Correlation between structure, protein composition, morphogenesis and cytopathology of *Glossina pallidipes* salivary gland hypertrophy virus

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The *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV) is a dsDNA virus with rod-shaped, enveloped virions. Its 190 kb genome contains 160 putative protein-coding ORFs. Here, the structural components, protein composition and associated aspects of GpSGHV morphogenesis and cytopathology were investigated. Four morphologically distinct structures: the nucleocapsid, tegument, envelope and helical surface projections, were observed in purified GpSGHV virions by electron microscopy. Nucleocapsids were present in virogenic stroma within the nuclei of infected salivary gland cells, whereas enveloped virions were located in the cytoplasm. The cytoplasm of infected cells appeared disordered and the plasma membranes disintegrated. Treatment of virions with 1 % NP-40 efficiently partitioned the virions into envelope and nucleocapsid fractions. The fractions were separated by SDS-PAGE followed by in-gel trypsin digestion and analysis of the tryptic peptides by liquid chromatography coupled to electrospray and tandem mass spectrometry. Using the MaxQuant program with Andromeda as a database search engine, a total of 45 viral proteins were identified. Of these, ten and 15 were associated with the envelope and the nucleocapsid fractions, respectively, whilst 20 were detected in both fractions, most likely representing tegument proteins. In addition, 51 host-derived proteins were identified in the proteome of the virus particle, 13 of which were verified to be incorporated into the mature virion using a proteinase K protection assay. This study provides important information about GpSGHV biology and suggests options for the development of future anti-GpSGHV strategies by interfering with virus–host interactions.

INTRODUCTION

The *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV) is a rod-shaped, enveloped virus measuring 50 nm in width and 1000 nm in length (Garcia-Maruniak et al., 2009). The virus has a circular dsDNA genome of 190,032 bp and contains 160 putative protein-coding ORFs (Abd-Alla et al., 2010a). GpSGHV is a member of the newly established family *Hytrosaviridae*, genus *Glossinavirus* and species *Glossina hytrosavirus* (Abd-Alla et al., 2009). To date, hytrosaviruses (SGHVs) have been identified that infect the tsetse fly *Glossina pallidipes* (Jaenson, 1978), the housefly *Musca domestica* (MdSGHV) (Coler et al., 1993) and probably the narcissus bulb fly *Merodon equestris* Fabricius (Amargier et al., 1979). Recently, a similar virus has been reported in the accessory gland filaments of the braconid wasp *Diachasmimorpha longicaudata* (Luo & Zeng, 2010). GpSGHV and MdSGHV induce similar gross pathology in their hosts, most notably the characteristic hypertrophy of salivary glands of the adult insects and a reduction in reproductive fitness (Abd-Alla et al., 2010b; Lietze et al., 2007). Whilst MdSGHV causes symptomatic infections in the housefly (Lietze et al., 2011b, 2012), tsetse flies infected by GpSGHV exhibit both asymptomatic and symptomatic infections, with the asymptomatic state being the most widespread in the fly colonies (Abd-Alla et al., 2007).
GpSGHV negatively affects laboratory colonies of G. pallidipes, often leading to colony collapse (Abd-Alla et al., 2007, 2010b). Maintenance of healthy, productive fly colonies is vital to tsetse fly and trypanosomiasis eradication campaigns through the sterile insect technique, thus creating an urgent need to develop antiviral strategies to manage GpSGHV infections. During membrane feeding, one viraemic fly deposits up to $10^7$ viral genome copies in the form of virus particles into a blood meal, which are infectious per os to healthy G. pallidipes (Abd-Alla et al., 2010b). Although it is unknown how the virus gets into salivary glands, it is assumed that ingested virions enter via the midgut, transverse the haemolymph-filled haemocoel to reach the glands where they replicate (Garcia-Maruniak et al., 2009) and reside until transmission to new tsetse hosts.

The proteome of the GpSGHV particle consists of 61 virally encoded proteins (Kariithi et al., 2010). However, the localization and function of these proteins and their respective contribution to the virus ultrastructure and infection process are unknown. It is particularly important to determine GpSGHV envelope proteins (likely to be involved in the virus entry process) and to know which virion proteins contribute to cytoplasmic trafficking of the virus, as these proteins are possible targets for the development of antiviral strategies against GpSGHV. Enveloped viruses often contain numerous host-derived proteins, some of which are specifically incorporated into mature virions (reviewed by Cantin et al., 2005). One would reason that GpSGHV virions also contain proteins of cellular origin, which reflect the morphogenesis and egress processes. In this paper, the structure of GpSGHV virions is detailed and a comprehensive repertoire of viral and cellular proteins with their localization within the virion is presented.

**RESULTS AND DISCUSSION**

**Signature features of GpSGHV virions in infected host cells**

Electron microscopy of cryo-sections of G. pallidipes hypertrophied salivary glands showed nucleocapsids embedded in a chromatin-like network of electron-dense filaments (or virogenic stroma) within the nuclei of infected cells (Fig. 1a, panel i), presumably induced by

![Fig. 1. Electron micrographs of infected salivary glands and GpSGHV virions. (a) Cryo-sections through hypertrophied salivary gland cells. Nucleocapsids (Nc) were embedded in virogenic stroma (Vs) present in the nucleus (i), whilst enveloped virions (V) were observed in the cytoplasm (Cy) (ii) and in the lumen (Lum) of the glands (iii). Note that the infected cytoplasm appeared jumbled, the plasma membrane appeared disintegrated (indicated by arrows) and the lumen was full of enveloped virions. Bars, 200 nm (i, ii); 1 μm (iii). (b) Electron micrographs of negatively stained GpSGHV enveloped virions. The surface of a mature virion consists of regular helically arranged surface projections (i). The top, middle and bottom views of the virion particle shown in (i) are also shown (ii–iv, respectively). A cryo-section through a group of virions in an infected cell cytoplasm is shown (v). The inset shows a high-magnification image of a cross-section through a virion particle, revealing the nucleocapsid, tegument (Tg), envelope (Env) and surface projections (Sp). Bars, 100 nm (i–iv); 200 nm (v).](https://www.microbiologyresearch.org/content/94/2/194)
GpSGHV infection. This is similar to the cytopathology of baculoviruses, where studies have demonstrated that packaging of virus particles occurs in the virogenic stroma, where empty capsids assemble in the pockets between chromatin-like filaments and the capsids then fill with DNA acquired from the stroma (Fraser, 1986; Young et al., 1993).

Another question is how GpSGHV acquires its envelope. Enveloped viruses have been shown to acquire their envelopes through various mechanisms. For instance, white spot syndrome virus (WSSV, family Nimaviridae) acquires its envelope within the nucleus (Xie et al., 2006), whilst herpesviruses are enveloped by budding either through the nuclear membrane or into trans-Golgi membranes (Johnson & Baines, 2011). Other herpesviruses are enveloped entirely in the cytoplasm (Tandon & Mocarski, 2011). Many enveloped vertebrate (e.g. ortho- and paramyxoviruses and retroviruses) and invertebrate viruses (baculovirus budded virus) acquire their envelopes by budding through the plasma membrane. Our results showed that naked GpSGHV nucleocapsids were most abundant in the nucleus, whilst enveloped virions were restricted to the cytoplasm (Fig. 1a, panel ii), suggesting a cytoplasmic envelopment of the plasma membrane. These observations suggest that virus budding through the plasma membrane occurs via the endoplasmic reticulum–Golgi system. This is in agreement with recent evidence that nucleocapsids of the related MdSGHV egress from the nucleus via a nucleopore complex for cytoplasmic envelopment (Boucias et al., 2012).

The lumen of infected salivary glands was filled with closely packed arrays of rod-shaped, enveloped virions (Fig. 1a, panel iii). Our previous reports have demonstrated that virions shed into the saliva are infectious per os to healthy flies during membrane feeding in tsetse colonies (Abd-Alla et al., 2010b; Kariithi et al., 2011). The infected cells appeared to be in disarray and extended into the adjoining lumen, with the plasma membranes disintegrated (Fig. 1a, panel iii). None of the examined sections showed evidence of virus budding through the plasma membrane into the salivary gland lumen. These observations suggest that GpSGHV virions egress from the infected cell via disintegration of the plasma membrane. This is remarkably different from MdSGHV, where recent studies have demonstrated that the particles migrate to and bud out of the plasma membrane bordering the salivary gland lumen (Boucias et al., 2012; Lietze et al., 2011a).

Negative-staining electron microscopy and electron tomography of enveloped virions extracted from freshly excised glands showed a helical arrangement of elongated surface projections, ~13 nm in length and with a periodicity of 15 nm (Fig. 1b, panels i–iv). Surface projections have also been reported in vesicular stomatitis virus (VSV) (Cartwright et al., 1969) and in several poxviruses (Hiramatsu et al., 1999). In VSV, the surface projections are composed of cellular material and virus-specific antigens, and enzymic removal of these substructures prevents virus attachment to susceptible cells (Cartwright et al., 1969). De Giuli et al. (1975) suggested that the surface projections in some strains of Rous sarcoma virus are essential for the interaction with cellular receptors to permit initiation of the infectious process. It is likely that the surface projections observed in GpSGHV are made up of polymeric structures of viral proteins, for which the 44 kDa proteins encoded by ORF96 and ORF97, two of the most abundant viral proteins (Table 1; see also Fig. 4), are possible candidates. Additionally, host-derived proteins (see below) may also be present in the GpSGHV surface projections.

The GpSGHV virion contains an internal core with a mean diameter of 40 nm, which is separated from the envelope by an electron-dense proteinaceous matrix of ~10 nm thick (Fig. 1b, panel v). We propose to call this amorphous structure the GpSGHV tegument. Almost 50% of the identified GpSGHV structural proteins are found in the tegument (Table 1), similar to the situation in human cytomegalovirus (Varnum et al., 2004). The GpSGHV nucleocapsid core consists of a thin dense layer surrounding a central, higher-density area, suggesting that the core is not hollow. Assuming an equal distribution of the superhelical DNA in the nucleocapsid, the superhelicity of GpSGHV DNA (190 kb, 900 nm long nucleocapsid) is approximately half that of the average baculovirus (130 kb, 300 nm long nucleocapsid) (Jehle et al., 2006).

Purification and fractionation of GpSGHV virions

For a comprehensive analysis of the full set of virion proteins, the purity and integrity of the virus preparations are critical. Initial purification of GpSGHV particles using a sucrose gradient resulted in total loss of the virus envelope, leading to the erroneous conclusion that GpSGHV is a non-enveloped DNA virus consisting of 12 polypeptides (Odindo et al., 1986). Although purification of the virus was later improved by the use of Nycodenz gradient centrifugation (Abd-Alla et al., 2007; Kariithi et al., 2010), the integrity of the virus particles was still compromised. In the current study, an improved GpSGHV purification protocol was developed using the high-molecular-mass sugar Ficoll, prepared in an organic buffer (HEPES) and supplemented with protease inhibitors at high pH (pH 8.0) (Kariithi et al., 2012). This protocol resulted in preservation of the rod shape of the GpSGHV virions with intact envelope surrounding the virus particles (Fig. 2a). Treatment of these particles with 1% NP-40/137 mM NaCl buffer resulted in efficient fractionation of the envelope and nucleocapsid components (Fig. 2b, c). Silver staining of SDS-PAGE gels of NP-40-treated virions showed four dominant bands in the nucleocapsid fraction (130, 55, 43 and 30 kDa) and two in the envelope fraction of ~40 and 26 kDa (Fig. 2d, indicated with asterisks). Several other protein bands observed in the intact virions were associated with either the nucleocapsid or the envelope fraction with varying intensities. A high-molecular-mass smear was observed close to the top of the resolving gel in the envelope fraction (Fig. 2d) and may be an indication of covalently modified glycoproteins.
Table 1. Structural GpSGHV proteins identified by LC-MS/MS

Phosphorylated proteins are indicated with asterisks (see text for details). EPV, entomopoxvirus; ezrA, septation ring formation regulator ezrA; GBD-FH3, Rho GTPase-binding/formin homology 3 domain; GV, granulovirus; HDAC, histone deacetylase; NLS-BP, nuclear localization signal binding protein; NPV, nuclear polyhedrosis virus; PPase-tensin, pyrophosphatase tensin-type domain profile; PUM, Pumilio RNA-binding repeat profile; RGD, Arg-Gly-Asp/cell-attachment sequence; SCG, serine-cysteine-glycine; SP, signal peptide; NUMOD3, nuclease-associated modular DNA-binding domain 3; TM, transmembrane domain.

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<th>Coverage (%)</th>
<th>No. peptides identified</th>
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<td>81.4</td>
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<td>7</td>
<td>PIF-0 (P74), Spodoptera pectinicornis NPV</td>
<td>SP; isoleucine-rich activation motif (aa 648–686)</td>
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<td></td>
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<td>24</td>
<td>Homology to cellular proteins and/or proteins in other viruses</td>
<td>Signature domain(s)/motifs (amino acid regions harbouring the motif/domain)</td>
<td>Functional annotation (references)</td>
<td>Homology to cellular proteins and/or proteins in other viruses</td>
<td>Export of macromolecules from nucleus to cytoplasm (Görlich &amp; Kutay, 1999); Gene regulation (Gwack <em>et al.</em>, 2001; Kuo &amp; Allis, 1998; Zhu <em>et al.</em>, 1999)</td>
</tr>
</tbody>
</table>
Western blots using polyclonal antibody directed against the product of ORF10 showed multiple bands in both the nucleocapsid and envelope fractions, the most prominent of which were present in the nucleocapsid fraction (Fig. 3a). Western blot analysis confirmed the presence of the marker for the GpSGHV envelope (the P74 protein) in the envelope fraction (Fig. 3b).

Identification and characterization of GpSGHV virion structural proteins

A total of 45 virion proteins were identified by liquid chromatography coupled to electrospray and tandem mass spectrometry (LC-MS/MS) analysis of the envelope and nucleocapsid fractions (see Table 1 for details). Of the 45 GpSGHV proteins, ten were found only in the envelope fraction, 15 in the nucleocapsid fraction only and 20 were measurably present in both fractions. Nine of the identified proteins had potential transmembrane domains (TMs) and 15 had predicted signal peptides (SPs), of which seven were identified in the envelope fraction. The giant viral protein encoded by ORF62 (4373 aa) was the least abundant protein measured. Of the 20 proteins associated with both fractions, five (encoded by ORF50, -10, -94, -46 and -86) were found to be much more abundant in the nucleocapsid fraction than in the envelope fraction (Fig. 4). Similarly, five other proteins (encoded by ORF96, -97, -69, -64 and -112) were much more abundant in the envelope than in the nucleocapsid fraction.

Three of the GpSGHV envelope proteins were homologues of the baculovirus occlusion-derived virus envelope proteins P74, PIF-1 and PIF-2, which in baculoviruses are essential in the initial stages of oral infection of the host (reviewed by Slack & Arif, 2006). They are highly likely to have similar roles in oral infections of GpSGHV in tsetse midguts. After ingestion, hytrosaviruses find their way to the salivary glands, thereby causing distinct hypertrophy of the gland tissues (Garcia-Maruniak et al., 2009). It is not known how these viruses induce hyperplasia of the infected glands. However, it is noteworthy that the GpSGHV PIF-1 sequence contains an epidermal growth factor (EGF)-like domain, SP, TMs and multiple tyrosine kinase phosphorylation sites (Table 1). EGF-like domains are known to initiate a tyrosine kinase-mediated signalling cascade that culminates in recruitment of the evolutionally conserved mitogen-activated protein kinase (MAPK) pathway and results in growth/differentiation signals (reviewed by Alroy & Yarden, 1997). Interestingly, it has been demonstrated that the fowlpox virus ORF FPV211 product, which contains these structural features, contributes to the hyperplasia of fowlpox virus-infected tissues (Afonso et al., 2000). Whether PIF-1 plays a role in tsetse salivary gland hyperplasia remains to be investigated.

Motif analyses using ExPASy revealed several features in the virion protein sequences, among which were an arginine-glycine-aspartate (RGD) motif/cell-attachment sequence, bipartite nuclear localization signals, a P-loop
nucleotide-binding motif (ATP/GTP-A2), nebulin repeats and regions enriched in specific amino acids such as proline, serine, isoleucine/leucine, asparagine and glutamine. In addition to the signature domains and predicted structures, 14 of the identified viral proteins had homologues in other viruses, whilst four showed similarity to known cellular proteins (Table 1).

Analysis of the phosphorylation status of GpSGHV structural proteins by Western blotting showed six major signals in the intact virus sample (marked with asterisks in Fig. 5; two signals >170 kDa, and signals of approximately 43, 38, 30 and 15 kDa. Of these, four signals were also visualized in the nucleocapsid fraction (Fig. 5). Several minor bands were also observed in the intact virion preparation, some of which were also observed in the nucleocapsid fraction. Only two signals were observed in the envelope fraction (~44 kDa). Bioinformatics analysis of the GpSGHV proteins in the structural components indicated that at least six GpSGHV tegument proteins (encoded by ORF38, -67, -69, -93, -96 and -97) are likely to be phosphorylated (data not shown). Based on the molecular sizes of the proteins identified by LC-MS/MS, phosphorylation of these proteins was confirmed by Western blot analysis using mouse anti-phosphoserine/threonine/tyrosine mAbs (marked with asterisks in Table 1; Fig. 5). In conclusion, the majority of the phosphorylated viral proteins were localized in the tegument. Although no common sequences/motifs have been identified to direct proteins into the viral tegument, it has been suggested for herpesviruses that phosphorylation facilitates incorporation of proteins into the viral tegument (Kalejta, 2008), with the majority of tegument proteins being phosphorylated (Shenk & Stinski, 2008). Viral phosphoproteins may have a significant influence on the assembly (Sen & Todaro, 1977; Sen et al., 1977) and uncoating of virions (Lackmann et al., 1987; Witt et al., 1981), on the interaction between viral proteins and host DNA (Scheidtmann et al., 1984) and on transcriptional regulation (Hsu & Kingsbury, 1985; Hsu et al., 1982; Kingsford & Emerson, 1980). Antiviral interventions could be
directed against incorporation of these proteins into the tegument, which may arrest virus morphogenesis.

**Cellular proteins in GpSGHV virions**

Fifty-one host (cellular) proteins were identified in the GpSGHV virion proteome. Of these, eight were measurable only in the nucleocapsid fraction, including several 26S/60S ribosomal proteins, histone H3-II, phage terminase (*Sodalis glossinidius* strain morsitans) and vesicle coat complex COPI-e (see Table S1, available in JGV Online, and Fig. 4). Similarly, five of the cellular proteins were measurable only in the envelope fraction, including cargo transport protein EMp24, a major outer-membrane lipoprotein (*Sodalis glossinidius* strain morsitans), F0F1-type ATP synthase-β and an uncharacterized membrane-trafficking protein. Other cellular proteins were detected in both the envelope and nucleocapsid fractions with varying abundances (see Fig. 4). Furthermore, enzymic codes could be assigned to 22 of the 51 cellular proteins. The identified cellular proteins could be divided into nine categories by their (putative) functions (Table S1).

Treatment of purified GpSGHV particles with proteinase K (PK) removed many of the proteins associated with the virus (Fig. 6a). After passing the PK-treated sample through a 20% Ficoll cushion followed by LC-MS/MS, none of the ten virion envelope proteins was detectable, in contrast to the majority of the tegument proteins. Based on the molecular masses, the major viral proteins that disappeared after PK treatment (marked with asterisks in Fig. 6a, lane 2) included proteins encoded by ORF45 (201 kDa), ORF38 (137 kDa), ORF107 (60 kDa), ORF47 and -97 (~50 kDa), ORF-69 and -85 (~30 kDa), and ORF-68 and -101 (~12 kDa). When observed by transmission electron microscopy, the PK-treated virus particles were devoid of intact envelopes (compare Fig. 6b and c). Thirteen host proteins could still be identified in the sample that was passed through the 20% Ficoll cushion. Of these proteins, six (heat-shock cognate-70 family proteins, histone H2A, myosin, tubulin, actin and glyceraldehyde-3-phosphate dehydrogenase) have been demonstrated to be incorporated in viruses (Table 2 and references therein). Western blot analysis of the PK-treated samples with antibodies against cellular host proteins produced a clear

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**Fig. 4.** Abundance distribution of GpSGHV-encoded proteins (■) and virion-associated host-derived cellular proteins (▲) identified by LC-MS/MS. Large ovals enclose the proteins that were measured only in the envelope or nucleocapsid fraction, whilst the rest were detectable in both fractions. Numbers correspond to the GpSGHV ORFs. IBAQ, Intensity-based absolute quantification. See Table S1A for the identities of the cellular proteins detected in the envelope and nucleocapsid fractions.
signal with anti-myosin IgG (Fig. 6d). Western blot analysis using antibodies against the other tested cellular host proteins showed either a negative signal (actin) or a weak signal (ubiquitin and tubulin). Limitations of virus quantities, however, precluded optimization of the protocol for detection of these host proteins.

**Fig. 5.** Western blot analysis of the phosphorylation status of GpSGHV structural proteins in intact virions and the nucleocapsid and envelope fractions. Major phosphorylated proteins in the intact virus sample and the nucleocapsid fraction are indicated by asterisks, whilst minor ones are indicated by arrows. Lane M, marker (kDa).

**Fig. 6.** PK protection assay of purified GpSGHV virions. (a) Silver-stained SDS-polyacrylamide gel of non-treated (–PK), PK-treated (+PK) and PK-treated samples that were passed through a 20% Ficoll cushion to remove PK and free-floating peptides (+PK-purity). Viral proteins that disappeared after PK treatment are indicated by asterisks in lane 2. (b, c) PK treatment followed by 20% Ficoll purification resulted in naked nucleocapsids, as shown by comparison of (b) and (c). (d) Western blotting with rabbit polyclonal anti-myosin antibody detected myosin in the (+PK-purity) fraction (see Table 2). Bars, 200 nm (b, c). Lane M, marker (kDa).
### Table 2. Verification of incorporation of cellular proteins in mature GpSGHV virions

Purified virions were either treated with 0.08 μg PK per 1 μg virion protein or treated and purified through a 20% Ficoll cushion followed by LC-MS/MS analysis. HIV-1, Human immunodeficiency virus 1; Mo-MuLV, Moloney murine leukemia virus; SV-40, simian virus 40; WNV, West Nile virus.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Host molecule</th>
<th>UniProt protein ID</th>
<th>Mass (kDa)</th>
<th>Unique peptides</th>
<th>Incorporated in other viruses</th>
<th>Virus (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein synthesis</strong></td>
<td>Elongation factor 1-α</td>
<td>D3TNV8</td>
<td>50.4</td>
<td>6</td>
<td>No</td>
<td>HIV-1 (Cimarelli &amp; Luban, 1999), WNV (Blackwell &amp; Brinton, 1997)</td>
</tr>
<tr>
<td></td>
<td>Elongation factor 2</td>
<td>D3TP87</td>
<td>94.5</td>
<td>5</td>
<td>No</td>
<td></td>
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<tr>
<td><strong>Protein processing machinery</strong></td>
<td>Hsc70-4</td>
<td>D3TPL1</td>
<td>60.8</td>
<td>4</td>
<td>Yes</td>
<td>HIV-1 (Gurer et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Hsc70-3</td>
<td>D3TRH2</td>
<td>72.6</td>
<td>10</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td><strong>Transcription regulation</strong></td>
<td>Histone H2A</td>
<td>D3TPW0</td>
<td>15.0</td>
<td>2</td>
<td>Yes</td>
<td>SV-40 (Chen et al., 1979)</td>
</tr>
<tr>
<td><strong>Cytoskeleton</strong></td>
<td>Myosin (heavy chain)</td>
<td>D3TQW0</td>
<td>87.3</td>
<td>30</td>
<td>Yes</td>
<td>HIV-1 (Ott et al., 1996, 2000b)</td>
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<tr>
<td></td>
<td>Tubulin-α</td>
<td>D3TGW7</td>
<td>49.9</td>
<td>11</td>
<td>Yes</td>
<td>Mo-MuLV (Wang et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Actin 5C</td>
<td>D3TQK0</td>
<td>41.8</td>
<td>3</td>
<td>Yes</td>
<td>Mo-MuLV (Nermut et al., 1999; Wang et al., 2003)</td>
</tr>
<tr>
<td><strong>Tubulin-β</strong></td>
<td></td>
<td>D3TR0</td>
<td>50.2</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cellular metabolism</strong></td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>D3TRU0</td>
<td>35.7</td>
<td>2</td>
<td>Yes</td>
<td>HIV-1 (Ott et al., 2000a)</td>
</tr>
<tr>
<td></td>
<td>Endoplasmic reticulum glucose-regulated protein</td>
<td>D3TS03</td>
<td>82.1</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protein disulfide isomerase</td>
<td>D3TRE3</td>
<td>57.4</td>
<td>3</td>
<td>No</td>
<td>HIV-1 (Ott 2002)</td>
</tr>
<tr>
<td><strong>Ion transport</strong></td>
<td>Porin</td>
<td>D3TRY2</td>
<td>30.4</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Implications of finding host proteins in GpSGHV

The results presented in this study clearly showed that GpSGHV virions can contain numerous cellular proteins. Cellular host proteins incorporated into or onto virus particles have been demonstrated to play specific or supplementary roles in virus life cycles. Actin and myosin have been reported to be incorporated, for instance, into Mo-MuLV (Nermut et al., 1999) and HIV-1 (Ott et al., 1996, 2000b). In HIV-1, inhibition of the interaction between the Gag protein and actin and myosin markedly reduced the amount of virus released from infected cells (Ott, 1997). Glyceraldehyde-3-phosphate dehydrogenase has been reported to be co-incorporated with actin inside GpSGHV virions (Ott, 1997). Glyceraldehyde-3-phosphate dehydrogenase between the Gag protein and actin and myosin markedly reduces the expression of gene expression (Ott et al., 2000b). Translation elongation factor-1α has been proposed to target WNV RNA to a microenvironment for efficient virus replication (Blackwell & Brinton, 1997) and play roles in the packaging of HIV-1 into nascent virions (Cimarelli & Luban, 1999). Heat-shock protein 70 family members have been demonstrated to be bona fide proteins of the primate lentiviral virions and have been proposed to play roles in virus assembly and egress (Gurer et al., 2002). Finally, virions of SV40 have been demonstrated to contain biosynthetically active histone H2A protein (Chen et al., 1979). Taken together, it seems likely that the cellular host proteins in GpSGHV virions may have specific or auxiliary roles in the virus life cycle. Further investigations are needed to determine whether the incorporated cellular proteins are distributed over infectious and non-infectious GpSGHV virions. These data provide important leads towards an understanding of the process of GpSGHV assembly.

Conclusions

The GpSGHV virion has a rod-shaped protein nucleocapsid core surrounded by a proteinaceous tegument, an outer envelope and helical surface projections. The tegument proteins of GpSGHV comprise almost half of the total virion proteins. In addition, the virus contains numerous virion-associated cellular proteins, some of which appear to be specifically incorporated into the mature virion. The presence of cellular proteins in GpSGHV virions may be a reflection of their requirement in the infection process or may be remnants of interactions between the virus and host proteins. The GpSGHV progeny nucleocapsids translocate to the cytoplasm where envelopment is orchestrated. Cytoplasmic assembly of the virus particles induces cellular damage, which culminates in disintegration of the cell plasma membrane as the mature virions egress from the infected cell. Finally, the data presented in this study may offer new directions in antiviral strategies based on virus-host interactions. Potential strategies have been reviewed recently by Kariithi et al. (2012) and include blocking of the initial attachment of GpSGHV to the tssetse midgut receptors using either antibodies against envelope proteins or competing peptides as reported for Autographa californica multicapsid nucleopolyhedrovirus in Heliothis virescens (Sparks et al., 2011), GpSGHV-specific gene silencing using RNA interference, inhibition of GpSGHV DNA polymerase (ORF79) by commercially available drugs (Abd-Alla et al., 2011) and blocking of RGD-directed cell adhesion as has been demonstrated for adenovirus (Bai et al., 1993).

METHODS

Electron microscopy of hypertrophied salivary glands. Hypertrophied salivary glands were freshly dissected from adults of a laboratory colony of G. pallidipes flies (IAEA Laboratories) and immediately fixed (4 h at 4°C) in 2% paraformaldehyde/3% glutaraldehyde in 0.1 M phosphate/citrate buffer (pH 7.2). The glands were washed, infiltrated with 2.3 M sucrose in 0.1 M phosphate/citrate buffer (16 h at 4°C) and cryo-fixed by plunging into liquid ethane at −160°C using a Reichert KF80 plunger. Cryo-sections (80 nm in thickness) were cut at −110°C with a Leica Ultratome S microtome equipped with an FCS cryo-system, mounted on Formvar-coated copper grids (100 mesh), negatively stained with 3% ammonium molybdate (pH 6.5) and air dried. Images were recorded with a Gatan 4K CCD camera on a JEOL 2100 transmission electron microscope equipped with a LaB6 filament operating at 200 kV.

Electron microscopy of virus particles. Salivary glands were squashed gently in 1:1-diluted fixative and extracts incubated on Formvar- and carbon-coated copper grids (100 mesh) and stained with 1% uranyl acetate (pH 3.7). Similar specimens were prepared from purified virus suspensions (see below). For electron tomography, gold fiducial markers of 10 nm were included in the virus extract and a series of 2×-binned images was recorded with SerialEM (Mastronarde, 2005) at tilt angles from −65 to +65° with increments of 1°. The series of tilted projection images were converted into three-dimensional tomograms using the IMOD program (Kremer et al., 1996).

Virus purification. Three replicate extractions were conducted on 25 pairs each of hypertrophied salivary glands dissected from adult G. pallidipes. The glands were disrupted immediately by two strokes of a glass/Teflon homogenizer (on ice) in 1 ml homogenization buffer [50 mM HEPES (pH 8.0), 10 mM Ficoll PM400 (GE Healthcare), 2 mM EDTA and protease inhibitors (Roche)]. The volumes were brought to 2 ml and clarified by centrifugation three times (7500 g for 10 min at 4°C). The supernatants were pooled and layered onto 5 ml of a 10–40% (w/v) Ficoll PM 400 discontinuous density gradient and ultracentrifuged (110 000 g at 4°C). The resultant pellet was collected, resuspended in 50 mM buffer (pH 8.0) and ultracentrifuged (60 000 g for 60 min at 4°C). The resultant pellet was allowed to dissociate into 1 ml 50 mM HEPES (pH 8.0) overnight at 4°C. The integrity of the purified virions was checked by negative staining using a JEOl 2100 transmission electron microscope (Kariithi et al., 2010).

Fractionation of virions into envelope and nucleocapsids. Purified virions were incubated (30 min at room temperature) in 250 μl reaction volumes with lysis buffer [1% NP-40, 50 mM Tris/HCl (pH 8.0), 137 mM NaCl, 10% glycerol and 2 mM EDTA]. The NP-40-treated virions were layered onto a 5 ml 10–60% (v/v) glycerol discontinuous gradient and ultracentrifuged (110 000 g for 1 h at 4°C). The envelope fraction was collected from the top 2 ml of the gradient. To ensure complete removal of virion envelopes, the...
pellet containing the nucleocapsids was resuspended and subjected to another round of 1% NP-40 extraction and glycerol ultracentrifugation. The purity of nucleocapsids was checked by negative-staining transmission electron microscopy. The envelope fraction was precipitated with trichloroacetic acid (overnight at 4°C). The precipitated proteins were recovered by centrifugation (20,000 g for 15 min) and the trichloroacetic acid was neutralized by three washes with ice-cold acetone. The pellets were dried and resuspended in 10 mM Tris/HCl (pH 8.0).

**Identification of GpSGHV structural proteins by LC-MS/MS.** Portions of the envelope and nucleocapsid fractions were treated with lysis buffer [8 M urea, 4 mM CaCl₂, 0.2 M Tris/HCl (pH 8.0)] and separated by SDS-PAGE (12% acrylamide). The gel was stained with a Colloidal Staining Kit (Invitrogen). The middle sections of entire gel lanes were excised and the gel sections cut into approximately 1 mm² pieces. In-gel trypsin digestions were performed and the resultant peptides were analysed by LC-MS/MS (Kariithi et al., 2010). The data generated by LC-MS/MS were analysed by the MaxQuant software package version 1.2.2.5 (Cox & Mann, 2008; Cox et al., 2011) with the following constructed databases: a Glossina morsitans morsitans database (http://www.sanger.ac.uk/resources/downloads/vectors/glossina-morsitans-morsitans.html), a GpSGHV ORF database (http://www.uniprot.org/) and a contaminant database containing sequences of common contaminants (BSA: NCBI protein accession no. P02769, bovine serum albumin precursor; trypsin: P00760, bovine; trypsin: P00761, porcine; keratin K2C1: P04264, human; keratin K1Cl: P35527, human; keratin K22E: P35908, human; keratin K2C9: P35527, human; trypsin: P00760, bovine; trypsin: P00761, porcine; keratin K22E: P35908, human; keratin K1C9: P35527, human). Peptides and proteins with at least two peptides of which at least one was identified were considered for further analysis. The peptides were identified with the MaxQuant software using default settings for the Andromeda search engine (Cox et al., 2011), except that extra variable modifications were set for deamidation of N and Q. Peptides and proteins with a false discovery rate of <1% and at least two peptides or at least one unique were accepted for further analyses. Student’s t-test of the identified proteins was performed in the Perseus module version 1.2.0.17 on normalized peak abundances (Cox & Mann, 2011). The normalized protein abundances were used to construct a scatter plot to determine protein distribution in the GpSGHV structural components.

**Localization of GpSGHV envelope and nucleocapsid proteins by Western blotting.** The envelope and nucleocapsid fractions of purified GpSGHV virions were separated by SDS-PAGE (12% acrylamide) and transferred onto Immobilon-P (Millipore) membranes by semi-dry electrophoresis transfer. Membranes were blocked by overnight incubation with 5% non-fat milk powder and 0.05% Tween 20 in TBS buffer [10 mM Tris/HCl (pH 8), 150 mM NaCl (TBS-T) at 4°C]. The membranes were then incubated (1 h at room temperature) with rabbit polyclonal antibodies against proteins encoded by GpSGHV ORF10 or ORF1 generated in rabbits as the primary antibodies (diluted 1:1000), washed three times with TBS-T and further incubated (1 h at room temperature) with alkaline phosphatase-conjugated polyclonal goat anti-rabbit IgG antibody (diluted 1:2000; Sigma) as the secondary antibody. Blots were developed with nitro-blue tetrazolium/BCIP (Sigma).

**Verification of incorporation of cellular proteins into GpSGHV virions.** Purified GpSGHV virions were incubated for 30 min at 37°C with 0.08 μg PK (Innitrogen) (μg total protein)-¹ (Moerdijk-Schauwecker et al., 2009). PK activity was stopped by the addition of PMSF to a final concentration of 5 mM, followed by incubation on ice for 15 min. Contaminating vesicles that commonly co-purify with enveloped viruses (Ott et al., 1996) were removed by passing a portion of the PK-treated virions through a 5 ml 20% Ficoll cushion by ultracentrifugation (60,000 g for 1 h at 4°C). The untreated and PK-treated samples were separated by SDS-PAGE (12% acrylamide), followed by in-gel trypsin digestion and LC-MS/MS analysis as described above. A portion of the PK-treated sample was subjected to Western blotting using mouse anti-tubulin-α mAb (clone DM1A; Sigma), mouse cytoplasmic actin mAb (clone 10-b3; Sigma), mouse IgG anti-ubiquitin mAb (clone P4D1; Santa Cruz Biotechnology) or rabbit anti-myosin polyclonal antibody (clone H-300; Santa Cruz Biotechnology) as the primary antibody (diluted 1:3000). Alkaline phosphatase-conjugated anti-mouse IgG (diluted 1:2000; Sigma) was used as the secondary antibody using the conditions described above.

**Functional and structural characterization of GpSGHV proteins.** Identified viral proteins were annotated using Blast2GO software version 2.5.0 (Conesa et al., 2005), whilst the protein motifs were analysed using the ExPaSy PROSITE database (http://www.expasy.org), and the numbers of TM helices were predicted by TMHMM version 2.0. Signal peptide sequences were predicted using SignalP 3.0. The phosphorylation potential of the identified proteins was predicted by the NetPhos 2.0 CBS Prediction server with a threshold value set at 0.7. Confirmation of the phosphorylation status of identified viral proteins was performed by Western blotting using mouse anti-phospho-serine/threonine/tyrosine mAbs (diluted 1:1500; Thermo Scientific) and anti-mouse IgG alkaline phosphatase (diluted 1:3000; Sigma) as the primary and secondary antibodies, respectively, following the supplier’s instructions.

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**REFERENCES**


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