural host, experiments that we are planning to perform in the future.

**METHODS**

**Viruses.** All viruses used in the study were recovered from the infectious bacterial artificial chromosome (BAC) clone of the EHV-1 strain Ab4 (Goodman et al., 2007). Viruses were reconstituted after
transfecting BAC DNA into human embryonic kidney (293T) cells, as described previously (Azab et al., 2009, 2010a; Rudolph et al., 2002). Supernatants and infected cells were collected 48 h after transfection and high titre stocks of each virus were produced by passaging the transfection product on equine dermal (ED) cells.

**Cells.** ED were grown in Iscove’s modified Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 20 % FBS, 1 % of non-essential amino acids (Biochrom) and 1 % of 100 mM sodium pyruvate (Biochrom). PBMCs were isolated from heparinized blood collected from healthy horses by density-gradient centrifugation over Histopaque 1077 (Sigma), following the manufacturer’s instructions. After two washing steps, cells were suspended in RPMI 1640 supplemented with 10 % FBS, 0.3 mg glucose ml⁻¹, non-essential amino acids (Biochrom) and 1 % penicillin/streptomycin.

**BAC mutagenesis.** The generated BACs were maintained in Escherichia coli GS1783 (a kind gift from Dr Greg Smith, Northwestern University, Chicago, IL, USA), which harbours the recombi- 

**Plasmids.** The entire wt (gB_wt) and mutated (gBA518RRR521, gB_518AAA521, gB_544AAA547, gB_495AAA498/518AAA521 and gB_495AAA498/518AAA521/544AAA547) genes, where sequences encoding an HA tag were fused to the gB C-terminus, were PCR-amplified using the primers listed in Table 1, digested with the appropriate restriction enzymes and cloned into the expression vector pCDNA3 (Invitrogen). Correct amplification and insertion were confirmed by Sanger sequencing (LGC Genomics). Transfection of the pDNA-gB_wt, pCDNA-gB_A518RRR521, pCDNA-gB_518AAA521, pCDNA-gB_544AAA547, pCDNA- 
gB_495AAA498/518AAA521 and pCDNA-gB_495AAA498/518AAA521/544AAA547 into ED cells was performed using Nucleofector (Lonza) (Distler et al., 2005).

**Western blot analysis.** For Western blot analyses, pellets of infected or transected ED cells were suspended in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris (pH 7.4), 1 % Triton X-100, 0.25 % Na-deoxycholate, 150 mM sodium chloride, 1 mM EDTA] with the Complete EDTA-free protease inhibitor cocktail (Roche). Sample buffer [1 % Tres/HCl (pH 6.8), 0.8 % SDS, 0.4 % glycerol, 0.15 % β-mercaptoethanol, 0.004 % bromophenol blue] was added, the mixture was heated at 95 °C for 5 min and proteins were separated by 10 % SDS-PAGE as described before (von Einem et al., 2007). Expression of gB was detected with either anti-HA antibody or anti-EHV-1 gB mAb 3F6 (1 : 1000), which recognizes a linear epitope located between aa 107 and 171 of EHV-1 gB, thereby only targeting the N-terminal cleavage fragment and uncleaved gB (Allen & Yeargan, 1987). Bound HA and 3F6 antibodies were detected with anti-rabbit and anti-mouse IgG peroxidase conjugate, respectively. Reactive bands were visualized by enhanced chemiluminescence (ECL Plus; Amersharm).

**Virus growth assays.** To determine differences in viral replication *in vitro*, single-step growth kinetics were conducted as described previously (Azab et al., 2010). Briefly, confluent ED cells were infected with an m.o.i. of 1. After 1 h of adsorption at 4 °C and 1 h of

---

**Table 1. Oligonucleotide primers used in this study**

Underlined sequences indicate the template binding region of the primers for PCR amplification with PEPkan-S, upper-case bold letters indicate the nucleotides that were mutated, restriction enzyme sites are given in lower-case bold letters, sequences in italics indicate additional bases that are not present in the EHV-1 and bold underlined sequences indicate the HA tag.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Product</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-F</td>
<td>Δ518RRR521</td>
<td>aaccccaaatcgaaccattaaatatattttatttttaaggg</td>
</tr>
<tr>
<td>P2-R</td>
<td>Δ518RRR521</td>
<td>cttgaggtctcactgacagagaaagtctgattgtttagtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P3-R</td>
<td>518AAA521</td>
<td>aacatatactaatccacggctgcgcgtctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P4-R</td>
<td>518AAA521</td>
<td>ctgatctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P5-F</td>
<td>495AAA498</td>
<td>gcgtatctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P6-R</td>
<td>495AAA498</td>
<td>tcttcatctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P7-F</td>
<td>544AAAA547</td>
<td>tcttcatctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P8-R</td>
<td>544AAAA547</td>
<td>tcttcatctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P7r-F</td>
<td>544AAAA547</td>
<td>tcttcatctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P8r-R</td>
<td>544AAAA547</td>
<td>tcttcatctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P9-F</td>
<td>gB</td>
<td>aaccccaaatcgaaccattaaatatattttatttttaaggg</td>
</tr>
<tr>
<td>P10-R</td>
<td>gB-HA</td>
<td>aaccccaaatcgaaccattaaatatattttatttttaaggg</td>
</tr>
<tr>
<td>P11</td>
<td>Sequencing</td>
<td>ctgatctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P12</td>
<td>Sequencing</td>
<td>ctgatctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P13</td>
<td>Sequencing</td>
<td>ctgatctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P14</td>
<td>Sequencing</td>
<td>atcaggtctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P15</td>
<td>Sequencing</td>
<td>atcaggtctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P16</td>
<td>Sequencing</td>
<td>atcaggtctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
<tr>
<td>P17</td>
<td>Sequencing</td>
<td>atcaggtctcactgacagagaaagtcaggtctgctgcttggggctaaccattaccaattctgattag</td>
</tr>
</tbody>
</table>

*Downloaded from www.microbiologyresearch.org by IP: 54.70.40.11 On: Sat, 22 Dec 2018 07:43:23*
incubation at 37 °C, cells were washed and treated with citrate buffer (pH 3). Infected cells and supernatant were collected separately at the indicated times post-infection (p.i.) and stored at −80 °C. Viral titres were determined by titration on ED cells.

Plaque size measurements were conducted by infecting ED cells for 1 h at 37 °C in an m.o.i. of 0.01, followed by removal of the virus suspension and overlay with DMEM containing 0.5 % methylcellulose (Sigma). After 72 h p.i., the diameters of 100 fluorescent plaques for each virus were measured using ImageJ software v1.32j (http://rsb.info.nih.gov/ij). The obtained values were normalized and compared with the values of parental viruses, which were set at 100 %.

Three independent experiments were used to calculate average plaque sizes and standard deviations.

Pharmacological inhibitors. Furin and convertase inhibitors (at 400 μM dissolved in DMSO; Calbiochem) were used to inhibit furin cleavage of gB. ED cells were pre-treated with the inhibitors for 60 min at 37 °C before infection with the viruses (m.o.i.=1) for 24 h in the presence of the drugs. Cells were then lysed and gB expression was detected by Western blotting. Cell morphology was carefully inspected to ensure that the inhibitors did not cause an adverse effect when used.

Glycosylation analysis of gB. For deglycosylation, cell lysates were digested with peptide-N-glycosidase (PNGase) F or endoglycosidase H (Endo H) according to the manufacturer’s instructions (New England BioLabs). After deglycosylation, lysates were subjected to SDS-PAGE and Western blot analysis as described above.

Infection assay. For evaluating the infection rates of the different PBMC subpopulations, 1 × 10⁵ PBMCs were infected with an m.o.i. of 1 with parental and mutant EHV-1 at 37 °C for 1 h. PBMCs were treated and washed twice as described above, followed by incubation at 37 °C. The infected PBMCs were stained at 24 h.p.i. with primary mouse antibodies against equine CD14 (monocytes), CD3 (T-lymphocytes) or IgM (B-lymphocytes) (Goodman et al., 2007). After washing, cells were labelled with a secondary Alexa Fluor 647-conjugated goat anti-mouse IgG antibody (Invitrogen). The labelled PBMCs were then analysed by means of flow cytometric analyses of 10 000 cells. A FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Treestar) were used to detect GFP expression (encoded by the used viruses under HCMV IE promoter control) and Alexa Fluor 647 staining in infected PBMCs.

Immunoprecipitation assay. Infected ED cells were suspended in RIPA buffer with the Complete EDTA-free protease inhibitor cocktail and incubated for 1 h on ice with occasional mixing. For immunoprecipitation, the cell lysates were pre-cleared for 30 min at 4 °C with 100 μl protein G-Sepharose beads (Pierce). Anti-EHV-1 gB mAb 3F6 was then added to the samples and incubated overnight at 4 °C, followed by 2 h of incubation with 200 μl protein G-Sepharose beads at 4 °C. Beads were washed three times with PBS containing 0.2 % Tween-20 (Carl Roth). The immunoprecipitates were subjected to SDS-PAGE as described above.

Protein sample preparation and mass spectrometry. After SDS-PAGE separation, excised protein bands were washed with 50 % (v/v) acetonitrile in 50 mM ammonium bicarbonate, shrunk by dehydration in acetonitrile and dried in a vacuum centrifuge. Disulfide bonds were reduced by incubation in 60 μl 10 mM DTT in 50 mM ammonium bicarbonate for 45 min at 56 °C. Alkylation was performed by replacing the DTT solution with 55 mM iodoacetamide in 50 mM ammonium bicarbonate. In-gel digestion with trypsin, chymotrypsin, AspN and elastase was performed overnight as described before (Czapalla et al., 2003). Peptides were extracted using 20 ml 0.5 % (v/v) trifluoroacetic acid (TFA) in acetonitrile and the separated liquid was dried under vacuum. The samples were reconstituted in 6 ml 0.1 % (v/v) TFA and 5 % (v/v) acetonitrile in water. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses were performed using an Orbitrap Elite hybrid mass spectrometer (Thermo Fisher) equipped with an UltiMate 3000 LC (Dionex) as described recently (Thaa et al., 2013). In brief, LC separations were performed on a capillary column (Acclaim PepMap100, C18, 2 μm, 150 mm × 75 μm i.d., Dionex) at an eluent flow rate of 200 nl min⁻¹ using an acetonitrile 0.1 % formic acid in water gradient. Mass spectra were acquired in a data-dependent mode with one MS survey scan with a resolution of 60 000 (Orbitrap Elite) and MS/MS scans of the 15 most intense precursor ions in the linear trap quadrupole.

For data processing, MS and MS/MS spectra were used to search against a custom made database containing all proteins of the SwissProt 2010_7 database (521 024 sequences; 183 901 752 residues) including the full-length EHV-1 gB sequence. For all searches, the following variable modifications were considered: carboxyamidomethyl for cysteine, oxidation of methionine and Asn/Asp amino acid exchanges. For identification of gB peptides, the processed MS/MS spectra were compared with the theoretical fragment ions of gB peptides using the Mascot server version 2.2.2 (Matrix Science Ltd). The maximum of two missed cleavages was allowed for tryptic and chymotryptic cleavages and up to six missed cleavages were allowed for the digestion with AspN and elastase. The mass tolerance of precursor and sequence ions was set to 10 p.p.m. and 0.35 Da, respectively.

Statistical analysis. Statistical analyses (described in context) were performed using GraphPad Prism 5 (Intuitive Software for Science). Normally distributed datasets, determined with the Shapiro-Wilks test, were analysed with one-way ANOVA. Datasets that were not normally distributed were analysed with Kruskal–Wallis one-way analysis of variance for two or more samples that are independent or Friedman test for repeated measures.

ACKNOWLEDGEMENTS

Antibodies against the different equine PBMC subpopulations were kindly provided by Bettina Wagner, Cornell University, NY, USA. We also thank Dana Teschner, horse clinic, Free University of Berlin, for providing horse blood for PBMC isolation. We thank Michaela Zeitlow and Atika Hadiati, Freie Universität Berlin, Germany, for helping with cloning and mutagenesis. This work was supported by the Elsa-Neumann Grant awarded to B. S. and a grant from DFG (AZ: 97/3-1 AO606893) to W. A. The authors declare no conflict of interest.

REFERENCES


Azab, W., Tsujimura, K., Maeda, K., Kobayashi, K., Mohamed, Y. M., Kato, K., Matsumura, T. & Akashi, H. (2010). Glycoprotein C of...
equine herpesvirus 4 plays a role in viral binding to cell surface heparan sulfate. *Virus Res* 151, 1–9.


