RESEARCH COMMUNICATION

Subcellular localization of the nonstructural protein NS3 of African horsesickness virus

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ABSTRACT

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The subcellular localization of the minor nonstructural protein NS3 of African horsesickness virus (AHSV) has been investigated by means of immunogold electron-microscopical analysis. NS3 was observed in perturbed regions of the plasma membrane of AHSV-infected VERO cells, and its presence appears to be associated with events of viral release. These events are budding, whereby released viruses acquire fragments from the host-cell membrane, as well as by the extrusion of nonenveloped particles through the cell membrane. The membrane association of NS3 was confirmed by its detection in the disrupted plasma membranes of cells infected with an NS3 baculovirus recombinant. The absence of NS3 on intact cell membranes suggests that the protein is not exposed extracellularly.

Keywords: African horsesickness virus, AHSV, nonstructural protein, NS3, subcellular localization

African horsesickness virus (AHSV), which causes high mortality in susceptible horses, belongs to the *Orbivirus* genus in the family Reoviridae. Like bluetongue virus (BTV), the prototype orbivirus AHSV consists of a segmented, double-stranded RNA genome which is encapsidated in a double-layered protein coat. Each genome segment encodes at least one viral polypeptide. In addition to the seven structural proteins (VP1–VP7), four nonstructural proteins (NS1, NS2, NS3 and NS3A) have been identified in AHSV-infected cells (Oellermann, Els & Erasmus 1970; Bremer 1976; Grubman & Lewis 1992; Laviada, Arias & Sanchez-Vizcaino 1993). The nonstructural proteins are thought to play an important role in the replicative cycle of these viruses.

The minor nonstructural proteins, NS3 and NS3A, are the gene products of the smallest genome segment of AHSV, namely segment 10 (S10) (Van Staden & Huismans 1991). When the nucleotide sequences of the S10 genes of several AHSV serotypes were compared, it was shown that the NS3 proteins of AHSV were less conserved than the cognate proteins within the BTV serogroup (Van Staden & Huismans 1991; Hwang, Yang, Chiou & Li 1992; De Sà, Zellner & Grubman 1994; Van Staden, Stoltz & Huismans 1995). However, a number of characteristics were found to be conserved amongst all orbivirus NS3 proteins. These included two hydrophobic regions which can form putative transmembrane domains (Lee & Roy 1986; Gould 1988; Van Staden & Huismans 1991; Moss, Jones & Nuttall 1992; Jensen, Cheney, Thompson, Mecham, Wilson, Yamakawa, Roy & Gorman 1994), suggesting a possible membrane association of NS3.

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An investigation of the subcellular localization of the NS3/NS3A proteins of BTV provided valuable information on the role that these proteins play in BTV morphogenesis. These proteins were located in the plasma membrane and intracellular smooth-surfaced vesicles of BTV-infected cells (Hyatt, Gould, Coupar & Eaton 1991). The possible involvement of NS3 in viral release was suggested by the presence of the NS3 protein in transient membrane fragments which are acquired by viruses during their release from infected cells. This was verified when expression of BTV structural proteins by recombinant baculoviruses resulted in the release of BTV-like particles from infected insect cells only when co-expressed with NS3 (Hyatt, Zhao & Roy 1993).

In contrast with BTV, little is known about the NS3 proteins of AHSV. The AHSV-3 NS3 gene has been characterized by Van Staden & Huismans (1991). When expressed in the baculovirus expression system, the protein was found to be cytotoxic, a property until then not known among orbivirus proteins (Van Staden *et al.* 1995). However, the importance of this characteristic, as well as the function of NS3 in AHSV morphogenesis, remains obscure. Since knowledge of the location of a viral protein in a cell often provides insight into its possible function in viral replication, the aim of this study was to determine the subcellular localization of AHSV NS3 and its possible involvement in virus release by means of immunogold electron microscopy.

As the release mechanisms of AHSV have never before been documented, the mode(s) of AHSV liberation from infected cells was investigated. VERO cells were grown to semi-confluency on plastic coverslips (Thermanox coverslips, Electron Microscopy Sciences, Washington) and infected with AHSV-3 at a multiplicity of infection (MOI) of at least ten plaque-forming units (PFU) per cell. The cultures were fixed and processed for transmission electron microscopy, without removal from the supports, according to standard techniques (fixation in 2,5% phosphate-buffered formaldehyde, dehydration in ethanol and embedded in L.R. White resin, visualization by contrasting in lead citrate and uranylacetate). At 20 h post infection (pi), two modes of viral release were observed. Some viruses budded from the cell surface (Fig. 1), thereby acquiring fragments from the plasma membrane. In other instances, the cell membrane seemed to be disrupted by the liberation of aggregates of non-enveloped viruses (not shown). It therefore appeared that AHSV, similarly to BTV, was released from infected cells by budding, as well as by a mechanism resembling extrusion.

To examine the possible involvement of the NS3 protein in AHSV release, the subcellular localization of NS3 was determined. At 20 h pi, unfixed AHSV-3-infected VERO cells on plastic coverslips were incubated with polyclonal anti-NS3 antibody (for 1 h at 4 °C) raised in rabbits to a denatured form of a bacterially expressed ß-galactosidase-NS3 fusion protein (Van Staden *et al.* 1995). Following several rinses in 1% serum albumin/phosphate-buffered saline (PBSA) and incubation with anti-rabbit gold, the samples were fixed in 1% phosphate-buffered glutaraldehyde, washed and post-fixed in 0,25% osmium tetroxide. These samples were infiltrated with DER resin, since standard embedding resins (e.g. Quetol) dissolved the Thermanox coverslips on which the

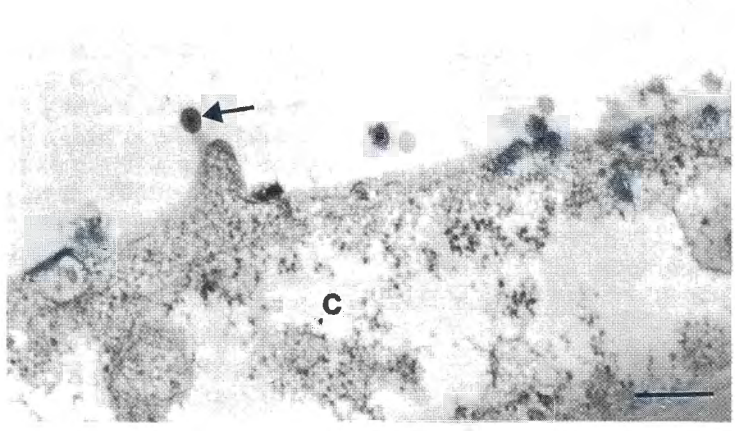

FIG. 1 Transmission electron micrograph showing the release of AHSV from infected VERO cells. The arrow indicates a single virus particle being released from the cell surface by budding

c = cytoplasm

Scale bar: 10 mm = 213 nm

cells were cultured. (Unfortunately DER resin produces electron micrographs of poor quality owing to low contrast.) When examined electron microscopically, the gold label was found to be associated with perturbed regions of the host-cell membrane. A good correlation existed between the presence of NS3 and sites of AHSV release. More specifically, gold particles were present on fragments of the host-cell membrane that had been disrupted by the egress of viruses (Fig. 2A and B). However, the intensity of labelling was low, as could be expected from the small quantities of NS3 present in AHSV-infected cells (Van Staden *et al.* 1995). Gold label was absent from virus particles and did not occur on the extracellular surface of intact host-cell membranes. The specificity of the NS3 labelling was verified by the use of controls, which included uninfected cells incubated with the anti-NS3 antiserum and infected cells incubated with monospecific serum to the nonstructural protein NS2 of AHSV. All control results were negative, with a complete absence of any cell- or virus-associated labelling.

Attempts to determine the intracellular localization of the NS3 protein by post-embedding procedures were unsuccessful. This could be due to masking of the weak NS3 signals by the fixation and resin-embedding procedures employed.

The membrane association of NS3 was also investigated in *Spodoptera frugiperda* (Sf9) cells infected with a baculovirus recombinant expressing the NS3 protein (Van Staden *et al.* 1995). Pre-embedding immunogold labelling of these cells with the NS3-specific antiserum presented results similar to those with AHSV-infected VERO cells. The only region where gold particles were detected was on the surface of cells in areas of localized plasma-membrane disruption (not shown). The absence of NS3 on intact host-cell membranes, together with the low intensity of labelling, appeared to indicate that the NS3 protein was not exposed extracellularly. This observation correlates with data obtained for BTV NS3 (Hyatt *et al.* 1991). The larger amounts of AHSV NS3 protein produced in recombinant baculovirus-infected insect

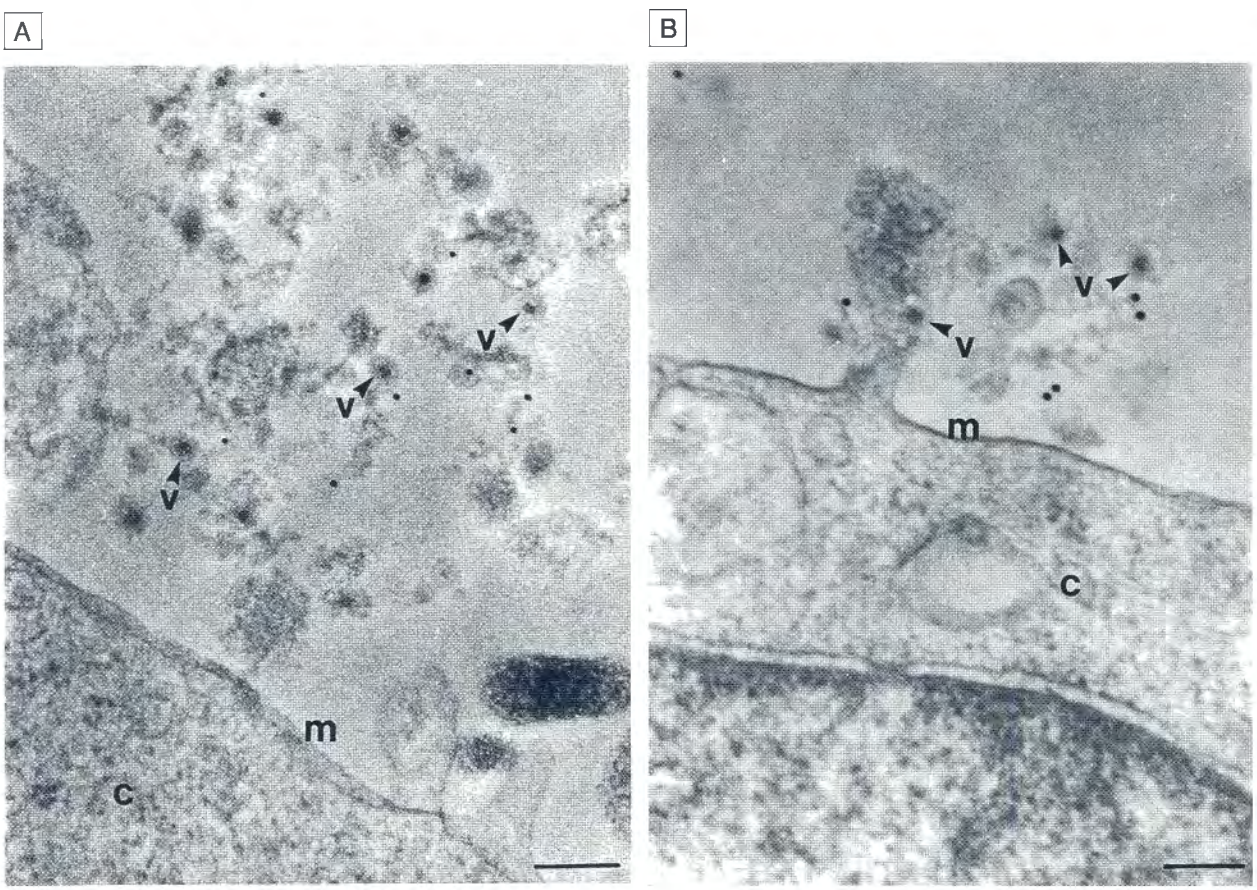

FIG. 2A and B Transmission electron micrographs of thin sections from pre-embedded, anti-NS3, immunolabelled, AHSV-infected VERO cells, showing the co-localization of membrane fragments containing NS3 (gold label) and released viruses (v). The low contrast of the electron micrographs is due to the DER resin used to embed the samples

m = plasma membrane, c = cytoplasm

Scale bars: A. 10 mm = 196 nm, B. 10 mm = 111 nm

cells allowed its detection after fixation and resin embedding. Following fixation in phosphate-buffered 2,5 % formaldehyde/0,1 % glutaraldehyde, infected cells were dehydrated in ethanol and embedded in L.R. White resin. Ultra-thin sections were sequentially incubated with anti-NS3-specific antibodies and Protein A-gold. The plasma membrane of infected cells labelled distinctly (Fig. 3A).

These results confirmed the plasma-membrane association of the NS3 protein, and additionally indicated that its localization is not dependent on the presence of AHSV particles. A rather unexpected and as yet unexplained result also seen in Fig. 3, was the intense labelling of baculoviruses. This was found to be entirely non-NS3 specific for the following reasons: a similar labelling pattern was obtained with pre-bled antisera that contained no NS3 antibodies (not shown); furthermore, recombinant baculoviruses expressing the NS2 protein of AHSV-9 (Uitenweerde, Theron, Stoltz & Huismans 1995) labelled similarly when probed with the NS3-specific antiserum (Fig. 3B).

The AHSV-3 NS3 protein was found to be cytotoxic when expressed in the baculovirus expression system (Van Staden *et al.* 1995). This was indicated by the observation that NS3 mRNA and protein synthesis were followed by an increased permeability of cells as determined by trypan-blue staining. The present investigation has indicated that the NS3 protein is associated with the host-cell membrane. It is possible that this association affects the permeability of the plasma membrane, resulting in some form of osmotic disregulation and eventually in cell death.

The rotavirus nonstructural protein NSP4, which has been proposed to share functional similarities with BTV NS3 (Wu, Chen, Iwata, Compans & Roy 1992) has also recently been shown to be cytotoxic. It was demonstrated that the expression of this protein resulted in a five-fold increase in the concentration of intracellular calcium of recombinant baculovirus-infected *Spodoptera frugiperda* cells (Tian, Hu, Schilling, Lindsay, Eiden & Estes 1994). Rotavirus NSP4 is, therefore, thought to be responsible for the increase in cytosolic calcium in rotavirus-infected cells which could result in cell lysis and the subsequent release of mature rotavirus particles during the late stages of the replication cycle (Tian *et al.* 1994).

The association of the NS3 protein with the cell membrane in regions where viral release occurs, strongly

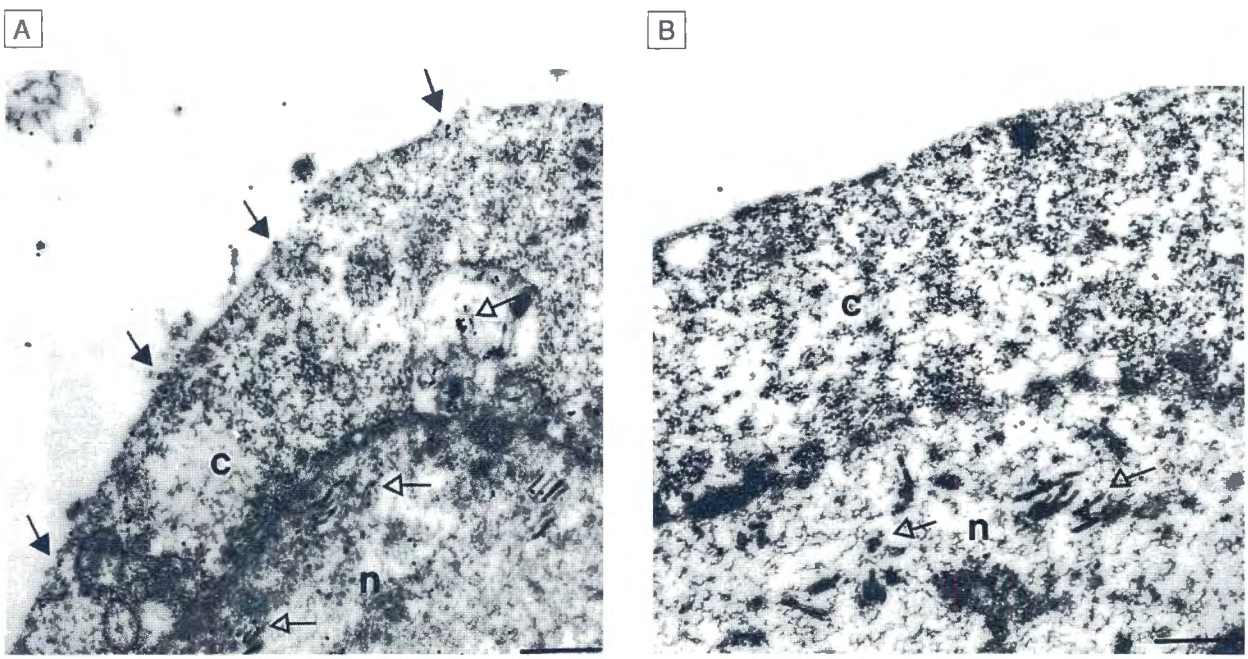

FIG. 3 Transmission electron micrographs of immunogold-labelled resin sections of recombinant baculovirus-infected Sf9 cells expressing AHSV NS3

c = cytoplasm, n = nucleus

A. Anti-NS3 serum reacted specifically with the plasma membrane of the cells (indicated by solid arrows). Non-NS3-specific labelling of baculoviruses occurred (indicated by open arrows)

Scale bar: 10 mm = 370 nm

B. Control cell immunolabelled with monospecific antiserum raised to the nonstructural protein NS2 of AHSV. Labelling of baculoviruses are depicted by open arrows

Scale bar: 10 mm = 333 nm

suggests the involvement of NS3 in this final stage of AHSV morphogenesis. However, this aspect needs to be investigated further.

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