Bluetongue Virus Outer Capsid Protein VP5 Interacts with Membrane Lipid Rafts via a SNARE Domain[†]

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Bluetongue virus (BTV) is a nonenveloped double-stranded RNA virus belonging to the family *Reoviridae*. The two outer capsid proteins, VP2 and VP5, are responsible for virus entry. However, little is known about the roles of these two proteins, particularly VP5, in virus trafficking and assembly. In this study, we used density gradient fractionation and methyl beta cyclodextrin, a cholesterol-sequestering drug, to demonstrate not only that VP5 copurifies with lipid raft domains in both transfected and infected cells, but also that raft domain integrity is required for BTV assembly. Previously, we showed that BTV nonstructural protein 3 (NS3) interacts with VP2 and also with cellular exocytosis and ESCRT pathway proteins, indicating its involvement in virus egress (A. R. Beaton, J. Rodriguez, Y. K. Reddy, and P. Roy, *Proc. Natl. Acad. Sci. USA* 99:13154–13159, 2002; C. Wirblich, B. Bhattacharya, and P. Roy *J. Virol.* 80:460–473, 2006). Here, we show by pull-down and confocal analysis that NS3 also interacts with VP5. Further, a conserved membrane-docking domain similar to the motif in synaptotagmin, a protein belonging to the SNARE (soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor) family was identified in the VP5 sequence. By site-directed mutagenesis, followed by flotation and confocal analyses, we demonstrated that raft association of VP5 depends on this domain. Together, these results indicate that VP5 possesses an autonomous signal for its membrane targeting and that the interaction of VP5 with membrane-associated NS3 might play an important role in virus assembly.

Bluetongue, a vector-borne disease of ruminants, is caused by a nonenveloped virus, bluetongue virus (BTV), a member of the genus *Orbivirus* within the family *Reoviridae*. Until the beginning of the last decade BTV was endemic in many tropical and subtropical areas of the world, but since 1998, it has significantly extended its range into southern Europe and, more recently, northern Europe (64, 78, 80, 95). BTV is one of the most economically important animal pathogens and can cause up to 75% mortality in sheep (28). Due to its serious impact on animal health, bluetongue is listed as a “notifiable disease” by the World Organization for Animal Health.

BTV particles are architecturally complex structures, composed of seven discrete proteins organized into two concentric capsids that encapsidate the genome of 10 double-stranded RNA (dsRNA) segments (79). The outer capsid is composed of two major structural proteins, VP2 and VP5, both of which are nonglycosylated and involved in cell attachment and virus penetration during the initial stages of infection (16, 24, 33, 34, 42, 43, 46, 62, 76, 89). After entry into cells, the virus is uncoated (VP2 and VP5 are lost), yielding the inner capsid (termed the “core”), composed of two major proteins (VP7 and VP3) and three minor proteins (VP2, VP4, and VP6), in addition to the dsRNA genome. The released core particles are the end point in virus disassembly, and they initiate transcription and replication of the viral genome. As in other members of the family, the assembly of newly synthesized inner

capsid, or “core,” components takes place within the virus-specific inclusion bodies (VIBs) in the cytoplasm of infected cells (40, 51, 58, 88). However, the assembly of the two outer capsid components, VP2 and VP5, onto the BTV core does not occur within these VIB structures (50, 65). Preliminary data suggest that the assembly of the outer capsid onto the cores occurs outside the VIBs in the cytosol, possibly prior to the trafficking of the mature virus particle to the plasma membrane for egress. Alternatively, it may be that the newly assembled core particles are transported to the plasma membrane for subsequent assembly of complete viral particles and egress. BTV also encodes three nonstructural proteins, one of which, NS3, the only virus-encoded glycosylated protein, has been observed attached to the plasma membranes of cells and has been demonstrated to be involved in virus release (48, 92). Hence, it is believed that cellular membranes may play an important part in BTV trafficking and egress.

Raft domains in cellular membranes have been implicated as relay stations in intracellular signaling and transport (1, 30, 35, 86). To date, more than 200 proteins have been found associated with the membrane rafts. They include proteins such as caveolin, flotillin, ganglioside M1 (G_{M1}), glycerophosphatidylinositol-anchored proteins, and members of the SNARE (soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor) superfamily (35, 81, 85). It has also been suggested that the cholesterol-enriched rafts play an active role in host-virus interactions. Raft domains are known to localize the structural proteins of many enveloped viruses (3, 61, 68, 84) and have been shown to play significant roles in the trafficking of enveloped viruses to the plasma membrane for assembly and/or egress via membrane fusion (10, 11, 31, 68, 84, 90). Rafts have also been implicated in intracellular trafficking

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and the assembly of rotavirus, a nonenveloped virus, although one of its major capsid proteins is glycosylated (14, 17, 18, 20, 21, 83). Recently, it has been hypothesized that the flotillin binding motif present in VP4, the outermost capsid protein of rotavirus, might be responsible for its interaction with rafts (14).

Thus, the interplay between viral and cellular proteins is emerging as an important event during virus assembly and egress from infected cells. In BTV-infected cells, virus particles have been found attached to vimentin intermediate filaments (27) and underneath the cell membrane, associated with the actin-rich cortical layer (26). Our recent investigation has also revealed that the outermost capsid protein, VP2, interacts with vimentin and that this association plays an important role in virus release from infected cells (5).

In this report, we have extended our studies on VP5, the second outer capsid protein of BTV, and demonstrate that VP5 interacts with raft domains. Additionally, the nonstructural protein NS3 also interacts with these domains in virus-infected cells. Moreover, we have identified a functional and conserved WHXL motif in the VP5 sequence that is identical to the plasma membrane docking motif present in cellular synaptotagmin (Sy1) protein. This is the first time such a motif has been reported in a nonenveloped-virus protein. Furthermore, the mutagenesis of this motif prevented the interaction of VP5 with raft domains, supporting its functional importance. Moreover, we show that disruption of the raft domains by cholesterol depletion also affects BTV replication in mammalian cells.

MATERIALS AND METHODS

Reagents. OptiPrep and Vectashield were obtained from Nycomed (Oslo, Norway) and Vector Laboratories (Burlingame, CA), respectively. Methyl beta cyclodextrin (m β cdx), sucrose, 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium, Triton X-100, and a cocktail of protease inhibitors were all acquired from Sigma (St. Louis, MO).

Buffers. TNET buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) and a cocktail of protease inhibitors were used to lyse cells. The sucrose and Optiprep gradients were made in TNET buffer without Triton X-100. In the pull-down experiments described below, NP-40 buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, and 1% NP-40) was used.

Antibodies. Antibodies against VP5, NS2, and NS3 were used as previously described (36, 43). Rabbit polyclonal anti-BTV serotype 17 (BTV-17) used in this study was generated previously by our laboratory. Rabbit polyclonal anti-caveolin was obtained from Abcam (Cambridge, United Kingdom). Mouse anti-flotillin 2 was acquired from BD Biosciences (New Jersey). The expression of cellular β -tubulin and actin was monitored by specific monoclonal antibodies obtained from Sigma (St. Louis, MO). Vimentin was detected with monoclonal antibody from DakoCytomation (Glosrup, Denmark). Cellular endoplasmic reticulum (ER) was labeled with rabbit polyclonal antibody raised against calnexin 2 from Sigma (St. Louis, MO). Goat anti-mouse, anti-guinea pig, and anti-rabbit immunoglobulin G (IgG) coupled to alkaline phosphatase were also acquired from Sigma (St. Louis, MO). Alexa Fluor 568-labeled phalloidin, Alexa 488-conjugated goat anti-guinea pig, and Hoechst were acquired from Molecular Probes. Tetramethyl rhodamine isothiocyanate-labeled goat anti-mouse and -rabbit were acquired from Sigma (St. Louis, MO). With the exception of anti-calnexin 2 antibody which was used at a 1:200 dilution, all primary antibodies for confocal microscopy were used at a dilution of 1:100. Alexa 488-conjugated goat anti-guinea pig was diluted to a final concentration of 10 μ g, whereas tetramethyl rhodamine isothiocyanate-labeled goat anti-mouse and anti-rabbit were used in dilutions of 1:64 and 1:400, respectively. For Western blot analysis in fractionation studies, anti-VP5, -NS2, -NS3, -caveolin, and -flotillin 2 were used at 1:1,000 dilution, while anti- β -tubulin, anti-actin, and anti-BTV-17 were used at 1:500 dilution. A 1:2,000 dilution of antibody was used to develop calnexin 2 in the immunoblots.

Cells and viruses. HeLa (human cervical epithelial) and BSR (baby hamster kidney) cells were incubated at 37°C in Dulbecco's modified Eagle's medium (Gibco, BRL) containing 10% fetal calf serum (FCS), 100 U penicillin/ml, and 100 μ g streptomycin/ml (Sigma-Aldrich Chemical Co., St. Louis, MO).

The BTV strains used were cell culture-adapted BTV-1 SA and BTV-17 propagated in BSR and HeLa cells, respectively. The viral titers were determined by plaque assay in BSR cells. For time course studies of viral infection, cell monolayers were washed with FCS-free growth medium and infected with BTV-1 and BTV-17 separately at an MOI of 1. Virus adsorptions were carried out for 1 h at 4°C, followed by incubation at 37°C in growth medium supplemented with 2% FCS for 2, 4, 8, 12, 16, 20, and 24 h.

Spodoptera frugiperda (Sf9) cells were maintained in shaking flasks using Insect Express serum-free medium (Lonza). In order to grow Sf21 cells, the medium was supplemented with 10% FCS. For pull-down experiments, insect cells were seeded in six-well plates and infected at an MOI of 2 to 5 with recombinant baculoviruses expressing NS3 (36) and VP5 (37) of BTV-10. At 30 h postinfection (p.i.), the cells were washed twice with cold phosphate-buffered saline (PBS) and used in pull-down assays as described below.

Plasmid. The full-length M5 and S10 genes from BTV-10 were amplified by PCR and ligated downstream of the Pol II polymerase promoter in the pCAG-GS (Common Access to Biological Resources and Information Consortium) vector. The QuickChange system (Stratagene) was used to generate amino acid substitution mutant M1, according to the manufacturer's protocol.

To express full-length VP5 with the M1 mutation at amino acids 414 to 417, the transfer vector pAcYM10.5 (62) expressing wild-type VP5 was mutated using the same primers and protocol that were used to introduce the mutation in the Pol II polymerase-containing plasmid. The orientations of all plasmid constructs were examined by restriction enzyme analysis, and the authenticity of each construct was confirmed by DNA sequencing (MWG Biotech).

Isolation of recombinant baculoviruses expressing mutant VP5 protein. Recombinant baculoviruses expressing the M1 mutant of VP5 were produced using standard baculovirus recombination procedures as described previously (54). The viruses were plaque purified and propagated in Sf21 cells as described elsewhere (54).

Transfection. HeLa cells were seeded in either 15-cm tissue culture plates or six-well plates and transfected when 70% confluent with Lipofectamine-Plus (Invitrogen) according to the manufacturer's recommendations. After further incubation for 30 h at 37°C, they were processed for membrane flotation or confocal microscopy assays as described below.

Extraction of cellular cholesterol. Increasing concentrations of m β cdx (5 to 20 mM) were titrated against HeLa cells for 1 h at 37°C. The trypan blue exclusion method was used to count the dead and total cells in three readings. The average percentage of dead cells was plotted against the amount of m β cdx in the medium. The standard error was also calculated (Sigma Plot 2000; Systat Software Inc.). Subsequently, the cells were infected or transfected, washed with PBS, and treated with 10 mM m β cdx for 1 h (86).

Raft membrane preparation. Raft membranes were prepared as described previously (83). Briefly, transfected or BTV-infected HeLa or BSR cells grown in 15-cm tissue culture plates were washed twice with cold PBS, scraped into 2 ml of cold TNET buffer, and incubated at 4°C for 30 min. Subsequently, the lysate was passed 10 times through a 23-gauge needle, adjusted to either 40% sucrose or OptiPrep, and placed underneath successive layers of 35% and 5% sucrose or 30% and 5% OptiPrep solutions, respectively. The discontinuous gradients were centrifuged in a Beckman SW41 rotor either at 180,000 $\times$ g for 18 h at 4°C (sucrose) or at 100,000 $\times$ g for at least 4 h at 4°C (OptiPrep). Fractions containing 500 μ l were collected from the top of the tube.

Immunoprecipitation experiments. Aliquots of cellular lysates from Sf9-infected cells were lysed in either TNET or NP-40 buffer and clarified by centrifugation for 5 min at 16,000 $\times$ g. The soluble extracts were incubated on a roller with anti-NS3 or -VP5 antibody (1:100) for 4 h at 4°C, followed by IgG-Sepharose beads at 4°C for 1 h. The beads were removed by spinning them at 16,000 $\times$ g for 1 min and were further washed five times with lysis buffer containing 0.1% Triton X-100. After the final wash, equal amounts of PBS and Laemmli sample buffer were added to the beads and heated for 15 min at 100°C. The samples were then centrifuged briefly, and equal aliquots of supernatant were run in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.

Analysis of the oligomeric nature of VP5. Sf9 cell monolayers were infected with recombinant baculoviruses (pAcNPV.VP5 or pAcNPV.VP5M1) at a multiplicity of infection (MOI) of 5. Cells were harvested at 30 h p.i., washed with PBS, and lysed in TNET buffer at 4°C for 10 min. The lysate was clarified by centrifugation for 10 min at 10,000 rpm (Sanyo Micro Centaur, Japson Bolton & Co Ltd., Watford, United Kingdom), and sample buffer was added to a final concentration of 1% SDS, 15% glycerol, 10 mM Tris-HCl (pH 6.8), 0.02%

FIG. 1. Comigration of BTV VP5 with raft fractions. (A) Wild-type VP5-transfected (top) and untransfected (bottom) HeLa cells were lysed with cold 1% Triton X-100, and the rafts were purified by density gradient fractionation. The lysates were run on SDS-PAGE and analyzed by Western blotting. Antibodies, molecular masses, and sucrose percentages are indicated on the right, left, and bottom, respectively. The pellet fraction is labeled P. The dotted arrows indicate localizations of different proteins in the raft fractions. (B) IFM colocalization of VP5 with caveolin (red) (a), actin (red) (b), tubulin (red) (c), calnexin (red) (d), and vimentin (red) (e). The expression of VP5 alone is shown in image f.

(wt/vol) bromophenol blue without 1% β -mercaptoethanol, without heating, and resolved by SDS-10% PAGE, followed by Western blotting.

Western blotting. In order to analyze infected cells and cellular fractions, the aliquots collected were mixed with Laemmli sample buffer, boiled, and separated by electrophoresis in either 10% or 15% SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes (Immobilon P; Millipore), and immunodetected.

Confocal microscopy. Mammalian cells seeded in six-well plates on 22-mm-diameter coverslips were either infected with BTV or transfected with VP5- and NS3-expressing plasmids. Subsequently, the cells were washed with cold PBS, fixed, permeabilized, and immunolabeled as described previously (5). Nuclei were stained using Hoechst 33258 at a dilution of 1:2,000 (Sigma). After being labeled, the samples were mounted using Fluoprep (Sigma) and analyzed with a Zeiss LSM 510 confocal microscope. The images were obtained using LSM 510 image browser software and processed using Photoshop Elements 2.0 software (Adobe).

Virus titration. To examine the effect of mβcdx on the virus yields at different times p.i., the cells were first infected as described previously and incubated for 8, 12, and 16 h at 37°C. Subsequently, at the end of each time p.i., the medium was discarded and the infected cells were treated with 10 mM mβcdx in fresh medium for 1 h at 37°C. Total viruses from both BTV-infected and treated cells were collected, and virus titers were determined by plaque assays on BSR cells as described previously (5). The total viral titer was determined and normalized to the titer obtained for infected but untreated cells. The mean and standard error of the reduction mediated by the inhibitor were calculated (Sigma Plot 2000; Systat Software Inc.).

RESULTS

Interaction of BTV outer capsid protein VP5 with raft domains in transiently transfected cells. The outer capsid of BTV consists of VP5, in addition to the receptor binding protein VP2. Due to its location on the virus particle, to date most studies of VP5 have been centered on its role during virus

entry into the host cell (33, 34). Since conceptually viruses without the lipid envelope are believed to egress via cell lysis, the trafficking of newly synthesized viruses and their surface proteins is less understood than that of the enveloped viruses. For BTV, some studies of the receptor binding protein VP2 (5, 26, 27) have been undertaken, but nothing is known about the trafficking of VP5 during virus assembly and egress. In the mature particle, VP5 is located in juxtaposition to VP2 and is slightly exposed on the surface of the particle (67). The secondary-structural analysis of VP5 has revealed that the protein consists of an N-terminal amphipathic helix, followed by a coiled-coil domain, which is connected by a flexible short peptide to a more globular domain (43). In order to determine the distribution of VP5 in mammalian cells in the absence of other BTV proteins, a plasmid expressing VP5 was generated and used to transfect BSR and HeLa cells. The cells were lysed in cold Triton X-100 30 h posttransfection and subsequently fractionated by equilibrium density centrifugation in sucrose density gradients as described in Materials and Methods. The gradient fractions were further analyzed by gel electrophoresis, followed by Western analysis (Fig. 1A). A population of VP5 in transiently transfected cells clearly floated in the 5%-to-35% interphase, indicating its association with the lipid raft, although a substantial amount of VP5 also accumulated in the non-lipid raft fractions (Fig. 1A top). To rule out the possibility that sucrose might have affected the buoyant density gradient, VP5-transfected cells were detergent extracted and floated on an Optiprep gradient. A similar flotation profile of VP5 in Optiprep gradient showed that the interaction of VP5 with

FIG. 2. Effect of cholesterol sequestration on cells. Shown is the average percentage of dead cells at each concentration of mβcdx. The cell viability was assessed by staining the cells with trypan blue. The amounts of mβcdx and percentages of dead cells are indicated on the x and y axes, respectively. The experiment was repeated three times, and the average value was plotted. Standard errors are indicated by the error bars.

Triton X-100-insoluble membranes was stable and not due to sucrose present in the gradient (results not shown). Caveolin (35) was used as a raft protein marker to confirm that the VP5-containing fractions corresponded to raft-containing fractions. The presence of calnexin 2, an ER-resident chaperone, in the denser fractions ruled out cross-contamination of the raft membranes by ER. Additionally, tubulin and actin were used as cytoskeletal markers. Although actin was predominantly present in the lower, denser fractions and in the cell pellet, a proportion of it also cosegregated with caveolin, a known raft marker, and floated in the 5%-to-35% sucrose interphase. Similar to calnexin 2, tubulin was present in the denser fractions. To exclude the possibility that transfection of VP5-expressing plasmid itself altered the detergent partitioning of rafts, we analyzed nontransfected control cells in parallel and confirmed the presence of caveolin in the 5%-to-35% interphase (Fig. 1A, bottom). The migration of actin in control untransfected cells was also similar to that in transfected cells. This indicated that the cofractionation of actin in the raft fraction was not due to expression of VP5 in transfected cells. As expected, the cytoskeletal protein tubulin and the ER marker calnexin 2 were found in the lower, denser fractions. Immunofluorescence microscopy (IFM) was subsequently used to confirm the intracellular distribution of VP5 in transiently transfected HeLa and BSR cells. At 30 h posttransfection, VP5 was found throughout the cytoplasm (Fig. 1B). Although both caveolin and a fraction of VP5 coextracted together in the raft

fraction, there was no colocalization between them (Fig. 1B, a). Further, there was also no colocalization between VP5 and actin (Fig. 1B, b). When probed for tubulin, VP5 cells showed partial colocalization with tubulin (Fig. 1B, c). Moreover, although flotation studies showed that a fraction of VP5 was present in the same fractions as calnexin 2, VP5 and calnexin 2 did not colocalize with each other (Fig. 1B, d). In addition, the intermediate filament protein vimentin also did not colocalize with VP5 (Fig. 1B, e). The detection of VP5 alone was used as a control (Fig. 1B, f). These experiments demonstrated that although VP5 has an inherent tendency to coextract with caveolin, they do not colocalize with each other.

Pharmacological extraction of cholesterol alters the cosegregation of VP5 proteins with the raft fraction in transfected cells. It is well established that cholesterol is an important component of rafts (8, 9) and that dynamic packing of sphingolipids and cholesterol results in the formation of rafts that move within the fluid bilayer (86). Beta-cyclodextrins are water-soluble cyclic oligosaccharides that have the capacity to remove cholesterol rapidly and efficiently from cell membranes (53). Initially, the effects of mβcdx on cell morphology (results not shown) and cell death were analyzed, and it was concluded that the cells could tolerate mβcdx at a 10 mM concentration for 60 min without any adverse effects (Fig. 2). Therefore, we used 10 mM mβcdx to treat cells expressing VP5, lysed and subjected to density gradient centrifugation as described in Materials and Methods. VP5-expressing cells not treated with mβcdx were used as controls. Cholesterol depletion with mβcdx clearly altered the migration of VP5 in the gradient, leading to a loss of VP5 in the raft fraction (Fig. 3, left). In comparison, VP5-expressing cells not treated with mβcdx showed coextraction of VP5 in the raft fractions (Fig. 3, right). In addition, compared to both control untreated cells (Fig. 1A, bottom) and VP5-expressing untreated cells (Fig. 3, right), cholesterol-depleting drugs also resulted in loss of caveolin from the raft fractions (Fig. 3, left). When flotillin 2 was used as an additional raft marker, mβcdx treatment also demonstrated a change in the migration of the protein (compare Fig. 3 left and right). Furthermore, the relocation of actin from the 5%-to-35% interphase to the denser fractions after mβcdx treatment (Fig. 3, left) confirmed results reported by Chichili and Rodgers (13) that the integrity of raft domains in the plasma membrane depends on its interaction with actin. These data further indicate that disruption of the raft domain dissociates VP5 from the membrane.

FIG. 3. Cholesterol is required for BTV VP5 raft association. HeLa cells expressing VP5 were treated with 10 mM mβcdx (left) or untreated (right) and fractionated in a sucrose gradient. Molecular masses, antibodies, and sucrose percentages are indicated at the left, center, and bottom, respectively. The pellet fraction is labeled P. The dotted arrows indicate localizations of different proteins in the raft fractions.

FIG. 4. VP5 expression in BTV-infected cells. VP5 in BTV-infected HeLa cells cofractionates with rafts. Cells infected with BTV-17 were analyzed by density gradient centrifugation 2, 4, 6, 8, 12, and 16 h p.i. Uninfected HeLa cells fractionated in parallel were used as controls. Antibodies, molecular masses, and sucrose percentages are indicated on the right, left, and bottom, respectively. The pellet fraction is labeled P. The dotted arrows indicate localizations of different proteins in the raft fractions.

Comigration of VP5 with raft proteins in BTV-infected cells. The above-mentioned studies established that the integrity of raft domains is important for VP5 localization. To determine if this association was also present in BTV-infected cells, HeLa cells infected with BTV were extracted in Triton X-100 at different times p.i. (Fig. 4). The lysate was layered onto a sucrose equilibrium density gradient, fractionated, and subsequently analyzed by Western blotting. Although at 2 h p.i. VP5 was mainly detected in the pellet fraction, faint traces could also be seen in the denser fractions (Fig. 4). The coextraction of VP5 with the raft marker caveolin at the 5%-to-35% sucrose junction was much clearer from 4 h p.i. (Fig. 4). Additionally,

from 8 h p.i. a substantial amount of VP5 also accumulated in the non-lipid raft fractions (35% to 40%) (Fig. 4). The cellular proteins actin, tubulin, caveolin, and calnexin were used as markers in the fractionation studies. In parallel, uninfected fractionated HeLa cells had a similar migration pattern of cellular proteins, demonstrating that BTV infection does not alter the migration patterns of these cellular proteins (Fig. 4). These findings confirm that newly synthesized VP5 rapidly associated with and accumulated in the raft-containing fractions in infected HeLa cells as early as 4 h p.i.

The biochemical data were further supported by IFM. VP5 had a punctate distribution in the cell cytoplasm and could also

FIG. 5. Immunofluorescence analysis of VP5 expression in BTV-infected cells. (A) Localization of VP5 (green) in infected HeLa cells with cellular cytoskeleton; vimentin (red), tubulin (red), and actin (red) are indicated. The time p.i. is given in each image. The arrowheads indicate distributions of labeled proteins. (B) Localization of vimentin, tubulin, and actin in control uninfected cells.

be seen in small patches at the plasma membranes of BTV infected cells (Fig. 5A). Since flotation data indicated that a fraction of VP5 in virus-infected cells comigrated in the denser gradient together with the cytoskeletal markers, colocalization of VP5 and the cytoskeleton in BTV-infected cells was therefore examined. Unlike VP2, which associates mainly with vimentin (5), there was no colocalization of VP5 with vimentin at the earlier times p.i., although from 12 h onward some colocalization of VP5 with vimentin was detectable (Fig. 5A, left

column). Additionally, VP5 was also aligned along vimentin filaments at earlier times p.i. Interestingly, at earlier times p.i., VP5 could also be visualized along the tubulin structures (Fig. 5A, middle column). Cells that were stained for actin showed accumulation of VP5 adjacent to the microfilament network (Fig. 5A, right column). Additionally, the specificity of each antibody set was corroborated using mock-infected cells (Fig. 5B). Our results indicated that VP5 is aligned against cytoskeletal tracks in infected cells.

FIG. 6. Cholesterol depletion affects BTV-1 production. (A) HeLa cells infected with BTV-17 (left) and BSR cells infected with BTV-1 (right) were analyzed for relative virus titers. Virus was harvested at 8, 12, and 16 h p.i. in untreated and mβcdx (10 mM)-treated cells. Control untreated samples are labeled 8c, 12c, and 16c. The error bars indicate the standard errors of three replicates of the experiments. The total titer for each time p.i. was normalized to 100% for untreated cells. (B and C) Cells infected with BTV-17 (left) or BTV-1 (right) and analyzed for expression of NS2 (B) or tubulin (C). The presence and absence of mβcdx is shown as + and -, respectively.

Drugs affecting lipid raft integrity perturb BTV assembly.

Two different BTV serotypes, BTV-17 and BTV-1, were used to assess the role of the lipid raft in the BTV replication cycle. The total virus titers were determined 8, 12, and 16 h p.i. in cells treated with mβcdx and were plotted as relative percentages of the titer in infected cells not treated with mβcdx (Fig. 6A). A significant reduction in the relative virus titer was observed in repeat experiments for both BTV-17 (Fig. 6A, left) and BTV-1 (Fig. 6A, right) infections. In BTV-17-infected cells, the virus titer in treated cells was on average 1.6 times less than in infected untreated cells at 8 to 12 h p.i. (Fig. 6A, left). At 16 h p.i., the proportion of reduction between treated and untreated cells increased to five times (Fig. 6A, left). Similar to that of BTV-17, the virus titer of BTV-1 in treated cells was two times less than in infected untreated cells at 8 to 12 h p.i. and six times less at a later time of 16 h p.i. (Fig. 6A, right). As a positive control to monitor BTV replication, we used NS2, a major BTV nonstructural protein that is involved in core particle assembly during virus replication (50, 52, 60, 65). Equal aliquots of infected cells at the same time points were blotted and analyzed for the presence of NS2 (Fig. 6B). A gradual increase in NS2 expression was observed 4 h p.i. in BTV-17-infected cells (Fig. 6B, left). When equal amounts of cells at each time point were assessed for NS2 production in BTV-1-infected cells, although expression of NS2 was observed from 2 h p.i., there was a sharp increase in NS2 signal at 10 h p.i., which was maintained for later times of viral infection (Fig. 6B, right). Additionally, when the infected cells were treated with mβcdx, there was no difference in the expression level of NS2 at each time p.i. (compare upper and lower panels in Fig. 6B). Thus, depletion of cholesterol with

mβcdx inhibits the virus titer but does not interfere with the replication cycle of BTV. Additionally, it can also be hypothesized that this decrease in the viral titer might be due to a restriction in viral assembly. Moreover, the levels of production of a cellular protein (tubulin) were also similar in treated and untreated cells for both BTV-17 (Fig. 6C, left) and BTV-1 (Fig. 6C, right) infections. This confirms that treating cells with mβcdx did not make any change in the production of cellular proteins and gives an indication of the amount of viral protein synthesis relative to cellular/total protein.

BTV nonstructural glycoprotein NS3 has been implicated in virus maturation and egress (4, 47, 48). NS3 also interacts directly with VP2 (4). Since the reductions in the viral titers in both BTV-17 and BTV-1 infections were similar at later times of virus infection, we also investigated the association of NS3 with lipid rafts. Our results indicated that the migration of NS3 was similar to that of VP5 in infected cells (Fig. 7). Flotillin 2 and actin were used as cellular controls. Additionally, treating the infected cells with mβcdx demonstrated loss of NS3 from the raft fractions (compare the left and right sides of Fig. 7, top and bottom). This change in migration of NS3 was similar to that of VP5 and the cellular proteins actin and flotillin 2. Taken together, these results demonstrate that disruption of rafts with cholesterol-depleting drugs specifically inhibits the association of viral proteins with rafts.

Interaction of VP5 with the BTV glycosylated nonstructural protein NS3. Although there are reports of studies investigating the interaction of outer capsid protein VP2 with NS3 (4), to date no data exist that demonstrate an interaction between VP5 and NS3. To investigate this, pull-down experiments were undertaken. For this study, previously generated recombinant

FIG. 7. Effect of cholesterol extraction on the migration of BTV proteins. Fractionation of BTV-infected BSR cells in the absence (left) and presence (right) of 10 mM mβcdx at 8 (top) and 16 (bottom) h p.i. Antibodies, molecular masses, and sucrose percentages are given at the center, left, and bottom, respectively. The pellet fraction is labeled P. The dotted arrows indicate localizations of different proteins in the raft fractions.

baculoviruses expressing VP5 and NS3 in *Sf9* cells were used (36, 37). For comparison, we also performed pull-down assays of infected cells with unlabeled IgG-Sepharose beads and uninfected cells with beads bound with antibody to either VP5 or NS3. As positive controls, lysates of *Sf9* cells coinfecting with recombinant baculovirus were analyzed for the expression of VP5 (Fig. 8A, left, lane 1) and NS3 (Fig. 8A, right, lane2). Beads loaded either with NS3 or VP5 antibody bound strongly to the other protein that was detected by the respective antibodies (Fig. 8A). Although IgG-Sepharose beads alone reacted to proteins in infected cells, the molecular weights of the precipitated proteins were lower than VP5 (Fig. 8A, left). Additionally, the absence of any protein with a molecular weight similar to that of VP5 or NS3 in uninfected cells further confirmed the specificity of this interaction. The interaction between VP5 and NS3 in transfected and infected cells was further confirmed by IFM. HeLa cells infected with BTV showed colocalization of VP5 and NS3 (Fig. 8B, left). In cells

transfected with plasmids expressing VP5 and NS3, colocalization was visualized only at or very close to the plasma membrane (Fig. 8B, right). Since NS3 interacts with both VP2 (4) and VP5, it is conceivable that NS3 forms a complex with BTV outer capsid proteins and plausibly keep the outer viral proteins in place for coating the cores during virus assembly.

Plasma membrane docking motif in VP5. The presence of VP5 in the raft domains prompted us to examine if the VP5 sequence possesses any vesicle or plasma membrane docking signals. When the VP5 sequences (526 amino acid residues) of 22 different serotypes were aligned, a conserved WHAL motif between amino acids 414 and 417 was not only observed in the sequence (data not shown), but the residue was present at the same position in all 22 serotypes (Fig. 9A). This motif is also present in almost the same position in the VP5 sequence of a closely related orbivirus, epizootic hemorrhagic disease virus serotypes 1 and 2 (Fig. 9A), and matched the conserved WHXL motif present in Syt1, an integral synaptic vesicle membrane

FIG. 8. Interaction of VP5 with NS3. (A) Pull-down analysis of the interaction between VP5 and NS3. Lysates were pulled down with antibody against NS3 (left) or VP5 (right) and labeled with VP5 (left) or NS3 (right) antibodies. The plus and minus signs represent infected and uninfected cells. Lanes 1 and 2 of both blots represent cellular lysates of *Sf9* cells that were either coinfecting with recombinant baculoviruses (lane 1) or uninfected (lane 2). Lanes 3 to 6 are the cellular precipitates. The presence of beads or antibodies is indicated at the bottom, while the respective molecular masses are indicated on either side. (B) Immunofluorescence of cells expressing VP5 (green) and NS3 (red) in infected (left), uninfected control (center), and cotransfected (right) cells.

FIG. 9. Mapping of a potential raft association domain in vp5. (A) Schematic showing the alignment of VP5 protein sequences belonging to serotypes 1, 10, and 17 of BTV and 1 and 2 of epizootic hemorrhagic disease virus. Amino acids that are completely conserved or conserved in charge are indicated by asterisks and colons, respectively. Amino acids targeted for mutagenesis are shown in boldface. (B) Distribution of wild-type VP5 (left) and M1 (right) in transiently transfected HeLa cells 48 h posttransfection.

protein that is a member of the SNARE superfamily (38). Sequence alignment of VP5 sequences belonging to BTV and epizootic hemorrhagic disease virus serotypes demonstrated that there is about 33% conservation in the amino acid composition (results not shown). To investigate the roles of the conserved amino acids in this motif, tryptophan, histidine, and lysine were mutated to a string of alanines (VP5_{WHAL-AAAA}) in full-length VP5 and the plasmid harboring the mutated

protein (M1) was generated and transfected in HeLa cells. Subsequent IFM analysis also demonstrated that compared to cells expressing wild-type VP5, the VP5 mutant was expressed as discrete patches in the cytosol and could no longer be seen along the plasma membrane (Fig. 9B).

HeLa cells were subsequently transfected with the mutant VP5 plasmid and analyzed in a sucrose gradient as described previously. Compared to cells expressing wild-type VP5 (Fig. 10A, left), the mutated protein could no longer be observed comigrating with caveolin in the raft domain (Fig. 10A, right). However, the migration of cellular proteins in cells expressing M1 was similar to that in control fractionations containing either native VP5 (compare Fig. 10A left and right) or non-transfected cells (compare Fig. 10A, right, with Fig. 1B). Misfolded proteins result in the formation of aggresomes whereby the normal cytoskeletal and ER distributions in cells are disrupted (49). In order to verify that the mutated VP5 protein did not result in the formation of aggresomes, cells expressing M1 were immunolabeled with antibodies against calnexin 2, vimentin, and microtubules (Fig. 10B). IFM analysis confirmed that the mutated protein did not alter the expression pattern of the cellular proteins, thus confirming the absence of aggresome formation (Fig. 10B, right). As a control, cells expressing unmutated VP5 were also stained for calnexin 2, tubulin, and actin (Fig. 10B, left). These results indicated that the WHXL domain is likely to play an important part in docking VP5 with plasma membranes.

The majority of mutant VP5 protein was associated with the pellet fraction, and it is possible that the expressed protein was not folded correctly. In order to address this possibility, the M1 mutation was introduced into the full-length VP5 protein and expressed in insect cells using the baculovirus expression system. The native VP5 forms trimers in solutions (43). Both native and mutant forms of VP5 were analyzed for their multimeric states by gel electro-

FIG. 10. Plasma membrane docking motif in VP5. (A) Fractionation of native (left) and mutated (right) VP5 in HeLa cells. Antibodies, molecular masses, and sucrose percentages are given at the center, left, and bottom, respectively. The pellet fraction is labeled P. The dotted arrows indicate localizations of different proteins in the raft fractions. (B) Distribution of native (left) and mutated (right) VP5 in HeLa cells.

FIG. 11. Trimerization of full-length VP5 carrying the M1 mutation. Shown is a Western blot of nondenatured samples for native (left) and M1 (right) VP5 variants. Note that trimerization is unaffected in the M5 mutant.

phoresis under nonreducing conditions (Fig. 11). In the absence of a reducing agent and heat, both native and mutant VP5 showed the presence of two bands. In addition to the lower band with an apparent molecular mass of ~ 59 kDa, an additional minor band with a molecular mass of ~ 180 kDa was detected (Fig. 11, lanes 2 and 3), consistent with the size of a VP5 trimer. Thus, the mutation that prevented the association of VP5 with the raft fraction did not prevent the formation of higher-order multimers of the VP5 protein. This suggests that the overall folding of the mutated protein was similar enough to that of the native protein and that multimerization was unaffected.

DISCUSSION

During BTV assembly, VIBs recruit core proteins (VP1, VP3, VP4, VP6, and VP7) and viral dsRNA to form virus cores (50, 52, 59, 60). However, the two outer capsid proteins do not localize or interact with the VIBs (50). Although our recent investigations have shown that one of the outer capsid proteins, VP2, interacts with vimentin and that this association plays an important role in virus release (5), it is still not clear to which cellular compartment the other outer capsid protein, VP5, localizes during virus replication.

In this study, we characterized for the first time association of VP5, a nonenveloped outer capsid orbiviral protein, with Triton X-100-resistant lipid rafts in both transiently transfected and BTV-infected mammalian cells. Among the different BTV serotypes, BTV-10 and BTV-17 are closely related to each other, and there is about 99.3% similarity in their amino acid compositions. Hence, in order to demonstrate that the VP5 proteins of both viruses behave similarly, transfection studies were carried out with VP5 of BTV-10, whereas BTV-17 was used for virus infection analysis. We showed by subcellular fractionation that disruption of rafts by the cholesterol-depleting reagent $m\beta cdx$ in transfected and infected cells altered the association of VP5 with raft domains. Additionally, the coextraction of VP5 with raft domains in BTV-17-infected cells was similar to that in transfected cells expressing BTV-10 VP5. Since disruption of lipid raft domains in infected cells by $m\beta cdx$ substantially decreased the viral titer, it demonstrated

that irrespective of the relatively large amount of non-raft-associated VP5 in density gradient centrifugation analysis, the raft-associated fraction is important for assembly onto the core particles to form the mature virus particle. Thus, although small, the fraction of VP5 associated with the raft fraction is quite significant. It is possible that, similar to changes in VP5 conformation during endocytosis, the protein also undergoes conformational changes during attachment to raft domains during later stages of virus assembly. The functional role of lipid rafts in BTV replication was further confirmed by determining the relative viral titers in two different mammalian cells in the presence and absence of $m\beta cdx$. In order to investigate whether the effects of $m\beta cdx$ were similar for all BTV serotypes, mammalian cells were infected with one of two distantly related serotypes, either BTV-10/17 or BTV-1. A decrease in the relative BTV titer upon exclusion of cholesterol from cells by $m\beta cdx$ demonstrated the importance of raft domains in BTV infection and indicated that rafts might act as scaffolds for the assembly of BTV particles. Additionally, the proportions of the decreases in the virus titers were similar for both serotypes. These results confirmed that, irrespective of the BTV serotypes used, removal of cholesterol from cells has similar effects on virus infection. Additionally, the use of two different mammalian cells in the plaque reduction assay also confirmed that the effect of $m\beta cdx$ was not cell specific.

As a control for virus replication in the infected cells, aliquots of cell lysates at the different time points were analyzed for NS2, a nonstructural BTV protein that forms VIBs in infected cells. The appearance of NS2 as early as 4 (BTV-17) and 2 (BTV-1) h p.i. in infected cells indicated that VP5 lipid raft interaction plays an important role in the assembly of virus particles and not during virus entry and early protein synthesis. The BTV nonstructural protein NS3 interacts with VP2, the outermost viral capsid protein, and cellular exocytosis machinery (4, 92). It has been proposed that this interaction is responsible for virus egress in infected cells. Hence, we postulated that if rafts provide scaffolds for virus assembly, then NS3 should also be present in the raft domains. Indeed, our results demonstrated that, similar to VP5, raft integrity was also essential for NS3 expression and that cholesterol extraction might indirectly disrupt VP5/NS3 interaction by perturbing plasma membrane lipid raft integrity in cells.

It has been well documented that enveloped-virus structural proteins interact with rafts during virus replication (32, 55, 69, 70, 82, 90, 94). Although outer capsid proteins of nonenveloped viruses, like rotavirus (18, 20, 83) and polioviruses (63), also interact with lipid rafts or detergent-resistant membranes, the nature of such interactions might be different from BTV VP5 and lipid raft interaction. Compared to VP5, poliovirus capsid protein VP4 possesses an amino-terminal myristoylation site (63), while rotavirus outer capsid protein VP4 has a flotillin binding motif (15), both of which are implicated for their raft association. Moreover, compared to other viral proteins that have conserved caveolin binding motifs (15, 45), no such domains were observed in BTV VP5. Our IFM studies also demonstrated no colocalization between VP5 and caveolin. Therefore, the absence of myristoylation or a known raft protein binding motif in VP5 makes its lipid raft interaction unique and intriguing.

Immunofluorescence analysis of cells transfected with VP5-

expressing plasmid and BTV-infected cells demonstrated a difference in VP5 expression. In transfected cells, VP5 was distributed throughout the cells and also along the cellular margins. In comparison, VP5 had a punctate expression in BTV-infected cells. This difference in VP5 expression might be due to the expression of viral structural and nonstructural proteins in BTV-infected cells. Confocal imaging of VP5 in cells demonstrated that although VP5 does not directly interact with vimentin or tubulin filaments early in BTV infection, they were observed aligned along the filamentous structures. In cells, proteins are usually trafficked via cellular motors and cytoskeleton tracks to their target membranes before tethering can take place (12). In addition, it has also been documented that vimentin filaments are closely interlinked with the microtubule and might move along the tubular structures (41, 44, 57, 77, 93). Since a recent report demonstrated that the use of the microtubule-disrupting drug colchicine also reduced virus release in BTV-infected cells from 8 h p.i. (5), it is likely that, similar to the cellular proteins, VP5 also makes use of the cytoskeletal networks to traffic to the plasma membrane. Further experiments are currently being undertaken to isolate the cellular protein or motor that plays a role in this interaction. The colocalized condensed expression of vimentin and VP5 at later times p.i. might be due to the induction of apoptosis in infected cells, which is known to cause the fragmentation of vimentin filaments (66). In colocalization studies of BTV-infected cells with actin, VP5 was always found along the actin filaments at cellular junctions. Earlier investigations showed that BTV particles can be found in the cortical areas of the cells comprised of actin filaments (25, 26). Since reports have demonstrated that the BTV outer capsid protein VP2 interacts with NS3, we analyzed whether NS3 also interacts with VP5, the second outer capsid protein. Our observations demonstrated that VP5 directly interacts with NS3. Based on this, we hypothesized that lipid rafts provide a scaffold for virus assembly and that NS3 keeps the two outer capsid proteins in position for the final assembly of the virus cores in BTV replication. Immunofluorescence analysis of infected cells also demonstrated the presence of pore-like structures in the actin cytoskeletal network at cellular junctions. Since VP5 was always observed next to these pores, it might be hypothesized that in mammalian cells BTV makes use of the cellular junctions to traffic into uninfected neighboring cells. Given that during BTV infection of mammalian cells the majority of viruses are cell associated, this hypothesis provides an attractive model for direct cell-to-cell spread of BTV. Direct cellular spread of animal viruses through cellular junctions has been reported to a great extent in enveloped viruses, like the members of the alphaherpesvirus group (22, 23), human immunodeficiency virus (7, 19, 29, 71, 74, 75), and poxviruses (87), but not for nonenveloped viruses.

Sequence analysis of the gene encoding VP5 was carried out to identify a potential lipid raft- or membrane-targeting motif for docking or tethering to the plasma membrane. Interestingly, a completely conserved WHXL motif was observed toward the carboxyl-terminal end of VP5. This domain is highly conserved in a SNARE regulatory protein, SytI (38), and enables it to dock to neurexin, a raft-associated integral plasma membrane protein (30) that is predicted to play a role in extracellular signaling or cell adhesion in a variety of cells (2,

72). Additionally, similar to the proteins that function in vesicle tethering and docking (73), BTV VP5 also has a coiled-coiled domain (43). Indeed, in our studies, mutation of WHXL to a string of alanines also hampered the association of mutated VP5 protein with Triton X-100-resistant rafts. Further immunofluorescence studies also showed that, unlike native VP5, the mutated protein failed to localize to the plasma membrane and was found as patches in the cytoplasm. Additionally, the mutated protein also did not disrupt the normal cytoskeletal network in transfected cells, which confirmed that aggresomes were not formed in the cells. However, a study carried out by Fukuda et al. (39) demonstrated that mutation or deletion of the evolutionarily conserved WHXL motif in the C terminus of the synaptotagmin family also destabilizes the mutant protein. The resulting malformed proteins accumulated in the ER rather than being transported to other membrane structures and induced the formation of crystalloid ER. Nevertheless, in our studies, expression of the mutant protein in transfected cells did not change the distribution of the ER in cells to crystalloid structures. Additionally, when the same mutation was introduced in full-length VP5, the ability of the recombinant protein to assemble into trimeric VP5 structures was not perturbed. Thus, the WHXL mutation did not change the overall structure of the mutated protein.

Here, we have demonstrated for the first time that a protein from a nonenveloped virus uses membrane-docking signals from the SNARE group of proteins for targeting to the plasma membrane. Although papillomavirus minor capsid protein L2 has been found to contain a SNARE domain, it interacts directly with the SNARE protein syntaxin 18 in the ER membrane via its highly conserved DKILK motif present between residues 40 and 44 (6, 56) and not to the plasma membrane. Additionally this interaction is functionally important during virus entry and uncoating but not assembly and egress.

In this study, we have shown that VP5 interacts with raft domains in mammalian cells and that potential plasma membrane protein-interacting domains may take part in this association; however, to obtain direct confirmation, it will be necessary to isolate the plasma membrane protein that is involved in this association. As earlier investigations have shown that, in contrast to mammalian cells, BTV is released from insect cells by budding (91), additional experiments using insect cells will be utilized for future studies to confirm this hypothesis further.

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REFERENCES

- Anderson, R. G. 1993. Caveolae: where incoming and outgoing messengers meet. *Proc. Natl. Acad. Sci. USA* **90**:10909–10913.
- Baumgartner, S., J. T. Littleton, K. Broadie, M. A. Bhat, R. Harbecke, J. A. Lengyel, R. Chiquet-Ehrismann, A. Prokop, and H. J. Bellen. 1996. A *Drosophila* neurexin is required for septate junction and blood-nerve barrier formation and function. *Cell* **87**:1059–1068.
- Bavari, S., C. M. Bosio, E. Wiegand, G. Ruthel, A. B. Will, T. W. Geisbert, M. Hevey, C. Schmaljohn, A. Schmaljohn, and M. J. Aman. 2002. Lipid raft microdomains: a gateway for compartmentalized trafficking of Ebola and Marburg viruses. *J. Exp. Med.* **195**:593–602.
- Beaton, A. R., J. Rodriguez, Y. K. Reddy, and P. Roy. 2002. The membrane trafficking protein calpactin forms a complex with bluetongue virus protein NS3 and mediates virus release. *Proc. Natl. Acad. Sci. USA* **99**:13154–13159.
- Bhattacharya, B., R. J. Noad, and P. Roy. 2007. Interaction between Blue-

- tongue virus outer capsid protein VP2 and vimentin is necessary for virus egress. *Virol. J.* **4**:7.
6. Bossis, I., R. B. Roden, R. Gambhira, R. Yang, M. Tagaya, P. M. Howley, and P. I. Meneses. 2005. Interaction of tSNARE syntaxin 18 with the papillomavirus minor capsid protein mediates infection. *J. Virol.* **79**:6723–6731.
 7. Bourinbaier, A. S., and D. M. Phillips. 1991. Transmission of human immunodeficiency virus from monocytes to epithelia. *J. Acquir. Immune. Defic. Syndr.* **4**:56–63.
 8. Brown, D. A., and E. London. 1998. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* **14**:111–136.
 9. Brown, D. A., and E. London. 1998. Structure and origin of ordered lipid domains in biological membranes. *J. Membr. Biol.* **164**:103–114.
 10. Byland, R., and M. Marsh. 2005. Trafficking of viral membrane proteins. *Curr. Top. Microbiol. Immunol.* **285**:219–254.
 11. Campbell, S. M., S. M. Crowe, and J. Mak. 2001. Lipid rafts and HIV-1: from viral entry to assembly of progeny virions. *J. Clin. Virol.* **22**:217–227.
 12. Caviston, J. P., and E. L. Holzbaur. 2006. Microtubule motors at the intersection of trafficking and transport. *Trends Cell Biol.* **16**:530–537.
 13. Chichili, G. R., and W. Rodgers. 2007. Clustering of membrane raft proteins by the actin cytoskeleton. *J. Biol. Chem.* **282**:36682–36691.
 14. Chwetoff, S., and G. Trugnan. 2006. Rotavirus assembly: an alternative model that utilizes an atypical trafficking pathway. *Curr. Top. Microbiol. Immunol.* **309**:245–261.
 15. Couet, J., S. Li, T. Okamoto, T. Ikezu, and M. P. Lisanti. 1997. Identification of peptide and protein ligands for the caveolin-scaffolding domain. Implications for the interaction of caveolin with caveolae-associated proteins. *J. Biol. Chem.* **272**:6525–6533.
 16. Cowley, J. A., and B. M. Gorman. 1987. Genetic reassortants for identification of the genome segment coding for the bluetongue virus hemagglutinin. *J. Virol.* **61**:2304–2306.
 17. Cuadras, M. A., B. B. Bordier, J. L. Zambrano, J. E. Ludert, and H. B. Greenberg. 2006. Dissecting rotavirus particle-raft interaction with small interfering RNAs: insights into rotavirus transit through the secretory pathway. *J. Virol.* **80**:3935–3946.
 18. Cuadras, M. A., and H. B. Greenberg. 2003. Rotavirus infectious particles use lipid rafts during replication for transport to the cell surface in vitro and in vivo. *Virology* **313**:308–321.
 19. Danis, C., J. Deschambeault, S. Do Carmo, E. A. Cohen, E. Rassart, and G. Lemay. 2004. The tyrosine-based YXXO targeting motif of murine leukemia virus envelope glycoprotein affects pathogenesis. *Virology* **324**:173–183.
 20. Delmas, O., M. Breton, C. Sapin, A. Le Bivic, O. Colard, and G. Trugnan. 2007. Heterogeneity of Raft-type membrane microdomains associated with VP4, the rotavirus spike protein, in Caco-2 and MA 104 cells. *J. Virol.* **81**:1610–1618.
 21. Delmas, O., A. M. Durand-Schneider, J. Cohen, O. Colard, and G. Trugnan. 2004. Spike protein VP4 assembly with maturing rotavirus requires a post-endoplasmic reticulum event in polarized Caco-2 cells. *J. Virol.* **78**:10987–10994.
 22. Dingwell, K. S., C. R. Brunetti, R. L. Hendricks, Q. Tang, M. Tang, A. J. Rainbow, and D. C. Johnson. 1994. Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. *J. Virol.* **68**:834–845.
 23. Dingwell, K. S., and D. C. Johnson. 1998. Herpes simplex virus gE/gI facilitates cell-to-cell spread and binds to components of cell junctions. *J. Virol.* **72**:8933–8942.
 24. Eaton, B. T., and G. S. Cramer. 1989. The site of bluetongue virus attachment to glycoporphins from a number of animal erythrocytes. *J. Gen. Virol.* **70**:3347–3353.
 25. Eaton, B. T., and A. D. Hyatt. 1989. Association of bluetongue virus with the cytoskeleton. *Subcell. Biochem.* **15**:233–273.
 26. Eaton, B. T., A. D. Hyatt, and S. M. Brookes. 1990. The replication of bluetongue virus. *Curr. Top. Microbiol. Immunol.* **162**:89–118.
 27. Eaton, B. T., A. D. Hyatt, and J. R. White. 1987. Association of bluetongue virus with the cytoskeleton. *Virology* **157**:107–116.
 28. Erasmus, B. J. 1975. Bluetongue in sheep and goats. *Aust. Vet. J.* **51**:165–170.
 29. Fais, S., M. R. Capobianchi, I. Abbate, C. Castilletti, M. Gentile, P. Cordiali Fei, F. Ameglio, and F. Dianzani. 1995. Unidirectional budding of HIV-1 at the site of cell-to-cell contact is associated with co-polarization of intercellular adhesion molecules and HIV-1 viral matrix protein. *AIDS* **9**:329–335.
 30. Faivre-Sarrailh, C., F. Gauthier, N. Denisenko-Nehrbass, A. Le Bivic, G. Rougon, and J. A. Girault. 2000. The glycosylphosphatidyl inositol-anchored adhesion molecule F3/contactin is required for surface transport of paranodin/contactin-associated protein (caspr). *J. Cell Biol.* **149**:491–502.
 31. Finzi, A., A. Orthwein, J. Mercier, and E. A. Cohen. 2007. Productive human immunodeficiency virus type 1 assembly takes place at the plasma membrane. *J. Virol.* **81**:7476–7490.
 32. Fleming, E. H., A. A. Kolokoltsov, R. A. Davey, J. E. Nichols, and N. J. Roberts, Jr. 2006. Respiratory syncytial virus F envelope protein associates with lipid rafts without a requirement for other virus proteins. *J. Virol.* **80**:12160–12170.
 33. Forzan, M., M. M. Marsh, and P. Roy. 2007. Bluetongue virus entry into the cells. *J. Virol.* **81**:4819–4827.
 34. Forzan, M., C. Wirblich, and P. Roy. 2004. A capsid protein of nonenveloped Bluetongue virus exhibits membrane fusion activity. *Proc. Natl. Acad. Sci. USA* **101**:2100–2105.
 35. Foster, L. J., C. L. De Hoog, and M. Mann. 2003. Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc. Natl. Acad. Sci. USA* **100**:5813–5818.
 36. French, T. J., S. Inumaru, and P. Roy. 1989. Expression of two related nonstructural proteins of bluetongue virus (BTV) type 10 in insect cells by a recombinant baculovirus: production of polyclonal ascitic fluid and characterization of the gene product in BTV-infected BHK cells. *J. Virol.* **63**:3270–3278.
 37. French, T. J., J. J. Marshall, and P. Roy. 1990. Assembly of double-shelled, virus-like particles of bluetongue virus by the simultaneous expression of four structural proteins. *J. Virol.* **64**:5695–5700.
 38. Fukuda, M., J. E. Moreira, V. Liu, M. Sugimori, K. Mikoshiba, and R. R. Llinas. 2000. Role of the conserved WHXL motif in the C terminus of synaptotagmin in synaptic vesicle docking. *Proc. Natl. Acad. Sci. USA* **97**:14715–14719.
 39. Fukuda, M., A. Yamamoto, and K. Mikoshiba. 2001. Formation of crystalloid endoplasmic reticulum induced by expression of synaptotagmin lacking the conserved WHXL motif in the C terminus. Structural importance of the WHXL motif in the C2B domain. *J. Biol. Chem.* **276**:41112–41119.
 40. Grimes, J. M., J. Jakana, M. Ghosh, A. K. Basak, P. Roy, W. Chiu, D. I. Stuart, and B. V. V. Prasad. 1997. An atomic model of the outer layer of the bluetongue virus core derived from X-ray crystallography and electron cryomicroscopy. *Structure* **5**:885–893.
 41. Gyoeba, F. K., and V. I. Gelfand. 1991. Coalignment of vimentin intermediate filaments with microtubules depends on kinesin. *Nature* **353**:445–448.
 42. Hassan, S. H., and P. Roy. 1999. Expression and functional characterization of bluetongue virus VP2 protein: Role in cell entry. *J. Virol.* **73**:9832–9842.
 43. Hassan, S. H., C. Wirblich, M. Forzan, and P. Roy. 2001. Expression and functional characterization of bluetongue virus VP5 protein: role in cellular permeabilization. *J. Virol.* **75**:8356–8367.
 44. Helfand, B. T., L. Chang, and R. D. Goldman. 2004. Intermediate filaments are dynamic and motile elements of cellular architecture. *J. Cell Sci.* **117**:133–141.
 45. Hovanessian, A. G., J. P. Briand, E. A. Said, J. Svab, S. Ferris, H. Dali, S. Muller, C. Desgranges, and B. Krust. 2004. The caveolin-1 binding domain of HIV-1 glycoprotein gp41 is an efficient B cell epitope vaccine candidate against virus infection. *Immunity* **21**:617–627.
 46. Huismans, H., N. T. Van der Walt, M. Cloete, and B. J. Erasmus. 1983. The biochemical and immunological characterization of bluetongue virus outer capsid polypeptides, p. 165–172. *In* R. W. Compans and D. H. L. Bishop (ed.), *Double-Stranded RNA Viruses*. Elsevier, New York, NY.
 47. Hyatt, A. D., A. R. Gould, B. Coupar, and B. T. Eaton. 1991. Localization of the non-structural protein NS3 in bluetongue virus-infected cells. *J. Gen. Virol.* **72**:2263–2267.
 48. Hyatt, A. D., Y. Zhao, and P. Roy. 1993. Release of bluetongue virus-like particles from insect cells is mediated by BTV nonstructural protein NS3/NS3A. *Virology* **193**:592–603.
 49. Johnston, J. A., C. L. Ward, and R. R. Kopito. 1998. Aggresomes: a cellular response to misfolded proteins. *J. Cell Biol.* **143**:1883–1898.
 50. Kar, A. K., B. Bhattacharya, and P. Roy. 2007. Bluetongue virus RNA binding protein NS2 is a modulator of viral replication and assembly. *BMC Mol. Biol.* **8**:4.
 51. Kar, A. K., M. Ghosh, and P. Roy. 2004. Mapping the assembly of Bluetongue virus scaffolding protein VP3. *Virology* **324**:387–399.
 52. Kar, A. K., N. Iwatani, and P. Roy. 2005. Assembly and intracellular localization of the bluetongue virus core protein VP3. *J. Virol.* **79**:11487–11495.
 53. Kilsdonk, E. P., P. G. Yancey, G. W. Stoudt, F. W. Bangert, W. J. Johnson, M. C. Phillips, and G. H. Rothblat. 1995. Cellular cholesterol efflux mediated by cyclodextrins. *J. Biol. Chem.* **270**:17250–17256.
 54. King, L. A., and R. D. Possee (ed.). 1992. *The Baculovirus expression system: a laboratory guide*. Chapman and Hall, London, United Kingdom.
 55. Laliberte, J. P., L. W. McGinnes, M. E. Peebles, and T. G. Morrison. 2006. Integrity of membrane lipid rafts is necessary for the ordered assembly and release of infectious Newcastle disease virus particles. *J. Virol.* **80**:10652–10662.
 56. Laniosz, V., K. C. Nguyen, and P. I. Meneses. 2007. Bovine papillomavirus type 1 infection is mediated by SNARE syntaxin 18. *J. Virol.* **81**:7435–7448.
 57. Liao, G., and G. Gundersen. 1998. Kinesin is a candidate for cross-bridging microtubules and intermediate filaments. Selective binding of kinesin to dytrotinated tubulin and vimentin. *J. Biol. Chem.* **273**:9797–9803.
 58. Limn, C.-H., N. Stauber, K. Monastyrskaya, P. Gouet, and P. Roy. 2000. Functional dissection of the major structural protein of bluetongue virus: identification of key residues within VP7 essential for capsid assembly. *J. Virol.* **74**:8658–8669.
 59. Lymperopoulos, K., R. Noad, S. Tosi, S. Nethisinghe, I. Brierley, and P. Roy. 2006. Specific binding of Bluetongue virus NS2 to different viral plus-strand RNAs. *Virology* **353**:17–26.

60. Lympopoulos, K., C. Wirblich, I. Brierley, and P. Roy. 2003. Sequence specificity in the interaction of Bluetongue virus non-structural protein 2 (NS2) with viral RNA. *J. Biol. Chem.* **278**:31722–31730.
61. Manie, S. N., S. Debreyne, S. Vincent, and D. Gerlier. 2000. Measles virus structural components are enriched into lipid raft microdomains: a potential cellular location for virus assembly. *J. Virol.* **74**:305–311.
62. Marshall, J. J., and P. Roy. 1990. High level expression of the two outer capsid proteins of bluetongue virus serotype 10: their relationship with the neutralization of virus infection. *Virus Res.* **15**:189–195.
63. Martin-Belmonte, F., J. A. Lopez-Guerrero, L. Carrasco, and M. A. Alonso. 2000. The amino-terminal nine amino acid sequence of poliovirus capsid VP4 protein is sufficient to confer N-myristoylation and targeting to detergent-insoluble membranes. *Biochemistry* **39**:1083–1090.
64. Mehlhorn, H., V. Walldorf, S. Klimpel, B. Jahn, F. Jaeger, J. Eschweiler, B. Hoffmann, and M. Beer. 2007. First occurrence of *Culicoides obsoletus*-transmitted Bluetongue virus epidemic in central Europe. *Parasitol. Res.* **101**:219–228.
65. Modrof, J., K. Lympopoulos, and P. Roy. 2005. Phosphorylation of Bluetongue virus nonstructural protein 2 is essential for formation of viral inclusion bodies. *J. Virol.* **79**:10023–10031.
66. Mortola, E., R. Noad, and P. Roy. 2004. Bluetongue virus outer capsid proteins are sufficient to trigger apoptosis in mammalian cells. *J. Virol.* **78**:2875–2883.
67. Nason, E., R. Rothnagel, S. K. Muknerge, A. K. Kar, M. Forzan, B. V. V. Prasad, and P. Roy. 2004. Interactions between the inner and outer capsids of Bluetongue virus. *J. Virol.* **78**:8059–8067.
68. Nguyen, D. H., and J. E. Hildreth. 2000. Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. *J. Virol.* **74**:3264–3272.
69. Ono, A., and E. O. Freed. 2001. Plasma membrane rafts play a critical role in HIV-1 assembly and release. *Proc. Natl. Acad. Sci. USA* **98**:13925–13930.
70. Panchal, R. G., G. Ruthel, T. A. Kenny, G. H. Kallstrom, D. Lane, S. S. Badie, L. Li, S. Bavari, and M. J. Aman. 2003. In vivo oligomerization and raft localization of Ebola virus protein VP40 during vesicular budding. *Proc. Natl. Acad. Sci. USA* **100**:15936–15941.
71. Pearce-Pratt, R., D. Malamud, and D. M. Phillips. 1994. Role of the cytoskeleton in cell-to-cell transmission of human immunodeficiency virus. *J. Virol.* **68**:2898–2905.
72. Peles, E., M. Nativ, M. Lustig, M. Grumet, J. Schilling, R. Martinez, G. D. Plowman, and J. Schlessinger. 1997. Identification of a novel contactin-associated transmembrane receptor with multiple domains implicated in protein-protein interactions. *EMBO J.* **16**:978–988.
73. Pfeffer, S. R. 1996. Transport vesicle docking: SNAREs and associates. *Annu. Rev. Cell Dev. Biol.* **12**:441–461.
74. Phillips, D. M., and A. S. Bourinbaier. 1992. Mechanism of HIV spread from lymphocytes to epithelia. *Virology* **186**:261–273.
75. Phillips, D. M., X. Tan, M. E. Perotti, and V. R. Zacharopoulos. 1998. Mechanism of monocyte-macrophage-mediated transmission of HIV. *AIDS Res. Hum. Retrovir.* **14**:S67–S70.
76. Pierce, C. M., P. V. Rossitto, and N. J. MacLachlan. 1995. Homotypic and heterotypic neutralization determinants of bluetongue virus serotype 17. *Virology* **209**:263–267.
77. Prahlad, V., M. Yoon, R. D. Moir, R. D. Vale, and R. D. Goldman. 1998. Rapid movements of vimentin on microtubule tracks: kinesin-dependent assembly of intermediate filament networks. *J. Cell Biol.* **143**:159–170.
78. Purse, B. V., P. S. Mellor, D. J. Rogers, A. R. Samuel, P. P. C. Mertens, and M. Baylis. 2005. Climate change and the recent emergence of Bluetongue in Europe. *Nat. Rev. Microbiol.* **3**:171–181.
79. Roy, P., and R. Noad. 2006. Bluetongue virus assembly and morphogenesis. *Curr. Top. Microbiol. Immunol.* **309**:87–116.
80. Saegerman, C., D. Berkvens, and P. S. Mellor. 2008. Bluetongue epidemiology in the European Union. *Emerg. Infect. Dis.* **14**:539–544.
81. Salaun, C., G. W. Gould, and L. H. Chamberlain. 2005. The SNARE proteins SNAP-25 and SNAP-23 display different affinities for lipid rafts in PC12 cells. Regulation by distinct cysteine-rich domains. *J. Biol. Chem.* **280**:1236–1240.
82. Sanderson, C. M., R. Avalos, A. Kundu, and D. P. Nayak. 1995. Interaction of Sendai viral F, HN, and M proteins with host cytoskeletal and lipid components in Sendai virus-infected BHK cells. *Virology* **209**:701–707.
83. Sapin, C., O. Colard, O. Delmas, C. Tessier, M. Breton, V. Enouf, S. Chwetzoff, J. Ouanich, J. Cohen, C. Wolf, and G. Trugnan. 2002. Rafts promote assembly and atypical targeting of a nonenveloped virus, rotavirus, in Caco-2 cells. *J. Virol.* **76**:4591–4602.
84. Scheiffele, P., A. Rietveld, T. Wilk, and K. Simons. 1999. Influenza viruses select ordered lipid domains during budding from the plasma membrane. *J. Biol. Chem.* **274**:2038–2044.
85. Schnitzer, J. E., D. P. McIntosh, A. M. Dvorak, J. Liu, and P. Oh. 1995. Separation of caveolae from associated microdomains of GPI-anchored proteins. *Science* **269**:1435–1439.
86. Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature* **387**:569–572.
87. Smith, G. L., B. J. Murphy, and M. Law. 2003. Vaccinia virus motility. *Annu. Rev. Microbiol.* **57**:323–342.
88. Tanaka, S., and P. Roy. 1994. Identification of domains in bluetongue virus VP3 molecules essential for the assembly of virus cores. *J. Virol.* **68**:2795–2802.
89. Urakawa, T., T. J. French, Y. Adachi, A. Fukusho, H. LeBlais, M. Flamand, P. Mertens, and P. Roy. 1994. Synthesis of recombinant baculoviruses expressing the outer capsid protein VP2 of five BTV serotypes and the induction of neutralizing antibodies to homologous and heterologous BTV serotypes. *Virus Res.* **31**:149–161. (Erratum, *Virus Res.* **34**:93 and **34**:187, 1994.)
90. Vincent, S., D. Gerlier, and S. N. Manie. 2000. Measles virus assembly within membrane rafts. *J. Virol.* **74**:9911–9915.
91. Wechsler, S. J., L. E. McHolland, and W. J. Tabachnick. 1989. Cell lines from *Culicoides variipennis* (Diptera: Ceratopogonidae) support replication of bluetongue virus. *J. Invertebr. Pathol.* **54**:385–393.
92. Wirblich, C., B. Bhattacharya, and P. Roy. 2006. Nonstructural protein 3 of bluetongue virus assists virus release by recruiting ESCRT-I protein Tsg101. *J. Virol.* **80**:460–473.
93. Yoon, M., R. D. Moir, V. Prahlad, and R. D. Goldman. 1998. Motile properties of vimentin intermediate filament networks in living cells. *J. Cell Biol.* **143**:147–157.
94. Zhang, J., A. Pekosz, and R. A. Lamb. 2000. Influenza virus assembly and lipid raft microdomains: a role for the cytoplasmic tails of the spike glycoproteins. *J. Virol.* **74**:4634–4644.
95. Zientara, S., C. Sailleau, G. Dauphin, C. Roquier, E. M. Rémond, F. Lebreton, S. Hammoumi, E. Dubois, C. Agier, G. Merle, and E. Bréard. 2002. Identification of bluetongue virus serotype 2 (Corsican strain) by reverse-transcriptase PCR reaction analysis of segment 2 of the genome. *Vet. Rec.* **150**:598–601.