Localization of the non-structural protein NS3 in bluetongue virus-infected cells

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The localization of the blue tongue virus (BTV) non-structural proteins NS3 and NS3a has been identified using immunoelectron microscopical techniques. NS3 and NS3a have been observed in the plasma membrane of BTV- and recombinant vaccinia virus (expressing NS3)-infected cells. The NS3 protein was associated with areas of membrane perturbation. There was a good correlation between the presence of NS3 and NS3a and BTV release. The NS3 protein was associated with membrane fragments and the inability to detect it on the extracellular aspect of intact cells suggested that the protein was not exposed extracellularly. Electron microscopical and biochemical evidence suggested that fragments of plasma membrane containing NS3 and NS3a were released from infected cells. Collectively, the data indicate that NS3 and NS3a may be involved in the final stages of BTV morphogenesis, i.e. the release of BTV from infected cells.

Bluetongue virus (BTV) is the type species of the Orbivirus genus in the Reoviridae family. The virus has an icosahedral core containing 10 dsRNA genome segments each of which encodes at least one protein. The core consists of two major proteins VP7 and VP3, and three minor proteins VP1, VP4 and VP6. The outer diffuse layer of the virus is made up of proteins VP2 and VP5. Non-structural proteins NS1, NS2 and NS3 are also synthesized in BTV-infected cells. RNA segment 10 codes for the non-structural protein NS3 and the related protein, NS3a (Mertens et al., 1984; van Dijk & Huismans, 1988). These proteins are produced in very small amounts in infected cells (van Dijk & Huismans, 1988) and sequence data suggest that NS3 and NS3a arise by translation starting from either of two in-frame initiation codons present on the same mRNA (Gould, 1988). Apart from one report where small amounts of NS3 and NS3a were found associated with purified virus (Mertens et al., 1984), no information is available on the location or function of these proteins.

Sequencing data from RNA segment 10 (Gould, 1988) revealed a high degree of amino acid sequence conservation between the NS3 proteins of BTV serotypes 1 (Australia) and 10 (U.S.A.) as well as regions of nucleic acid sequence conservation. The high degree of amino acid conservation suggests a conserved biological function(s). There exist two putative transmembrane domains at amino acids 118 to 148 and 156 to 181 and two potential glycosylation signals at amino acid positions 63 to 65 and 150 to 152. The location of the second potential glycosylation site lies between the two hydrophobic domains. The molecular structure of NS3 appears to be similar to that of NS28, a non-structural glycoprotein found in the rough endoplasmic reticulum (RER) membrane of rotavirus-infected cells (Ericson et al., 1983; Kabcenell & Atkinson, 1985). The final stages of rotavirus morphogenesis occur within the lumen of the RER (Holmes, 1983), and recent evidence (Au et al., 1989; Meyer et al., 1989) has shown that NS28 may function as a receptor for subviral particles to mediate their budding through the RER. The morphogenesis of BTV differs significantly from that of rotaviruses. In BTV-infected cells infectious virus particles originate from virus inclusion bodies and their morphogenesis does not involve the RER. However, BTV does interact with plasma membranes where virions are released from infected cells either by budding, whereby they acquire a membrane, or by a process of extrusion through the cell membrane (Hyatt et al., 1989). The mechanisms underlying these processes are unknown. The similarity in size and structure of BTV NS3 and rotavirus NS28 suggests that information on the ultrastructural location of NS3 in BTV-infected cells may help determine whether it, like NS28, is involved in virus morphogenesis. The results described here indicate that NS3 can be localized in the plasma membrane of BTV-infected cells at sites where viruses are budding or being extruded.

BTV-1 (Australia) was used to infect tissue culture cells which were subsequently examined by transmission and scanning electron microscopy. Cells were fixed 18 h post-infection in 0.1% phosphate-buffered glutaraldehyde, washed in PBS (wash buffer) and incubated with
Fig. 1. Backscattered scanning electron micrograph of a BTV-infected cell. Silver-enhanced protein A-gold particles (arrows) indicate the presence of NS3. The nucleus (Nu) and fat droplets (F) are indicated. The bar represents 10 µm.

Fig. 2. Transmission electron micrograph of a thin section taken from a pre-embedded, immunolabelled BTV-infected cell. Viruses (V) and gold-labelled membrane fragments containing NS3 (arrows) are indicated. Note that gold label is not associated with intact plasma membrane. The bar represents 200 nm.

Fig. 3. Transmission electron micrograph of a gold-labelled Lowicryl section showing the co-localization of NS3 (gold label) and budding BTV. The bar represents 200 nm.

Mouse polyclonal antibody against NS3 (Martyn et al., 1990) for 1 h at room temperature (RT). Following several rinses in wash buffer and incubation with Protein A-gold (1 h, RT) the samples were processed for either transmission electron microscopy (Hyatt et al., 1989) or silver enhancement and scanning electron microscopy (Hyatt et al., 1989). Fig. 1 shows the distribution of NS3 on the surface of BTV-infected cells. The location of label indicates a non-uniform distribution of the non-structural protein. When examined by thin section electron microscopy, the gold label was associated with regions of the plasma membrane where BTV is extruded (Fig. 2) or budding (Fig. 3). Specifically, labelling is confined to areas of the host cell membrane that have been disrupted by the egress of virus and is restricted to membrane fragments associated with the cell surface or budding virions. Immunogold labelling was not observed on the extracellular aspect of intact BTV-infected host cell plasma membranes (Fig. 2).

Thin sections from Lowicryl K4M blocks (prepared as described by Hyatt, 1991) of BTV-infected cells were labelled with polyclonal NS3 antibody (as above), biotinylated anti-mouse antibody and streptavidin-gold. Labelling was restricted to areas of plasma membrane perturbation, budding (Fig. 3) and intracellular viral
aggregates (Fig. 4); no labelling was observed in association with the RER. The label associated with viral aggregates was restricted to those found in close association with structures which can be identified in conventionally fixed Spurr’s resin sections as smooth membrane-limited vesicles. In conventionally fixed specimens, the viruses can be observed either near or occasionally within or budding into the vesicles (data not shown). The precise location of the label in the Lowicryl sections was difficult to define. Gold label appears not to be associated with virus particles but with an ill-defined matrix associated with either the aggregates or the vesicles. The poor immunoresolution was attributed to the bridged labelling protocol which was in turn made necessary by the low levels of intracellular NS3.

A recombinant vaccinia virus expressing NS3 was constructed by insertion of the NS3 coding sequence into the thymidine kinase gene of vaccinia virus (WR strain) under the control of the vaccinia P7.5 promoter (Boyle et al., 1985; Martyn et al., 1990). Pre-embedding immunogold labelling of intact cells infected with the vaccinia virus–NS3 construct showed results similar to those with BTV-infected cells. Gold labelling occurred on the surface of infected cells but only in areas of localized plasma membrane disruption where vaccinia viruses were leaving the cell (Fig. 5). Unlike BTV-infected cells, where there was a good correlation between the sites of virus release and immunogold labelling; not all areas of membrane perturbed by vaccinia virus release were labelled. These results indicate that although NS3 is associated with the plasma membrane, its localization is not dependent on the presence of BTV particles.

It should also be noted that the levels of NS3 and NS3a within BTV-infected cells are very low (van Dijk & Huismans, 1988) thus the amount of accessible antigenic mass of NS3 and NS3a available for immunogold labelling within any of the above BTV-infected cells must similarly be small. To facilitate the level of labelling of BTV- and vaccinia virus-infected cells, 5 nm gold probes were used instead of 10 nm and 15 nm probes. The smaller probes increased the intensity of labelling by decreasing the effect of steric hindrance. It should therefore be noted that intensive gold labelling of NS3 was unlikely.

Controls for the above experiments consisted of infected and uninfected cells being processed for scanning electron microscopy, pre-embedding immunolabelling and post-embedding immunolabelling. These preparations were used to access the specificity of the gold labelling. Infected cells were incubated with non-relevant antisera, namely a monoclonal antibody to NS2 and mouse polyclonal antiserum generated to a recombinant vaccinia virus expressing VP5. Uninfected cells were incubated with mouse polyclonal antisera against NS3. All samples were negative, and examples of some of these are shown in Fig. 6. The control experiments illustrate that the labelling of NS3 in association with BTV- and vaccinia virus-infected cells is specific.
Fig. 6. Electron micrographs of control cells. (a) Backscattered scanning electron micrograph of a silver-enhanced uninfected SVP cell incubated with mouse polyclonal antiserum raised against NS3 and protein A gold. The nucleus (Nu) is indicated. The bar represents 10 µm. (b) Thin section of a BTV-infected cell embedded in Lowicryl K4M, and incubated with a mouse monoclonal antibody to NS2, biotinylated anti-mouse antibody and strepavidin-gold. A virus inclusion body (VIB), virus aggregates (VA) and virus tubules (VT) are shown. Note there is no labelling on the virus aggregates. The bar represents 200 nm. (c) Thin section of a cell infected with vaccinia virus–NS3 constructs. Whole cells were incubated with mouse polyclonal antibody raised to VP5 and Protein A–gold. The bar represents 100 nm.

The results in Fig. 2 suggest that fragments of plasma membrane containing NS3 may be released from infected cells. Evidence for the presence of membrane-associated NS3 in the culture medium came from experiments in which BTV-infected cells were labelled with $^{35}\text{S}$methionine and the culture medium was layered over a sucrose column. Following centrifugation, proteins in the top and bottom portions (including the medium/sucrose interface) of the culture medium were precipitated with methanol and analysed by electrophoresis in a 12% gel. [35S]Methionine-labelled intracellular virus particles were prepared (Eaton et al., 1991) and the proteins were analysed (lane 5). The positions of viral structural and non-structural proteins are indicated by the products of an in vitro RNA translation reaction.

Fig. 7. Release of NS3 and NS3a from BTV-infected cells. SVP cells were infected with BTV-1 at an m.o.i. of 5 p.f.u./cell and were labelled in 4 ml methionine-free MEM containing 50 µCi/ml $^{35}\text{S}$methionine from 18 to 22 h post-infection. After labelling, cells in the culture medium were removed by low speed centrifugation and the clarified medium was either left untreated (lanes 2 and 3) or made 1% in NP40 (lanes 1 and 4). Treated and control samples were layered over 40% sucrose in STE (100 mM-NaCl, 10 mM-Tris pH 7.2, 1.5 mM-EDTA) and centrifuged in a Beckman SW41 rotor at 300000 g for 1.5 h. Proteins in the top three-quarters (lanes 1 and 2) and bottom quarter including the medium/sucrose interface (lanes 3 and 4) were precipitated with methanol and analysed by electrophoresis in a 12% gel. [35S]Methionine-labelled intracellular virus particles were prepared (Eaton et al., 1991) and the proteins were analysed (lane 5). The positions of viral structural and non-structural proteins are indicated by the products of an in vitro RNA translation reaction.

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sedimenting structure that failed to enter the sucrose column. Virus particles are sedimented through the sucrose column under the conditions of centrifugation used in these experiments. Treatment of the culture medium with NP40 reversed this and markedly reduced the proportions of NS3 and NS3a that sedimented rapidly (lane 4) and increased the more slowly sedimenting NS3 and NS3a (lane 1). These results indicate that the non-structural proteins NS3 and NS3a in the culture medium of BTV-infected cells were present in a detergent-sensitive structure and are presumably associated with membranes. No other virus proteins in the culture medium of infected cells displayed a similar degree of sensitivity to NP40 treatment.

This report describes the localization of NS3 and NS3a within and the release of these proteins from BTV-infected cells. NS3 is associated with both host plasma membranes and intracellular smooth-surfaced vesicles. The data presented here indicate that there is a correlation between the association of virus particles with the cell membrane and the presence of NS3. The ability of NS3 to bind to the plasma membrane of cells infected with a vaccinia virus recombinant expressing NS3 indicates that the protein does not require BTV to mediate binding and thus the association of BTV with cell membranes may be mediated by NS3 already inserted within membranes. The inability to detect NS3 on the surface of infected cells except where viruses are being released indicates that either the protein or at least its antigenic epitopes may not be exposed extracellularly. Although the localization of NS3 differs from that of NS28 in rotavirus-infected cells, there are similarities between the two proteins and specific morphological events. In rotavirus-infected cells, viruses bind to RER containing NS28 thereby passing into the lumen of the RER and acquiring a transient envelope. Within BTV-infected cells, viruses acquire transient envelopes from the regions of the plasma membrane which contain NS3; thus NS3 may facilitate the release of BTV from infected cells.

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