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Bluetongue Virus Assembly and Morphogenesis

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Abstract Like other members of the *Reoviridae*, bluetongue virus faces the same constraints on structure and assembly that are imposed by a large dsRNA genome. However, since it is arthropod-transmitted, BTV must have assembly pathways that are sufficiently flexible to allow it to replicate in evolutionarily distant hosts. With this background, it is hardly surprising that BTV interacts with highly conserved cellular pathways during morphogenesis and trafficking. Indeed, recent studies have revealed striking parallels between the pathways involved in the entry and egress of nonenveloped BTV and those used by enveloped viruses. In addition, recent studies with the protein that is the major component of the BTV viroplasm have revealed how the assembly and, as importantly, the disassembly of this structure may be achieved. This is a first step towards resolving the interactions that occur in these virus 'assembly factories'. Overall, this review demonstrates that the integration of structural, biochemical and molecular data is necessary to fully understand the assembly and replication of this complex RNA virus.

1 Introduction

Bluetongue virus (BTV) is the type species of the genus *Orbivirus* within the family *Reoviridae*. Orbiviruses are distinct from reoviruses and rotaviruses, both in the organisation of structural proteins and in the nonstructural proteins expressed in virus-infected cells. Despite many functional similarities, there is no primary sequence similarity between orbivirus proteins and the corresponding proteins of other genera within the family. In addition, orbiviruses express proteins that apparently have no functional equivalent in either reoviruses or rotaviruses. These differences between orbiviruses and the other two genera within the *Reoviridae* family may be a reflection of the difference in the mode of transmission of these three virus genera. Whereas reoviruses and rotaviruses are transmitted by the faecal-oral route, orbiviruses are arthropod-transmitted and also replicate in the arthropod host. Thus, in addition to facing the structural and enzymatic constraints imposed by the dsRNA genome common to all the members of *Reoviridae*, orbiviruses must be sufficiently flexible to replicate in two very different hosts. This difference in virus transmission is also reflected in very different physical characteristics. For example, orbivirus virions are more fragile than reovirus and rotavirus virions, with infectivity being lost in mildly acidic conditions and on treatment with detergents (Gorman et al. 1983).

BTV is transmitted by *Culicoides* spp., causing diseases in ruminants of economic importance in many parts of the world. Bluetongue disease in sheep, goats, cattle and other domestic animals as well as in wild ruminants (e.g. blesbuck, white-tailed deer, elk, pronghorn antelope, etc.) was first described in the late eighteenth century. In sheep, the disease is acute, and the mortality can be very high. To date, BTV has been isolated in tropical, subtropical and temperate zones of the world, and 24 different serotypes have been identified. Other orbiviruses infect a wide variety of vertebrates, including man (sometimes causing severe infection). Vector–virus interactions play a crucial role in vector-borne disease epidemiology. The spread of *Culicoides* species from endemic to non-BTV (and also related African horse sickness virus, AHSV, and Epizootic haemorrhagic disease virus, EHDV, of deer) regions of the world in the past raises the concern that these viruses represent an emerging threat for regions that are presently free from viral infection. As a result of its economic significance, BTV has been the subject of extensive molecular, genetic and structural studies.

Although BTV is well characterised at structural and molecular levels, to fully understand the BTV infection cycle it is important to understand the dynamic protein–protein and protein–RNA interactions between viral

components and proteins of the host cell. A complete understanding of virus infection of a cell, replication, synthesis and release of new virions can only be achieved through an approach that integrates structural, molecular and cell biology data. In this chapter, we present the current understanding of how the complex interplay between BTV and cellular proteins contributes to the assembly and morphogenesis of new virus particles within the host cell. *In vitro* studies using individual virus proteins and complexes of virus proteins will also be reviewed.

2
BTV Morphology, Cell Entry and Transcription

Like the other members of the *Reoviridae* described within this volume, BTV virions (550S) are architecturally complex structures composed of multiple layers of proteins that undergo incomplete disassembly upon entry into host cells. In the case of BTV, there are seven structural proteins (VP1-VP7) that are organised into an outer capsid and an inner capsid (commonly known as "core") and containing the ten double-stranded (ds) RNA segments of the viral genome (Fig. 1). The outer capsid is composed of two proteins, VP2 and VP5, and is necessary for cell attachment and virus penetration of the mammalian host cell during the initial stages of infection. Although a great deal is known about the structure of the viral core from atomic resolution structural data (Grimes et al. 1998), there is not yet an equivalent structure for the BTV outer capsid, or for any of the outer capsid proteins. However, recent biochemical analyses together with high-resolution cryoelectron microscopy (EM) structural studies of the BTV outer capsid have given some insights towards the role of each of these proteins in cell entry.

2.1
BTV Outer Capsid and Virus Entry into Cells

In mammalian cells, BTV entry proceeds via virus attachment to the cell, followed by endocytosis and release of a transcriptionally active core particle into the cytoplasm. (Grubman et al. 1983; Huismans et al. 1983, 1987; Eaton and Hyatt 1989; Hassan and Roy 1999; Hassan et al. 2001; Forzan et al. 2004). From our recent studies, it appears that unlike rotavirus, BTV does not use an alternate direct penetration process to enter mammalian cells (M. Forzan and P. Roy, unpublished data). The structural features of VP2 and VP5 correlate with their biological roles in the virus attachment and penetration of the endosomal vesicle, as discussed below.

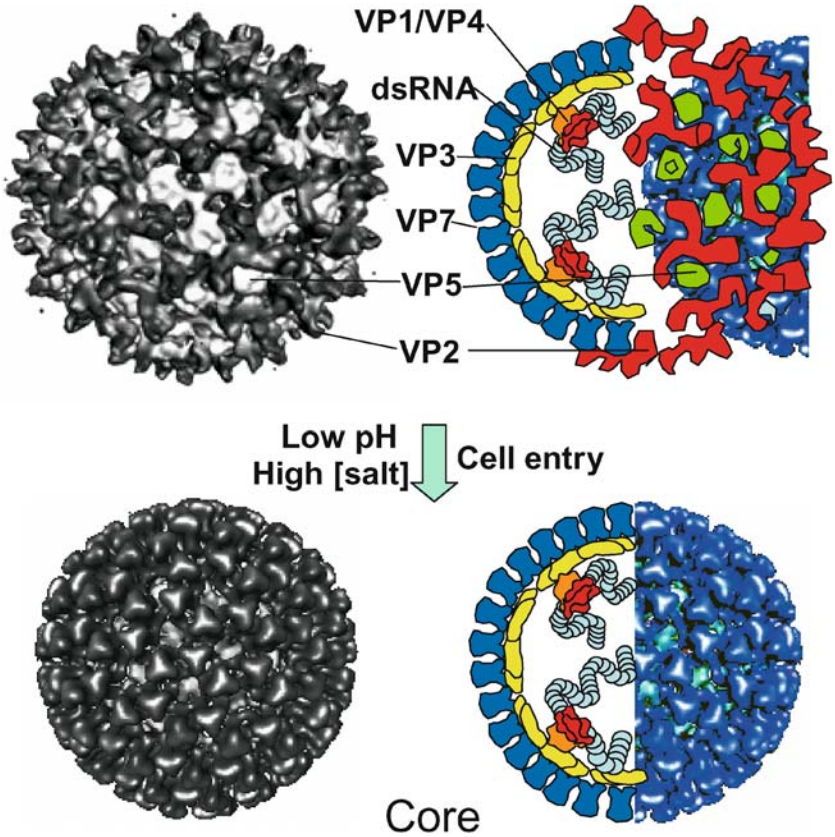

Fig. 1 The arrangement of proteins and nucleic acid in BTV virion (*top*) and core (*bottom*) particles: for each particle a cryo-EM reconstruction (*left*) and a diagramme (*right*) is shown. VP1 (polymerase) and VP4 (capping enzyme) co-localise to the five-fold axes of symmetry on the inner face of the VP3 layer in contact with the viral dsRNA. The transition from virus to core particle occurs during cell entry and can be mimicked *in vitro* by lowering pH or increasing salt concentration. (Adapted from Forzan et al. 2004; Nason et al. 2004)

The BTV outer capsid has an icosahedral configuration with a diameter of approximately 880 Å (Fig. 1). Cryo-EM analyses have revealed that the outer capsid is composed of a total of sixty triskelion spike-like structures formed by VP2 (110 kDa) trimers and 120 globular VP5 (60 kDa) trimers (Hewat et al. 1992b, 1994; Nason et al. 2004). The VP2 spikes extend up to 3 nm from the main body of the particle and have bent tips. The globular VP5 trimers, although also entirely exposed in the virion, are located more internally than

VP2. Both proteins make extensive contacts with the underlying outer layer of the core (VP7). Nevertheless both proteins, in particular VP2, are easily removed by high salt concentrations and/or in acidic pH (Verwoerd et al. 1972; Huismans et al. 1987).

The propeller-like VP2 spike contains the cell attachment sites of the virion. VP2 is responsible for eliciting neutralising antibodies, possesses haemagglutination activity and is the major serotype determinant of the virus. When VP2 is added to susceptible cell lines, it is rapidly internalised by endocytosis (Hassan and Roy 1999). Following internalisation, the clathrin coats of endocytic vesicles are rapidly lost, larger vesicles form, the membranes of which become rapidly destabilised allowing the penetration of the now uncoated core particle into the cytoplasm. This pathway of virus entry relies on specific conformational changes that occur to the BTV outer capsid in response to the changing environment of the virion as it enters the endocytic pathway. Additions of compounds that raise the endosomal pH and block the normal endosomal acidification process prevent virus particles from entering the cytoplasm (Hyatt et al. 1989; Forzan et al. 2004).

The globular outer capsid protein VP5 shares certain structural features with the fusion proteins of enveloped viruses. In its monomeric form, VP5 can be divided into an amino terminal coiled-coil domain and a carboxyl terminal globular domain with a flexible hinge region in between. In addition, the amino terminus of VP5 has the potential to form two amphipathic helices with the capacity for membrane destabilisation (Hassan et al. 2001; Forzan et al. 2004). Indeed, when VP5 is presented appropriately on the cell surface it induces cell-to-cell fusion, confirming that it has the capability to destabilise the cellular membranes. Critically, VP5 only exhibits its membrane-destabilising properties after it has undergone a low pH-triggered activation step, which mimics the endosomal environment encountered during cell entry and results in a change in the VP5 conformation. VP5 lacks the autocatalytic cleavage and N-terminal myristoyl group present in the entry proteins of reoviruses and rotaviruses and does not require proteolytic activation in contrast to some other viral fusion proteins (Colman and Lawrence 2003).

In summary, current data suggest a model in which VP2 makes initial contact with the host cell and triggers receptor-mediated endocytosis of the virus particle and then VP5 undergoes a low pH-triggered conformational change that results in the destabilisation of the endosomal membrane. It is likely that the change in conformation of VP5 that promotes membrane destabilisation, forming a protein layer with intrinsic outside-in curvature, weakens the contacts between VP5 and VP7, the surface layer of the core. This then allows core particles (470S), from which both outer-capsid proteins have been lost, to be released into the cytoplasm (Fig. 2).

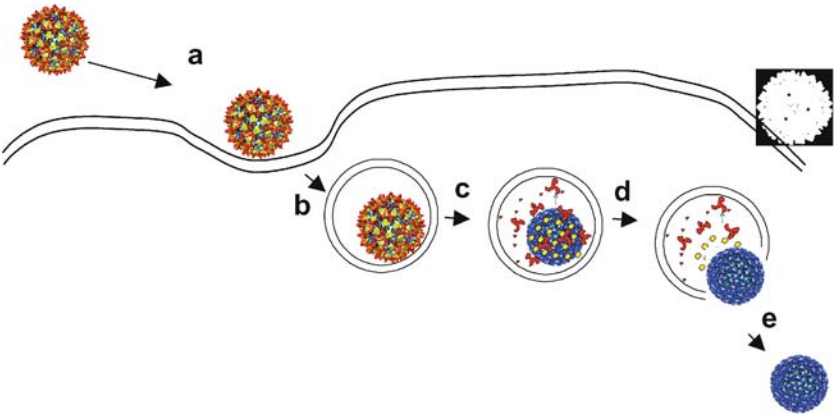

Fig. 2 Summary of structural changes which occur to the virus particle during BTV entry. (*a*) Virus particle binds to cellular receptor(s). (*b*) Receptor-mediated endocytosis of the virus particle. (*c*) Acidification of the endosome triggers the release of VP2 (*red*) from the particle and the activation of VP5, the viral fusion-like protein. (*d*) VP5 molecules (*yellow*) penetrate the endosomal membrane and are released from the particle. (*e*) The transcriptionally active core particle is released into the cytoplasm

2.2 BTV Core Particles and mRNA Synthesis

Upon release into the cytoplasm, BTV core particles become transcriptionally active and synthesise capped but not polyadenylated viral messenger-sense RNA (vmRNA) (Van Dijk and Huismans 1980, 1982; Mertens et al. 1987; Van Dijk and Huismans 1988). The transcriptionally active core is composed of 10 dsRNA genome segments, two major capsid proteins, VP3 and VP7, and three minor enzymatic proteins, VP1, VP4 and VP6. In this structure, the major proteins account for the overall morphology of the core and the minor proteins are responsible for the transcription of vmRNA. The fact that the enzymatic proteins necessary for transcribing vmRNAs from dsRNA template are contained within the core particle avoids any requirement for the viral core to disassemble completely. Thus, the viral dsRNA is sequestered away from cellular dsRNA surveillance systems which are linked to innate antiviral defences (Jacobs and Langland 1996; Samuel 1998). The newly synthesised, capped mRNA species are extruded from core particles into the cytoplasm and program the translation of virus proteins. A detailed review of the atomic structure of the BTV core is provided by in the chapter by Stuart and Grimes in this volume. Although the structural integrity of the core particle appears to be essential for maintaining efficient transcriptional activity, it has been

possible to use *in vitro* assays with each enzymatic protein of the core to delineate specific protein functions within the transcription complex.

Since transcription of the dsRNA genome of members of the *Reoviridae* occurs by a fully conservative process (Bannerjee and Shatkin 1970), it is logical that this process would involve a helicase protein either to unwind the dsRNA ahead of the transcriptase protein or to separate the parental and newly synthesised RNAs following transcription. For BTV, the 38-kDa minor core protein VP6 possesses helicase activity *in vitro* and exhibits physical properties characteristic of other helicases, including an oligomeric nature and an ability to form ring-like structures in the presence of BTV RNA (Stauber et al. 1997; Kar and Roy 2003).

The replication of viral dsRNA occurs in two distinct steps. First, plus-strand RNA (vmRNA) is transcribed, using the dsRNA genome segments as template, and extruded from the core particle (Bannerjee and Shatkin 1970; Cohen 1977; Van Dijk and Huismans 1980). Second, the plus-strand RNAs serve as templates for the synthesis of new minus-strand RNA at an undefined stage during the assembly of a new virus core particle. The largest minor core protein VP1 (150 kDa) has the ability to both initiate and elongate minus-strand synthesis de novo (Boyce et al. 2004). This does not require a specific secondary structure present at the 3′ end of the plus strand template. This polymerase activity was lost when a GDD motif (amino acids 287–289), characteristic of other RNA polymerases, was deleted (M. Boyce and P. Roy, unpublished observation). Studies of replicase activity in other members of the *Reoviridae* have used very short templates (Tao et al. 2002) or have required a particulate replicase in which the catalytic subunit is proposed to possess replicase activity only in the context of a subviral particle (Kohli et al. 1993; Chen et al. 1994; Patton 1996, 1997; Charpilienne et al. 2002). Such apparent discrepancy with the BTV polymerase may be attributable to the varied experimental systems used to assay replicase activity. Since the replicase activity associated with recombinant BTV VP1 is low, it is possible that the activity of VP1 is enhanced by other viral proteins present in the assembling core particle, although to date our studies have revealed little evidence for this hypothesis.

Although VP1 has been shown to be active as the viral RNA-dependent RNA polymerase it is not sufficient for the synthesis of the methylated cap structure found at the 5′ end of BTV vmRNA. Within the assembled core particle, VP1 is closely associated with VP4 (Nason et al. 2004). Recombinant, purified VP4 (76.4 kDa) is an enzyme that can synthesise type 1-like ‘cap’ structures *in vitro* that are identical to those found on authentic BTV vmRNA. The protein represents a model enzyme, which has methyltransferase, guanylyltransferase and RNA triphosphatase activities in a single protein. VP4 is unusual in

that it is capable of completing several distinct enzymatic reactions in the absence of any other viral protein (Martinez Costas et al. 1998; Ramadevi et al. 1998; Ramadevi and Roy 1998). This is notably different to other viral capping enzymes, e.g. those of vaccinia virus, where capping is dependent on a complex of 3 proteins (Venkatesan et al. 1980).

3 Synthesis of Viral Proteins and Assembly of Particles

Following virus entry, uncoating of the core and initiation of transcription, vmRNA are released into the cytosol and serve as both templates for viral dsRNA genome synthesis and messengers for the synthesis of viral proteins. The first virus-specific proteins are detectable at 2–4 h after infection, and the rate of protein synthesis increases rapidly until 11–13 h after infection, after which it slows down but continues until cell death (Huismans 1979). Most viral proteins are synthesised throughout the infection cycle and accumulate during infection until cell lysis. Infection of mammalian cells with BTV leads to a rapid inhibition of cellular macromolecular synthesis and the induction of an apoptotic response. The latter response appears to be triggered by the combination of two signals involving attachment of the virus to the cell and the activity of the membrane permeabilising protein, VP5 (Mortola et al. 2004).

In addition to the seven structural proteins of the virion, four nonstructural proteins, NS1, NS2, and NS3/NS3A are synthesised in BTV infected cells. In common with the corresponding proteins of many other viruses, the nonstructural proteins of BTV are key components of the infection machinery, modulating host–virus interplay as well as virus morphology. The two larger BTV NS proteins, NS1 and NS2, are produced at high levels in the cytoplasm and multimerise into discrete structures. While NS2 is clearly involved in virus replication and assembly processes (discussed below), a defined role for NS1 that multimerises into tubules is yet to emerge. However, in the absence of NS1 and NS2, all structural proteins except VP6 readily assemble when co-expressed using a heterologous expression system. Therefore, the viral structural proteins possess inherent properties to assemble, and neither NS1 or NS2 nor genomic RNAs are essential for capsid assembly. Nevertheless, it is clear that the assembly process is sequential and highly precise (see below).

In contrast to NS1 and NS2, the steady-state level of the small viral nonstructural proteins, NS3/NS3A, is highly variable (from being barely detectable to being highly expressed) and is dependent on the host cell species. The level of NS3/NS3A in different cell types appears to correlate with the efficiency of virus release with those cells producing the most NS3 releasing the

most virus. In this context, it is noteworthy that orbiviruses such as BTV and African horse sickness virus establish persistent infections in susceptible insect cells with little apparent cytopathic effect (CPE). In contrast, both viruses cause dramatic CPE in mammalian cells. While the majority of the progeny particles remain cell-associated in mammalian cell infection, as is commonly observed with reoviruses, there is clear evidence that virus particles can, in contrast to reoviruses, also leave host cells by budding at the cell membrane.

3.1 Virus Inclusion Bodies and NS2

For a number of animal and plant viruses, replication complexes, transcription complexes, replication and assembly intermediates, as well as nucleocapsids and virions accumulate in specific locations within the host cell in structures described as virus assembly factories or virus inclusion bodies (VIBs). During BTV infection, core particles become very rapidly associated with a matrix that gradually surrounds the particles to form virus inclusion bodies. As the infection progresses, these VIBs increase both in size and numbers. In addition to being the site of transcription, vmRNA and proteins can be identified within these VIBs; thus, they appear to be the site of BTV replication and of early viral assembly (Eaton et al. 1988; Hyatt and Eaton 1988; Brookes et al. 1993). This has largely been inferred from data on the localisation of incomplete virus particles within VIBs.

Expression of BTV NS2 without other viral proteins in both insect and mammalian cells results in the formation of inclusion bodies that are indistinguishable from the VIBs found in virus-infected cells (Thomas et al. 1990). Furthermore, purified recombinant NS2 possesses ssRNA binding activity *in vitro*, and BTV RNAs are preferentially bound over nonspecific RNAs (Theron and Nel 1997; Lymperopoulos et al. 2003; Markotter et al. 2004). These observations support the suggestion that NS2 may have a role in the recruitment of RNA for replication (Thomas et al. 1990). While the amino terminus, but not the carboxy terminus, of NS2 is essential for ssRNA binding, neither the amino (up to 92 residues) nor carboxy terminus (including the last 130 amino acids) of the protein is required for oligomerisation (Zhao et al. 1994). These observations are important because how the ten dsRNA segments that make up the viral genome are selectively recruited and packaged into newly assembling virus particles is one of the most enduring questions in the field. It is commonly believed that each of the viral segments must possess some specific sequence or RNA structure, which is recognised by one or more virally encoded proteins to facilitate these processes. In particular, one recent study using recombinant purified NS2 in combination with BTV RNA species and a range of specific and

nonspecific ssRNA competitors has demonstrated that NS2 has high affinity for specific BTV RNA structures that are unique in each RNA segment (Lymperopoulos et al. 2003). Intriguingly, the sequences that represent the putative binding partners for NS2 are neither the octanucleotides and hexanucleotides conserved at the 5′ and 3′ termini of all BTV RNA segments nor the potential panhandle structures (formed by the partial complimentary sequences of the 5′ and 3′ termini). Instead the sequences that are predicted to be bound by NS2 on the basis of this initial study are distributed throughout the coding and noncoding regions of the different genome segments (Lymperopoulos et al. 2003). Chemical and enzymatic structure probing of regions bound preferentially by NS2 revealed that the NS2 bound regions of the BTV RNA transcripts folds into unique hairpin-loop secondary structures. The NS2–hairpin interaction was further confirmed by using hairpin mutants that had a decreased affinity for NS2. No other RNA binding protein of any other member of *Reoviridae* has been shown to have RNA structural specificity so far. However, rotavirus NSP3 appears to bind a linear sequence found at the 3′ end of all rotavirus RNA segments (Poncet et al. 1993) and rotavirus VP1 also binds to the 3′ end of rotavirus RNA (Patton 1996). The significance of these interactions for genome packaging is at this stage unclear, and it is worth pointing out that, other than BTV, there are several systems where viral proteins recognise packaging signals that are contained in hairpin structures with no apparent sequence homology (Bae et al. 2001; Beasley and Hu 2002). Although the RNA binding activity of NS2 explains how BTV vmRNA are selected from the pool of cellular messages for incorporation into assembling virus particles, how only a single copy of each genome segment is included in newly formed core particles remains to be elucidated. The two most likely scenarios at this stage are either that NS2 brings the viral RNAs into close proximity, and intersegment RNA interactions allow the formation of an RNA complex that is the basis of core assembly, or that different RNA subsets that already interact are bound by the same NS2/NS2 complex. The latter hypothesis is consistent with the observations that NS2 may form decameric complexes (Butan et al. 2004) and that each NS2 protein subunit may have several RNA binding domains (Fillmore et al. 2002). Establishing the precise order of events and the molecular mechanisms of virus assembly will be an exciting area of future research.

In addition to a role in RNA selection, NS2 VIBs also act as the nucleation site for a number of the viral structural proteins that form the core structure. Since the VIB structure could alternately act both as a virus assembly factory and as a trap that prevents egress of newly assembled core particles from exiting infected cells, it is necessary that their formation is a dynamic, reversible process. Recent data on the nature of different phosphorylation variants of NS2 have begun to reveal how the reversible assembly/disassembly

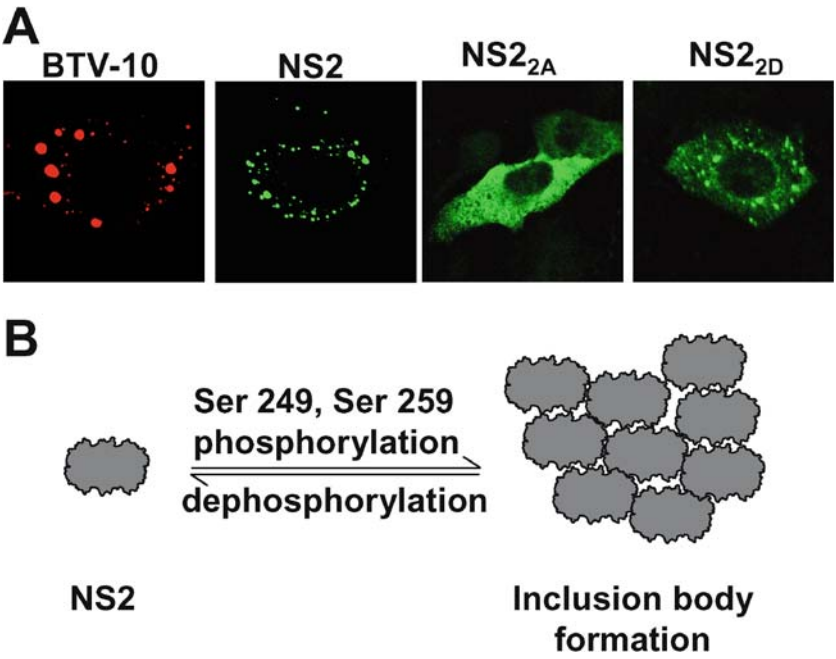

Fig. 3A, B Phosphorylation of BTV NS2 controls the assembly of virus inclusion bodies. **A** Confocal data showing the effect of mutating the phosphorylated serine residues of NS2 to alanine ($NS2_{2A}$) or aspartic acid ($NS2_{2D}$). **B** Cartoon showing how phosphorylation/ dephosphorylation of NS2 may control inclusion body integrity, and core release. (Modrof et al. 2005)

of bluetongue VIBs is achieved. NS2 is the only BTV encoded phosphoprotein and this phosphorylation is reproduced when NS2 is expressed in insect cells (Devaney et al. 1988; Thomas et al. 1990). Consistent with the role of phosphorylation to effect changes in protein–protein interactions of many other proteins, phosphorylation plays a key role in the formation of BTV VIBs. Mutagenesis of two normally phosphorylated serine residues in NS2 to alanine abrogates formation of distinct VIBs, while mutation of the same residues to aspartic acid, mimicking a constitutively phosphorylated state, results in the formation of normal inclusion body structures (Fig. 3; Modrof et al. 2005). Furthermore the expression of the nonphosphorylated mutant of NS2 was sufficient to disrupt the formation of VIBs in virus-infected cells, thus demonstrating the potential of the mutant to act in a dominant negative fashion and of the potential that phosphorylation could control BTV VIB stability (Modrof et al. 2005).

Recent data suggest that a first candidate cellular protein that interacts with NS2 is the protein kinase casein kinase II (CKII), which is able to phosphorylate NS2 *in vitro*. (Modrof et al. 2005). Additionally, both proteins co-localise intracellularly upon recombinant expression of NS2. Zetina et al. have reported that phosphorylation of the conserved motif (LS/SL)(D/E)(D/E)(D/E)X(D/E) could stabilise helix unfolding (Zetina 2001; Meggio and Pinna 2003). The sequence context of phosphorylated serine residue 249 ($_{248}$L**S**DDDDQ$_{254}$) in NS2 conforms to this conserved sequence except for the last residue. This indicates that phosphorylation of NS2 could be involved in stabilising its folding.

Intriguingly, it has recently been shown that rotavirus NSP5, which along with NSP2 is involved in the formation of inclusion bodies, is also phosphorylated at a CKII recognition site within its C-terminus (Eichwald et al. 2002). Phosphorylation and hyperphosphorylation of NSP5 result in widely variable migration patterns in SDS-PAGE (Afrikanova et al. 1996), but this is not the case for BTV NS2. Using NS2 expressed from different sources (mammalian cells, insect cells or bacteria) or NS2 alanine mutants which are not phosphorylated, the migration velocity of the proteins remained the same in denaturing PAGE. The question of whether rotavirus NSP5 phosphorylation is similar to BTV NS2 phosphorylation in affecting intracellular localisation is still unclear. A possible influence of NSP5 phosphorylation on localisation to VIBs had been suggested but is contradicted by recent investigations which showed that the deletion of the phosphorylated sites within the protein did not prevent NSP5 from localising with VIBs in infected cells (Poncet et al. 1997; Eichwald et al. 2002). For reoviruses, μNS and σNS have been suggested to be responsible for the formation of VIBs (Broering et al. 2002; Becker et al. 2003). However, these proteins are not known to be post-translationally modified.

As the formation of BTV VIBs, the centres for viral replication and early assembly, requires phosphorylated NS2, it is reasonable to assume that newly synthesised, unphosphorylated, NS2 might take on other functions prior to its association to VIBs. In this scenario, NS2 could bind the viral polymerase VP1 and other BTV core proteins in the cytoplasm and recruit these components to the VIBs on phosphorylation. In support of this hypothesis, we have recently obtained evidence that the phosphorylation of NS2 is important for VIB formation but not for the interaction with other viral proteins (Modrof et al. 2005). Following core assembly dephosphorylation of NS2 would allow disassembly of NS2 inclusions and the release of immature virus particles into the cytoplasm.

3.2 Assembly of the Viral Core Particle

While NS2 is intimately involved in the synthesis of infectious core particles, particularly at the level of RNA packaging, BTV structural proteins, with the possible exception of VP6, also have the inherent capacity to self-assemble into virus-like particles (VLPs) that lack the viral genome (French et al. 1990; French and Roy 1990). This has been exploited to explore atomic structures of the core for understanding of the protein–protein interactions that drive viral capsid assembly. The assembly of BTV capsids is especially intriguing as it requires a complex highly ordered series of protein–protein interactions and recent studies have focussed on the use of VLPs and core-like particles (CLPs), formed by expression of viral structural proteins in insect cells. Some of the key findings from a number of recent studies are summarised below.

3.2.1 VP3 Assembly and Minor Proteins

In the virion particle, 120 VP3 molecules are arranged as 60 dimers, each consisting of two different conformations of VP3 (A & B) on a T=2 icosahedral lattice (Grimes et al. 1998). A set of five VP3 AB dimers are arranged as decamers and 12 of these decamers are interconnected via the dimerisation domain in each molecule to form the final VP3 shell (Grimes et al. 1998). Such icosahedral organisation of the inner shell is shared by all members of *Reoviridae* and other viruses with segmented dsRNA genomes (Hewat et al. 1992a; Prasad et al. 1992, 1996; Grimes et al. 1998; Reinisch et al. 2000), emphasising the importance of their essential roles in the assembly process of these viruses. Moreover, the primary amino acid sequences of VP3 (100 kDa) across 24 BTV serotypes as well as other related orbiviruses (EHDV and AHSV) are highly conserved, highlighting the structural constraints that may govern virus assembly (Iwata et al. 1992, 1995; Roy 1996).

A key question in VP3 assembly is whether the decamer present in the final assembled particle is an identifiable intermediate in the assembly process or only arises upon assembly. To gauge this, a recent study deleted the dimerisation domain of VP3 and showed that subcore formation was abolished (Kar et al. 2004). The deletion of this domain, however, did not perturb decamer or dimer formation. Decamers were highly stable and due to their hydrophobic nature a higher order of decamers were particularly evident from the cryo-EM and dynamic light scattering experiments (Kar et al. 2004). These data suggest that decamers are probably the first stable assembly intermediates of the VP3 layer and subsequent core assembly and those decamer–decamer interactions via the dimerisation domain drive the assembly of the viral subcore.

The atomic structure of the BTV core shows that VP3 is associated with genomic dsRNA and indicates the possible locations of the internal proteins that form the transcription complex of the virion at the vertices of the five-fold axes of the decamers (Grimes et al. 1998; Gouet et al. 1999). However, the exact contribution of each of the internal proteins to the densities of the transcription complex was not resolved in the X-ray diffraction studies. High-resolution cryo-EM analysis has been used successfully to reveal the shape of the complex formed by two of these proteins, the polymerase VP1 and the capping enzyme VP4. This has been possible because of the use of CLPs consisting of VP1, VP3, VP4 and VP7 but not the genomic RNAs (Nason et al. 2004). Co-expression studies indicated that both VP1 and VP4 proteins are independently associated with the VP3 layer, although recent data suggest that VP4 protein has more direct contact with VP3 (Le Blois et al. 1991; Loudon and Roy 1992; Nason et al. 2004). In addition, VP1 and VP4 are very tightly associated with each other and form a stable complex that can be visualised by cryo-EM analysis. This complex directly interacts with the VP3 decamer in solution, supporting the cryo-EM analyses (Kar et al. 2004). However, BTV RNA was not able to bind VP3 decamers under conditions where the intact VP3 bound RNA very efficiently. The interaction of VP1 and VP4 in the absence of the BTV genome or VP3 and the association of VP1 and VP4 with VP3 decamers suggests that assembly of the BTV core initiates with the complex formed by these two enzymes which simultaneously associate with the VP3 decamers. VP3 decamers as assembly intermediates are most likely involved in the recruitment of the polymerase complex prior to completion of the assembly of the VP3 subcore. The viral genome, by contrast, wraps around the VP1–VP4 complex while the subcores are assembling.

Unlike VP1 and VP4, it has not been possible to confirm the location of VP6 in the core, although it is likely that VP6 is also located within the five-fold axes of the VP3 layer directly beneath the decamer together with VP1 and VP4. However, VP6 forms defined hexamers in the presence of BTV transcripts and assembles into distinct ring-like structures that could be isolated by glycerol gradient centrifugation. At present, it is not known if such structures are indeed present within the core and incorporated either together with VP1 and VP4 into the VP3 decamer intermediate stage or independently during the assembly of the 12 VP3 decamers.

3.2.2 VP7 Assembly

The mismatch between the number of subunits in the VP3 and VP7 layers poses an interesting problem as to how these layers reconcile to form an

intact icosahedral structure. In the absence of VP3, VP7 forms trimers but these trimers do not assemble as icosahedral particles. The construction of the T=13 icosahedral shell requires polymorphism in the association of the VP7 subunits, each of which has two domains that contribute to trimer formation. The lower helical domain controls both the formation of the VP7 lattice and its interaction with the scaffolding layer of VP3. Structural and comparative sequence information have guided investigation of how such a complex structure is achieved during virus assembly and what residues are required to form a stable capsid. Extensive site-directed mutagenesis in combination of various assembly assay systems have given insight into the order of the assembly pathway of VP7 and stable core formation that subsequently serve as a foundation for the deposition of the outer capsid.

Since the lower domains are in direct contact with the VP3 layer, and the interactions between the lower domains within the trimers are intensive, a series of VP7 mutations were generated focusing on the lower domain residues that appeared to be involved in intramolecular (within the VP7 subunit) and intermolecular (between the VP7 subunits) interactions. Another set of mutations was created to perturb the trimer–trimer interactions. The rationale behind these experiments was that since VP7 molecules oligomerise into trimers even when expressed in the absence of VP3, it is possible that attachment of preformed trimers onto the VP3 subcore and subsequent formation of the VP7 layer would be directed by side-to-side interaction of adjacent trimers. Interactions between neighbouring trimers at the two-fold axis appear to be through a thin band around the lower domains. A series of single and multiple substitution mutants of VP7 were created targeting these regions to examine their involvement in assembly (Limn et al. 2000). Another series of single or multiple site-specific substitution mutations have been introduced into the regions of the flat under-surface of the VP7 trimers that adhere closely to the VP3 surface in order to examine the VP7 and VP3 assembly (Limn and Roy 2003). The effects of these mutations on VP7 solubility, ability to trimerise and formation of CLPs in the presence of the VP3 scaffold were investigated.

In brief, the detailed analysis of an extensive range of targeted VP7 mutations not only precisely identified the critical residues responsible for formation of the VP7 layer, but also revealed that core assembly depends on trimer formation. Furthermore, the precise shape of the trimers drives the formation of the tight lattice of 260 VP7 trimers on the core surface. In terms of the overall assembly pathway of the BTV core, combination of cryo-EM and X-ray structures have revealed that of the 13 unique contacts made between the VP3 and VP7 shells in the atomic structure, the contact that aligns the VP7 trimer axis with the icosahedral three-fold axis of the VP3 layer is the strongest. This suggests that these trimers may nucleate the assembly of the

VP7 lattice on the VP3 subcore once the first trimer is anchored. It has been postulated that preformed hexamers of VP7 may propagate around the initial VP7 trimer forming a sheet that loosely wraps the VP3 layer (Grimes et al. 1998). However, the data obtained from mutagenesis studies did not support a gradient of trimer association from a single nucleation site, as mutations that destabilise the CLP particle still allow assembly of some VP7 lattice on the VP3 shell. More likely is an alternate model for assembly where multiple sheets of VP7 form around different nucleation sites. A likely pathway of core assembly is therefore that a number of strong VP7 trimer-VP3 contacts act as multiple preferred initiation sites and a second set of weaker interactions then fill the gaps to complete the outer layer of the core. There is a clear sequential order of trimer attachment on VP3 scaffold. The T trimers (of the P, Q, R, S, T trimers) which are at the three-fold axis of the icosahedron act as nucleation, while P trimers that are furthest from the three-fold axis and closer to the five-fold axis, are the last to attach (Nason et al. 2004). The distinction between those VP7 trimers that first occupy the subcore and those that follow is necessarily subtle as it must be sufficient to allow variation in packing yet not prevent the overall biological purpose of virus assembly.

3.3 Assembly of Outer Capsid

Following the assembly of the core particle, the next logical step in virus assembly is the addition of the viral outer capsid, consisting of the proteins VP2 and VP5. While at a structural level, recent cryo-EM experiments have aided our understanding of how the outer capsid proteins interact with the outer VP7 layer of the core, our understanding of where in the cell these proteins are added to the core is limited. Since the addition of VP2 and VP5 abolishes the transcription activity of the particle, addition of the outer capsid is likely to be a highly regulated process to prevent premature shut-off of virus transcription. Within BTV-infected cells, virus particles are found associated with the vimentin intermediate filaments (Eaton et al. 1987). Furthermore, the presence of both VP2 and VP5 was found to be necessary to direct VLP to the cytoskeleton of insect cells (Hyatt et al. 1993). However, our own recent studies have suggested that VP2 alone associates with vimentin intermediate filaments and disruption of these structures prevents the normal release of virus particles from cells. Given the importance of these structures for the assembly and egress of other viruses (Garcia-Barreno et al. 1988; Murti et al. 1988; Ferreira et al. 1994; Karczewski and Strebel 1996; Arcangeletti et al. 1997; Nedellec et al. 1998; Cordo and Candurra 2003), it may be that intermediate filaments are used to control the addition of the VP2 layer to the assembled core.

The assembly of VP2 and/or VP5 with core is easily mimicked using the baculovirus expression system (French et al. 1990; Le Blois et al. 1991; Loudon et al. 1991; Liu et al. 1992). Both proteins can be added to the core and assemble onto that structure independently of each other, implying that the two proteins are directly attached to the core. This has recently been confirmed by high-resolution cryo-EM studies that have revealed the separate regions of the VP7 trimers that are involved in attachment of VP2 and VP5 (Nason et al. 2004). The structural organisation of the outer layer represents a drastic mismatch with the underlying VP7 layer. The VP7 trimers with a triangular top portion serve as the platform for the deposition of the VP2 and VP5. Each triskelion motif (VP2 trimers) essentially interacts with four VP7 trimers by its underside in four places. The base of the triskelion interacts with the Q type VP7, whereas the propeller-like arms make three other connections with the P, R, and S VP7 trimers. By docking the X-ray structure of the VP7 trimer into the virion reconstruction it was possible to identify the VP7 residues that are in contact with VP2. The base of the VP2 trimer interacts exclusively with the upper flattish surface of the VP7 trimer, which includes amino acid residues 141–143, 164–166, 195–205, and 238–241 of the VP7(Nason et al. 2004). All the VP7 molecules of the core are covered by the connections that are made with the tips of the propeller except the VP7 at the icosahedral three-fold axis (T type). The top of the VP7 trimer at this position is thus clearly exposed to the exterior.

The globular densities (VP5 trimers) on the other hand, sit right above the type II and type III channels, between the VP7 trimers, making contacts with the sides of VP7 trimer which face these channels. Thus, the channels are filled with triangle-shaped densities that are connected to the VP5 trimers. The inner triangle-shaped density interacts mainly with the lower portion of the β-barrel domain of VP7 and includes residues 168–173, 210–215, and 226–234 (Nason et al. 2004).

Overall, the principal protein–protein contacts of the two outer capsid proteins of the BTV appear to be with the outer layer of the core rather than with each other. This may be related to the sequential attachment and endosomal penetration steps mediated by each protein respectively during virus entry into uninfected cells.

4
Egress of Progeny Virions

Once virions are newly assembled, they must be trafficked to the plasma membrane for release. The efficiency of BTV release varies between host cell types, with insect cells allowing a nonlytic release of virus while the

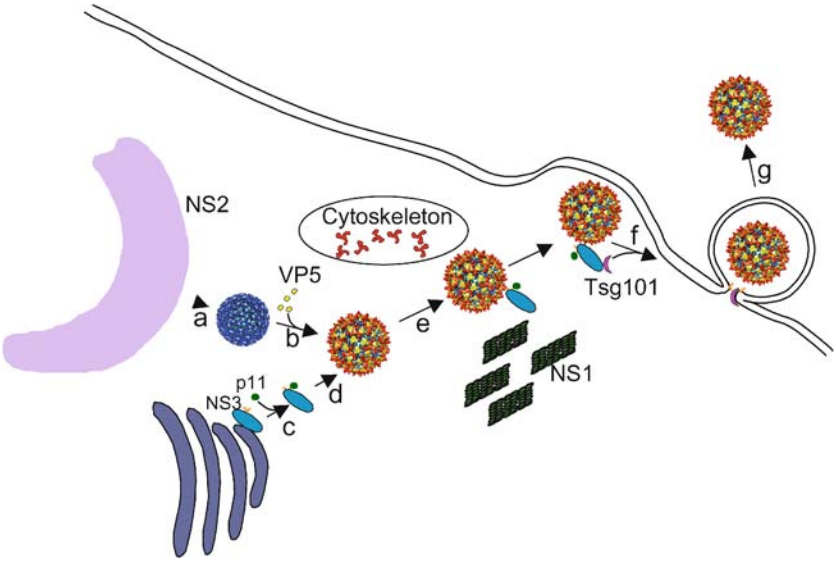

Fig. 4 Diagram summarising key interactions in the assembly and release of BTV: cores are released from NS2 inclusion bodies (*a*) and associate with outer-capsid proteins VP5 and VP2 (*b*). BTV NS3 associates with the intracellular trafficking protein p11 (*c*) and forms a bridge between this protein and newly assembled virus particles by a secondary interaction with VP2 (*d*). This leads to trafficking of the virus particle to the cell membrane (*e*) where interaction between the PSAP motif in NS3 and the cellular release factor Tsg101 results in the pinching off of vesicles containing virus particles (*f*) followed by release of mature virion particles from these vesicles (*g*)

majority of virus in mammalian cells remain cell-associated (Hyatt et al. 1989). However, even in mammalian cells that show substantial cytopathic effect as a result of BTV infection, the titre of virus in the culture supernatant increases significantly before the onset of dramatic CPE. Thus it is clear that there are defined mechanisms that traffic newly formed virus particles out of infected cells. Recent evidence has implicated the viral nonstructural proteins NS1 and NS3 in this process and has revealed intriguing parallels between the egress of nonenveloped BTV and enveloped viruses (Fig. 4) (Beaton et al. 2002; Owens and Roy 2004).

4.1 The Role of NS3 in the Intracellular Trafficking of Virus Particles

The only membrane proteins that are encoded by BTV are NS3 (229 aa) and its shorter form NS3A (216 aa), which lacks the N-terminal 13 amino acids of

NS3 (Van Dijk and Huismans 1988). Both proteins have been found associated with smooth-surfaced, intracellular vesicles (Hyatt et al. 1993) but do not form part of the stable structure of the mature virus. NS3/NS3A proteins comprise a long N-terminal and a shorter C-terminal cytoplasmic domain, which are connected by two transmembrane domains and a short extracellular domain (Wu et al. 1992; Bansal et al. 1998; Beaton et al. 2002). A single glycosylation site is present in the extracellular domain of BTV NS3 but is missing in NS3 of African horse sickness virus (AHSV), a closely related orbivirus, and therefore does not seem to be essential for the function of the protein (van Staden and Huismans 1991; Bansal et al. 1998). The NS3/NS3A proteins accumulate to only very low levels in BTV-infected mammalian cells, but in invertebrate cells the expression level of these proteins is high (French et al. 1989; Guirakhoo et al. 1995). The correlation between high level of NS3/NS3A expression and nonlytic virus release suggests a significant functional role for NS3 in virus egress from invertebrate cells. When NS3/NS3A is expressed using recombinant baculoviruses, it facilitates the release of baculovirus-expressed virus-like particles, VLPs (acting as surrogates for authentic virions) from host cells and NS3 protein is localised at the site of the membrane from which VLPs are released (Hyatt et al. 1993). This suggests that the integration of NS3/NS3A into the plasma membrane may release mature virions, a function that is similar to that described for NSP4, a nonstructural glycoprotein of rotaviruses, which interacts with viral double-layered particles to facilitate their transport across the rough endoplasmic reticulum of infected cells (Meyer 1989). NS3 of BTV and AHSV exhibit cytoxicity when expressed singly in mammalian or insect cells (French et al. 1989; van Staden et al. 1995). The cytoxicity of NS3 requires membrane association of the protein and depends on the presence of the first transmembrane domain, leading to the suggestion that NS3 might function as a viroporin, facilitating virus release by inducing membrane permeabilisation (Han and Harty 2004). Recent study showed that NS3 can indeed act as viroporin, causing permeabilisation of host cell membranes (Han and Harty 2004). It is possible that this permeabilisation activity facilitates local disruption of the plasma membrane allowing virus particles to be extruded through a membrane pore without acquiring a lipid envelope. Whether this process requires any of the cellular proteins that participate in the formation of an enveloped viral particle merits further investigation.

The first 13 amino acids of NS3 that are absent in NS3A have the potential to form an amphipathic helix. This cytoplasmic region of NS3 also specifically interacts with the calpactin light chain (p11) of cellular annexin II complex (Beaton et al. 2002). The complex has been implicated in membrane-related events along the endocytic and regulated secretory pathways including the trafficking of vesicles (for reviews see Creutz 1992; Raynal and Pollard 1994).

The interaction between NS3 with p11 is highly specific. Moreover, an NS3 peptide (a mimic of the sequences of the p11 binding domain) has inhibitory effects on virus release of progeny virions from BTV-infected insect vector cells (Beaton et al. 2002). Even though the exact physiological role of this interaction is still unknown, it is likely that interaction of p11 with NS3 may help direct NS3 to sites of active cellular exocytosis, or it could be part of an active extrusion process. Furthermore, there are some indications that cytoskeletal material was seen at sites of BTV egress (Hyatt et al. 1991), which may be annexin II being drawn through the membrane during the extrusion process, as it is still associated with NS3. The significance of this interaction to BTV egress becomes more apparent in the light of the observation that the other cytoplasmic domain of the protein, situated at the C-terminal end, interacts specifically with the BTV outer-capsid protein VP2.

In addition to its interaction with the p11 component of the annexin II complex, NS3 is also capable of interaction with Tsg101 (Wirblich et al. 2005), a cellular protein implicated in the intracellular trafficking and release of a number of enveloped viruses (Garrus et al. 2001; Freed 2004; Morita and Sundquist 2004). Tsg101 specifically interacts with a PTAP motif that is present in the late domain of the retroviral Gag protein. Other motifs present include the YPDL motif that interacts with the protein Alix, which functions downstream of Tsg101, and the PPxY motif, which plays a role in recruiting host ubiquitin ligases. The PTAP and PPxY motif have also been identified in proteins of other enveloped viruses, such as VP40 of Ebola virus, the matrix protein of VSV and the Z protein of Lassa virus, where they exhibit an equivalent function as the late domain motifs of retroviruses (Craven et al. 1999; Harty et al. 1999, 2000, 2001; Licata et al. 2003; Perez et al. 2003; Timmins et al. 2003; Yasuda et al. 2003). Interestingly, similar motifs are also present within the NS3 of BTV and certain other orbiviruses (Wirblich et al. 2005). Recent findings showed that both NS3 and NS3A of BTV and AHSV bind with similar affinity *in vitro* to human Tsg101 and also its orthologue from *Drosophila melanogaster*. This interaction is mediated by the conserved PSAP motif in NS3 of BTV and ASAP in the NS3 of AHSV. Mutation of the PSAP motif abolished binding of NS3 to Tsg101, but had little effect on binding to p11. The interaction of NS3 and Tsg101 also plays a role in orbivirus release, as knockdown of Tsg101 with siRNA inhibits release of BTV and AHSV from mammalian cells (Wirblich et al. 2005).

Like most other viral proteins which recruit Tsg101, NS3 also binds NEDD4-like ubiquitin ligases *in vitro* via a resident PPXY late domain motif (Yasuda et al. 2003; Blot et al. 2004; Heidecker et al. 2004; Sakurai et al. 2004). However, the late domain motifs in NS3 do not function as effectively as the late domains of other enveloped viruses. This appears to be mainly due to the presence of

a unique arginine at position 3 of the PPXY motif in NS3. The low activity of the NS3 late domain motifs can be reversed by converting the PPRY motif of NS3 into a more universal PPPY, rendering NS3 as effective as Gag in facilitating retroviral VLP release as the late domains of enveloped viruses. It was also apparent that PSAP motif of NS3 is as efficient as the PTAP motif in retrovirus VLP release (Wirblich et al. 2005).

Of all the viruses utilising conventional late domains that have been examined, BTV is the only one that encodes arginine at position three of the PPxY motif (Wirblich et al.y 2005). The reason for this may be that orbiviruses replicate in insects, whereas the other viruses do not. Hence, the arginine could be an adaptation to growth in insect cells. NS3 is also unusual among viral proteins containing a late domain since it also functions as a viroporin. While both activities seem to facilitate virus release, the relative contribution of the two activities could differ between different orbiviruses. This conclusion is consistent with our observation that cells infected with AHSV displayed a much stronger cytopathic effect at early times after infection than cells infected with BTV, which could reflect a higher intrinsic cytotoxic activity of AHSV NS3 or a higher expression level of NS3 in cells infected with AHSV (van Staden et al. 1995). In any case, particle release due to cell lysis caused by viroporin activity of NS3 presumably does not require the function of Tsg101.

Thus, orbivirus NS3 recruits the cellular protein Tsg101 to facilitate virus release from mammalian cells and presumably insect cells as well. The ability to usurp the vacuolar protein sorting pathway is likely to be more important in insect hosts as orbiviruses establish persistent infections in insect cells without causing significant cytopathic effect. While full clarification of this issue will have to await the availability of a reverse genetics system for BTV, it should be possible to identify insect proteins that interact with NS3 and to shed more light on the question of whether NS3 is better adapted to engage insect proteins so facilitating improved virus release.

4.2
The BTV Tubules in Infected Cells and NS1 Protein

One of the most striking intracellular morphologic features during BTV and other orbivirus infection is the formation of abundant tubular structures within the cytoplasm. Orbivirus tubules are biochemically and morphologically distinct from the microtubules and neurofilaments of normal cells. BTV tubules are formed by the 64-kDa protein NS1 (Huismans and Els 1979; Urakawa and Roy 1988), which is synthesised in large amounts, up to 25% of the virus-specified proteins, in infected cells. Following synthesis, the protein rapidly oligomerises into a high-molecular-weight tubular structure formed

by helically coiled ribbons of NS1 dimers with a diameter of 52 nm and lengths of up to 1,000 nm that sediment as 300–500S structures (Urakawa and Roy 1988). Early and abundant synthesis of NS1 and tubules suggests their involvement in virus replication and/or virus translocation.

Indeed our recent data suggests the role of this protein in virus replication. Intracellular expression of a single-chain antibody to the viral NS1 protein (scFv-αNS1) has demonstrated that high levels of NS1 in BTV-infected cells are critical to the morphology and trafficking of virus particles (Owens and Roy 2004). Four major changes were apparent: first, there was a tremendous reduction in virus-induced cytopathic effect (CPE); second, there was a more than tenfold increase in the amount of virus released into the culture medium; third, there was a shift from lytic release of virus to budding from the plasma membrane (Fig. 5); and forth, NS1 tubule formation was completely inhibited by scFv-αNS1 expression. Each of these changes, except for the lack of tubule formation, is reminiscent of what occurs during BTV infection of insect cells in culture. Based on these findings, we propose that the NS1 protein is a major determinant of pathogenesis in the vertebrate host, and that its mechanism of action is the augmentation of virus–cell association (but not transport of virus to the cell surface), which ultimately leads to lysis of the infected cell. It is possible that NS1 is somehow involved in virus trafficking. Differential virus release in different cells suggests the involvement of host proteins. Indeed a yeast two-hybrid analysis has identified NS1 interaction with the cellular protein SUMO-1 (R. Noad and P. Roy, unpublished data). This protein is conjugated to cellular targets as a post-translational modification involved in the intracellular trafficking. In addition, it appears that sumoylation of cellular

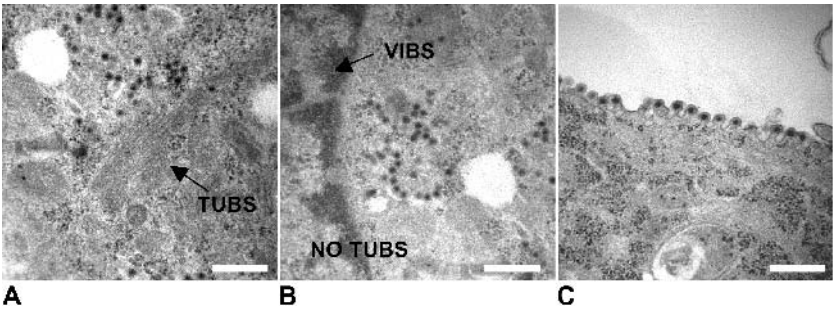

Fig. 5A–C Ultrastructural analysis of BTV Infected cells. **A** Normal BTV infection in BSR cells showing the presence of Tubules (*TUBS*). **B, C** Negatively stained cell section infected by BTV in the presence of anti-NS1 antibody. Note in **C**, virus particles are exiting cells by budding from the plasma membrane. *Bar*, 500 nm. (Owens and Roy 2004)

proteins is highly enhanced in BTV-infected cells. Upregulation of SUMO-1 has recently been shown to regulate dynamin-dependent protein trafficking within the cell (Mishra et al. 2004). Thus, it could be speculated that the role of NS1 is to control the trafficking of immature or mature virus particles in infected cells through the interaction with cellular proteins such as SUMO-1.

5 Concluding Remarks

Our understanding of the assembly of BTV within virus-infected cells continues to advance rapidly. The roles of the VP2 and VP5 proteins in virus entry have been elucidated and, surprisingly, reveal striking similarity to the entry mechanisms of enveloped viruses. Based on structure-informed mutagenesis of the major proteins of the core, we now have a clearer picture of the order in which VP7 trimers are assembled onto the underlying VP3 layer, which enhances the static model provided by the core structure. In addition, there remain a number of significant unanswered questions regarding the assembly of mature virus particles and the egress of virus from infected cells. In particular, the mechanism by which the virus manages to efficiently package its genome into the assembling particle and the route taken by newly assembled particles as they exit infected cells remain poorly understood. Recent studies have provided tantalising glimpses of the solution to these problems and have indicated that, as with virus disassembly during entry into the cell, interactions involved in these mechanisms are dynamic and complex.

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