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BRIEF REPORT

Membrane permeabilization of the African horse sickness virus VP5 protein is mediated by two N-terminal amphipathic α-helices

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Abstract The VP5 outer capsid protein of African horse sickness virus (AHSV) is cytotoxic when expressed in *Spodoptera frugiperda* (Sf-9) cells. Secondary structure analysis of the VP5 amino acid sequence of AHSV-9 identified two N-terminal amphipathic α-helices within the first 43 amino acids. Baculovirus expression of N- and C-terminal truncated VP5 proteins in Sf-9 cells indicated that the N-terminal 43 amino acids correlated with low levels of protein expression and with increased membrane permeabilization and cytotoxicity. Exogenous addition of chemically synthesized VP5 peptides indicated that both N-terminal amphipathic α-helices are required for membrane permeabilization of Sf-9 cells. These findings suggest that AHSV VP5 is a membrane-destabilizing protein.

African horse sickness virus (AHSV), a member of the genus *Orbivirus* in the family *Reoviridae*, is an arthropod-borne virus (*Culicoides* spp.) and the causative agent of African horse sickness (AHS), a highly infectious disease of equines with high mortality rates in horses [4]. Like bluetongue virus (BTV), the prototype orbivirus, AHSV consists of two concentric protein layers that encapsidate the genome of ten double-stranded (ds) RNA segments [14, 19]. The core particle is composed of two major (VP3 and VP7) and three minor (VP1, VP4 and VP6) structural proteins and is surrounded by the outer capsid, composed of the two major structural proteins VP2 and VP5 [19]. AHSV VP5 contains a neutralizing epitope and has been reported to play a supportive role to VP2 in enhancing the protective neutralizing activity of VP2 in horses [17, 18]. Apart from its immunogenicity, no functional studies have been undertaken on the VP5 protein of AHSV. Nevertheless, expression of the AHSV VP5 protein in *Escherichia coli* [18] and *Spodoptera frugiperda* (Sf-9) cells [8] has been reported to be cytotoxic, causing rapid cell lysis and resulting in low levels of protein expression. The basis of the apparent cytotoxicity has also not yet been investigated. Here, through the characterization of a series of VP5 deletion mutants and relevant peptides, based on the predicted structural features of VP5, we report that the viral protein possess membrane-permeabilizing activity. We furthermore show that this activity is linked to two N-terminal amphipathic α-helices, located within the first 43 amino acids of AHSV VP5.

The nucleotide sequence of the full-length cDNA copy of AHSV-9 genome segment M6, contained in recombinant plasmid pBSVP5 (kindly provided by Dr. W. Fick, Department of Genetics, University of Pretoria), was determined by automated sequencing procedures, and the deduced amino acid sequence was used in secondary structure analyses. The hydrophobicity profile of the VP5 protein was predicted with the algorithm of Kyte and Doolittle [16], whilst the PredictProtein server (http://www.predictprotein.org) was used for secondary structure analysis. The hydrophobic profile of the 505 residues of the AHSV-9 VP5 protein indicated a clear partition between two domains: an N-terminal domain (amino acids 1 to 220) and a C-terminal domain (amino acids 280 to 505), separated by a hydrophobic hinge region (amino acids 220 to 280) that is rich in alanine and glycine residues (Fig. 1a). Two amphipathic α-helices were also identified in the first

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43 residues at the N-terminus of VP5, which are immediately followed by a stretch of hydrophobic residues. Helical wheel representation of amino acids 1 to 22 (α-helix 1) and amino acids 23 to 43 (α-helix 2) of VP5, using the BioEdit version 7.0.4.1 software program [12], revealed that both helices have a net positive charge on their hydrophilic faces as a result of the clustering of positively charged lysine residues (K) (Fig. 1b). The latter may allow the helices to interact with negatively charged phospholipids present in cell membranes. Indeed, cationic amphipathic α-helices are motifs common to many polypeptides with membrane-destabilizing properties and have been implicated in the membrane-binding activities of viral fusion proteins [9, 26, 27].

Towards determining which domain(s) of the VP5 protein plays a role in its reported cytotoxicity [8, 18], full-length and different truncated VP5 fragments were generated by PCR amplification with oligonucleotides complementary to the relevant AHSV-9 VP5 coding sequence (GenBank accession no. U74489). In the case of BTV VP5, it has been reported that glutathione-*S*-transferase (GST) fusion proteins are expressed at higher levels than is untagged VP5 protein, suggesting that masking of the N-terminus allows higher levels of protein to stably accumulate [13]. Consequently, the PCR amplicons were ligated to pGEM®-T Easy Vector (Promega) and then cloned into the GST baculovirus transfer vector pAc-GHLT-B (BD Biosciences) using standard recombinant DNA methodologies [23]. The nucleotide sequence and orientation of cloned insert DNA was verified by automated sequencing procedures. Recombinant baculoviruses were obtained by co-transfecting Sf-9 cells with the respective recombinant baculovirus transfer vectors and linearized BaculoGold™ DNA according to the specifications of the manufacturer (BD Biosciences). A representation of the full-length and truncated VP5 fusion proteins used in this study is shown in Fig. 2a. The resultant parental and recombinant baculoviruses were used to infect Sf-9 cell monolayers (1×10^7 cells) at a multiplicity of infection (MOI) of 10 PFU/cell. The recombinant fusion proteins were expressed at maximal levels between 30 and 48 h postinfection, after which the expression levels declined significantly (results not shown). Expression of the respective VP5 fusion proteins, analyzed 48 h after infection by 12% SDS-PAGE, is presented in Fig. 2b. Subsequent immunoblot analyses indicated that the recombinant fusion proteins were recognized by a both an anti-AHSV-9 polvalent serum (results not shown) and a polyclonal anti-GST antibody (Fig. 2c). Compared to the full-length VP5 fusion protein, deletions from the N- and C-terminus, respectively, resulted in an increase and a

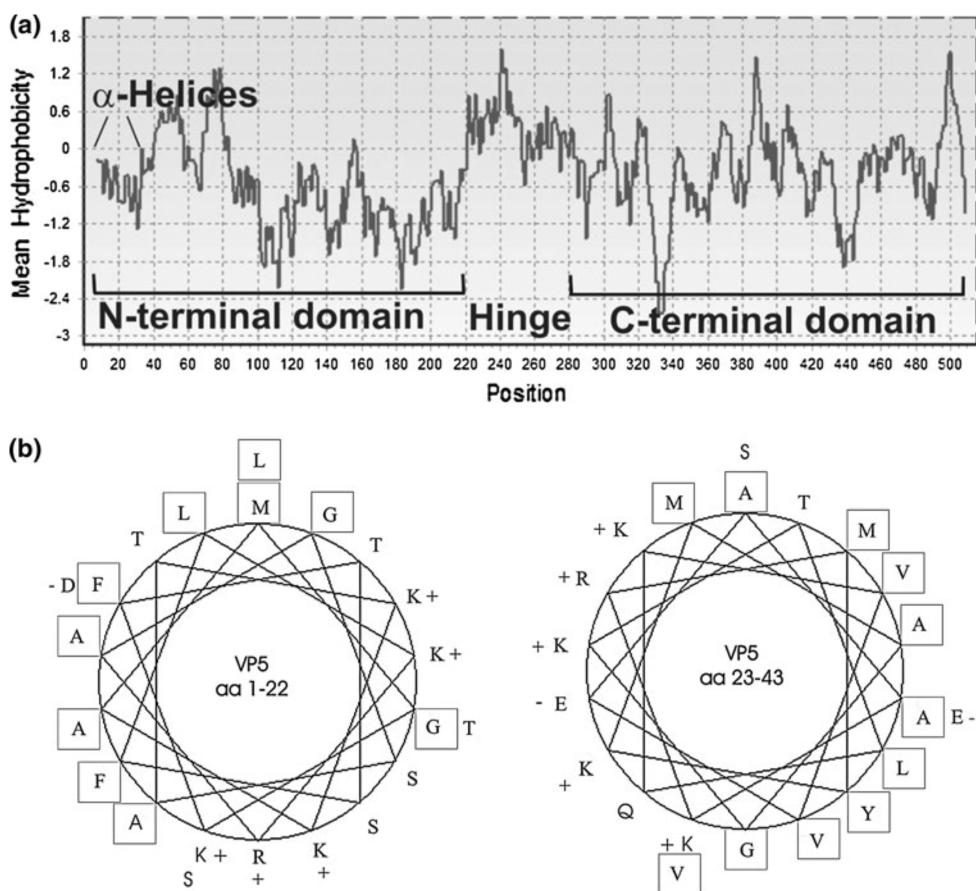

Fig. 1 Structural features and domains of the AHSV-9 VP5 protein. (**a**) Hydrophobicity profile of VP5, as predicted using the algorithm of Kyte and Doolittle [16] with a window setting of 13. (**b**) Helical wheel diagrams showing the amphipathic nature of each predicted α-helix at the N-terminus of VP5. Each panel represents an α-helix viewed along the helix axis. Hydrophobic amino acid residues are boxed

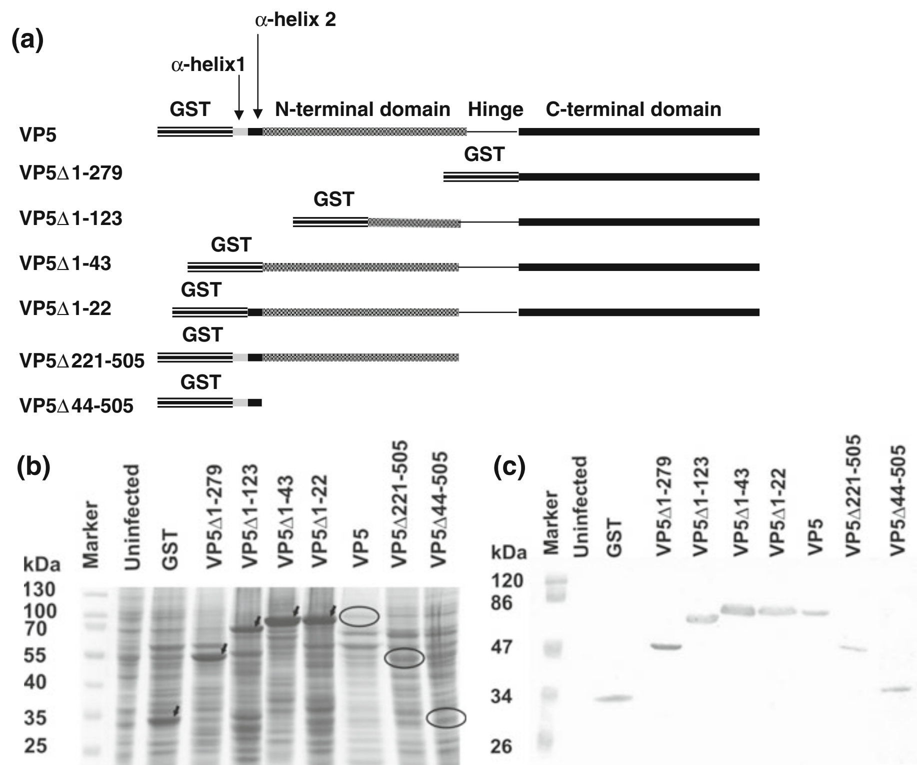

Fig. 2 Baculovirus expression of full-length and truncated VP5 fusion proteins in Sf-9 cells. (**a**) Schematic representation of the full-length and truncated VP5 proteins expressed as GST fusion proteins in Sf-9 cells. (**b**) SDS-PAGE analysis of Sf-9 lysates recovered after 48 h of infection by each of the recombinant baculoviruses. The low expression of the full-length and C-terminally truncated VP5 fusion proteins is indicated. (**c**) Immunoblot analysis of the SDS-PAGE gel was performed with polyclonal anti-GST antibodies. The sizes of molecular mass markers (kDa) are indicated to the left of the figure. Uninfected Sf-9 cells and GST-expressing baculoviruses were included as controls

decrease in the amount of VP5 fusion protein produced. This inverse correlation between the presence of the VP5 N-terminus and the level of expression observed suggested a sequence-specific effect rather than it being the result of differences in the size of the synthesized protein products (Fig. 2b and c).

The cytopathic effects of several viruses may result from the altered membrane permeability of the host cell due to the expression of a single viral polypeptide [5, 7, 11]. Therefore, the ability of the recombinant baculovirus-expressed full-length and truncated VP5 fusion proteins to permeabilize Sf-9 cells was evaluated, and cytotoxicity (cell leakage) was determined using the Cytotoxicity Detection Kit (Roche Applied Science). This quantitative assay measures levels of lactate dehydrogenase (LDH), a stable cytoplasmic enzyme that is released when the plasma membrane is damaged. The amount of LDH present in the supernatant is directly proportional to the number of lysed cells. This assay reveals low-level damage to cell membranes, gives values similar to those obtained using ^{51}Cr release assays [6, 15] and has been used successfully in other virus-induced cytotoxicity studies [13, 22]. Control samples were included in each experiment to correct for LDH in the culture medium (background control) and for spontaneous release of LDH from uninfected cells (low control). The maximum release of LDH from uninfected cells (high control) was measured after lysis of the cells with 2% (v/v) Triton X-100 in the medium. Three independent experiments, each consisting of triplicate samples, were performed. Sf-9 cells (1.5×10^4 cells/well) were infected with the respective recombinant baculoviruses at an MOI of 10 PFU/cell, and the cytotoxicity of each of the expressed full-length and truncated GST-VP5 proteins was determined at 30 h postinfection by measuring the amount of released LDH. Expression of the GST-tagged full-length VP5 protein, as well as expression of VP5 fusion proteins with C-terminal deletions (*e.g.*, VP5Δ221-505 and VP5Δ44-505) induced substantial release of LDH, with VP5Δ44-505 exhibiting the highest activity (cytotoxicity of *ca.* 98%). In contrast, expression of VP5 fusion proteins with deletions from the N-terminus (*e.g.*, VP5Δ1-279, VP5Δ1-123, VP5Δ1-43 and VP5Δ1-22) resulted in comparatively low levels of LDH release (Fig. 3a). Based on the low cytotoxicity (*ca.* 3.3%) associated with expression of the GST protein only, and considering that GST is unable to associate with liposomes [5], these results therefore indicated that the cytotoxicity observed with the VP5 fusion proteins was mediated by the VP5 component. Notably, the presence of the two amphipathic α-helices at the N-terminus of VP5 correlated strongly with increased cytotoxicity and thus membrane permeabilization.

To ascertain whether the two predicted N-terminal amphipathic α-helices individually or in combination trigger LDH release, four synthetic VP5 peptides were generated (GenScript Corp.). Peptide VP5(1-43), encompassing both

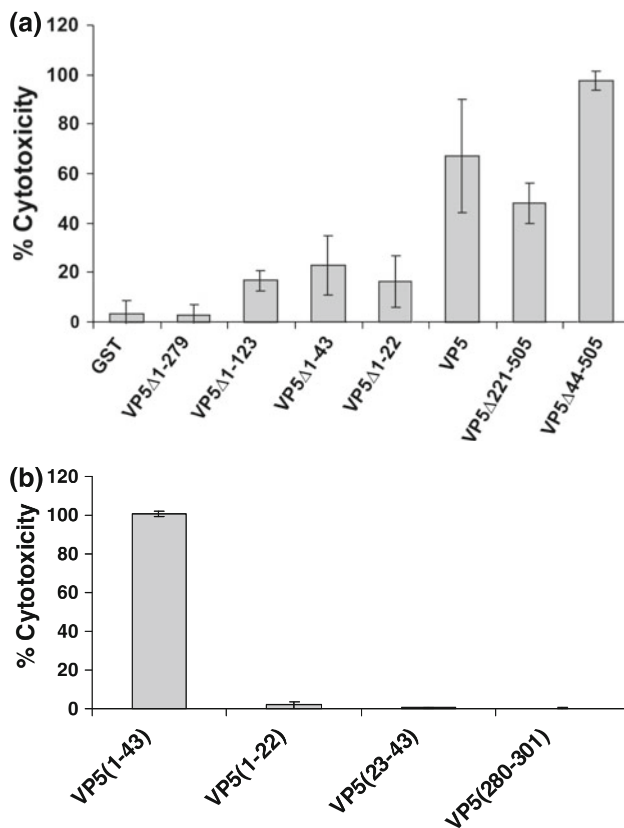

Fig. 3 Membrane permeabilization of Sf-9 cells by VP5. (**a**) Cell cytotoxicity of baculovirus-expressed full-length and truncated VP5 fusion proteins expressed in Sf-9 cells by recombinant baculoviruses at 30 h post-infection. Sf-9 cells infected with a baculovirus expressing GST only served as a control. (**b**) Cell cytotoxicity of three synthetic N-terminal peptides encompassing the amphipathic helices (VP5 [1-43], VP5 [1–22] and VP5 [23-43]) and a control C-terminal peptide (VP5 [280-301]). The peptides were dissolved in UHQ water, and 50 μM of each peptide was added Sf-9 cells, followed by LDH release assays at 24 h post-treatment. In both assays, the amount of LDH release was measured (OD_{492}) from triplicate wells, and used to calculate the percent cytotoxicity using the following equation: cytotoxicity (%) = [(experimental value) − (low control)/(high control − low control)] × 100. Bars show mean ± SD for three independent experiments

α-helices, had the sequence $_1$MGKFTSFLKRAGSATKKALTSD$_{22}$AAKRMYKMAGKTLQKVVESEV$_{43}$. Peptides VP5(1-22) and VP5(23-43) were composed of amino acids 1 to 22 (α-helix 1) and amino acids 23 to 43 (α-helix 2), respectively, of the above peptide sequence. The fourth peptide, designated VP5(280-301), had the sequence PHIIEKAMLKDKIPDNELAMAI and was composed of residues at the VP5 C-terminal region (amino acids 280-301). This peptide was used as a control, since baculovirus expression of the VP5 fusion protein VP5(280-505) resulted in neglible cytotoxicity (Fig. 3a). Sf-9 cell monolayers (1.5×10^4 cells) were incubated with 50 μM of each synthetic VP5 peptide for 24 h at 27°C, and the cell culture supernatants were then assayed for the amount of LDH released. The results indicated that the VP5(1-43) peptide caused substantial release of LDH (cytotoxicity of *ca.* 100%), whilst none of the other three VP5 peptides assessed showed any such effect (Fig. 3b). The dependence of cytotoxicity on peptide concentration has been documented previously, and high exogenous peptide concentrations appear to be required for the formation of multimeric peptide complexes capable of perturbing the membrane [20, 21, 24]. Thus, the relatively high concentration of VP5(1-43) peptide required for observing the cytotoxic effect might also be a reflection of the biophysical properties of the peptide. These data nevertheless indicated that both N-terminal amphipathic α-helices of VP5 were required to permeabilize the plasma membrane of Sf-9 cells. It appears that for AHSV VP5, α-helix 1 exerts its membrane-permeabilizing activity in concert with α-helix 2 and that both of these helices may be cooperatively involved in the formation of membrane-integral pores. These results are in contrast to those of BTV VP5, in which the most N-terminal α-helix (amino acids 1 to 20) exhibits a significantly higher permeabilizing activity than the adjacent α-helix (amino acids 22 to 41) [13].

Our data, based on the structural features and cytotoxic activity of the AHSV VP5 protein, indicate that VP5 acts as membrane-permeabilization protein. It is tempting to speculate that this property of VP5 may be of importance during the early stages of virus entry into susceptible host cells. In contrast to enveloped viruses [27], there is a paucity of information regarding the precise mechanism by which nonenveloped viruses, including AHSV, penetrate and deliver their genome across host-cell membranes in the absence of membrane fusion. However, short membrane-altering amphipathic or hydrophobic sequences have been reported in several nonenveloped viruses, *e.g.*, the gamma peptide of flock house virus [2], the NSP4 protein of rotavirus [3] and the V1 protein of adenovirus [28]. These polypeptides either cause membrane rupture of endosomes or are involved in the formation of pores through which the viral genome is transported to the cytoplasm [1, 25]. In this regard, it noteworthy that VP5 of BTV has been implicated in the translocation of the transcriptionally active core into the cytoplasm after cell binding and endocytosis [10, 13, 29]. Our current studies are therefore aimed at clarifying the functional importance of the VP5 protein in AHSV biology.

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