

Localization of viral proteins in cells infected with bovine viral diarrhoea virus

B. Grummer,¹ M. Beer,² E. Liebler-Tenorio³ and I. Greiser-Wilke¹

^{1,3}Institutes of Virology¹ and Pathology³, School of Veterinary Medicine Hannover, Buenteweg 17, 30559 Hannover, Germany

²Federal Research Centre for Virus Diseases of Animals, Institute for Diagnostic Virology, Boddenblick 5a, 17498 Insel Riems, Germany

Bovine viral diarrhoea virus (BVDV) is a member of the genus *Pestivirus* within the family *Flaviviridae*. In this report, protein localization studies were performed to assess the mechanism for the release of mature virus particles from infected cells. Since BVDV is an enveloped virus, budding from either intra- or extracellular membranes is feasible. A prerequisite for the latter mechanism is the integration of viral glycoproteins into the host cell membrane. Using monoclonal antibodies (MAbs) directed against the viral envelope glycoproteins E2 and E^{RNS}, no specific signals were detected on the surface of BVDV-infected cells by indirect fluorescence, confocal microscopy or fluorescence-activated cell sorter analyses. Furthermore, biotin-labelled cell surface proteins of virus-infected and non-infected cells were not detected by immunoprecipitation using MAbs directed against E^{RNS} and E2 or the non-structural protein NS2-3. None of these proteins was detected on the cell surface. In addition, to analyse the intracellular localization of the two viral glycoproteins E^{RNS} and E2 and the non-structural proteins NS2-3 and NS3, subcellular fractionation of virus-infected cells followed by radioimmunoprecipitation with the MAbs were performed. These results led to the conclusion that the BVDV envelope glycoproteins E^{RNS} and E2 as well as the non-structural proteins NS2-3 and NS3 were almost quantitatively associated with intracellular membranes. These findings indicate that BVDV is released by budding into the cisternae of the endoplasmic reticulum and that there seems to be no correlation between the location and function of the analysed proteins.

Introduction

Bovine viral diarrhoea virus (BVDV) is an enveloped, positive-stranded RNA virus that belongs to the genus *Pestivirus* within the family *Flaviviridae* (Wengler *et al.*, 1995). BVDV is a major virus pathogen of cattle. Due to the action of the virus in cell culture, a non-cytopathic (ncp) and a cytopathic (cp) biotype can be distinguished. *In vivo*, the two virus biotypes interact to cause the highly fatal mucosal disease in animals that are persistently infected with ncp BVDV (Bolin *et al.*, 1985; Brownlie *et al.*, 1984). *In vitro*, ncp BVDV has no immediate effect on infected cells, while cp BVDV induces vacuolation and cell death (Baker *et al.*, 1954; Gillespie *et al.*, 1960). This cell death is mediated by the induction of apoptosis (Zhang *et al.*, 1996; Grummer *et al.*, 1998). The mechanisms

that induce cp BVDV-mediated apoptosis are, as yet, unknown. The BVDV genomic RNA is about 12.5 kb in size and comprises the 5' and 3' untranslated regions (UTR), which flank a long open reading frame encoding a single viral polyprotein (Collett *et al.*, 1991; Wengler *et al.*, 1995). Translation initiation is mediated by the highly conserved 5'UTR, which functions as an internal ribosomal entry site (Poole *et al.*, 1995; Pestova & Hellen, 1999). The polyprotein is processed to yield 11 or 12 proteins (Wiskerchen & Collett, 1991; Wiskerchen *et al.*, 1991; Rumenapf *et al.*, 1993). The order of the proteins on the polyprotein is NH₂-N^{PRO}-C-E^{RNS}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Collett *et al.*, 1994; Elbers *et al.*, 1996). The structural proteins include the nucleocapsid protein (C) and the three envelope glycoproteins E^{RNS}, E1 and E2. In BVDV-infected cells, the expression of the non-structural protein NS3 correlates with cytopathogenicity; thus, the NS3 protein may function as a possibly important soluble factor in the molecular

Author for correspondence: Irene Greiser-Wilke.

Fax +49 511 953 8898. e-mail Irene.Greiser-Wilke@tiho-hannover.de

pathways of cp BVDV-induced cell death. In cells infected with ncp BVDV, the NS2-3 gene is expressed only as a readthrough product (Donis & Dubovi, 1987; Purchio *et al.*, 1984). Different mutations in the genomes of cp BVDV can lead to the expression of NS3, including gene duplications, insertion of host sequences, deletions or point mutations (Meyers *et al.*, 1989, 1991, 1992; Qi *et al.*, 1992; Greiser-Wilke *et al.*, 1993; Tautz *et al.*, 1993).

While considerable information is now available about genome structure and viral protein function, there is little and inconsistent information available about the structure and morphogenesis of the virion. Since BVDV is an enveloped virus, budding from the extracellular membrane could be one possible mechanism for the release of mature particles. A prerequisite for virus release by this mechanism is the integration of viral glycoproteins into the membrane of the host cell. In this study, the expression of viral proteins on the surface of infected cells and in soluble and insoluble subcellular fractions was analysed using different approaches. After labelling with monoclonal antibodies (MAbs), no expression of the viral glycoproteins E2 and E^{RNS} was detected on the surface of viable BVDV-infected cells by either confocal laser scanning microscopy (CFM) or FACS analysis. In addition, no viral proteins were immunoprecipitated with the specific MAbs from biotin-labelled cell surfaces of infected cells. Subcellular fractionation of virus-infected cells followed by radioimmunoprecipitation (RIP) with MAbs demonstrated that the two viral glycoproteins E2 and E^{RNS} as well as the non-structural proteins NS2-3 and NS3 were almost quantitatively associated with intracellular membranes. From these results, we conclude that BVDV is released by budding into the cisternae of the endoplasmic reticulum (ER). Due to the fact that all proteins analysed were membrane-bound or membrane-associated, it can be excluded that one or more of them function as soluble factors during BVDV-induced apoptosis. This is in agreement with the findings that in BVDV-infected cell cultures, only cells actively synthesizing viral proteins undergo apoptosis (Grummer *et al.*, 1998).

Methods

■ **Cells and viruses.** MDBK cells were infected at an m.o.i. of 1 with a plaque-purified clone of BVDV strain NADL (cp BVDV, genotype I), which was shown not to be contaminated with an ncp subpopulation by RT-PCR (Greiser-Wilke *et al.*, 1993), and strain 7443 (ncp BVDV, genotype I). Mock-infected cells were used as negative controls. As a positive control, BHK-21 cells infected with Semliki Forest virus (SFV) at an m.o.i. of 1 were included.

All cells were grown in Dulbecco's modification of Eagle's medium supplemented with 5% bovine serum that had been tested for the absence of BVDV and antibodies against BVDV. Cell cultures were maintained at 37 °C in a 5% CO₂ incubator.

■ **Monoclonal antibodies (MAbs).** The E2-specific MAb BVD/CA3, the E^{RNS}-specific MAb BVD/C42 and the NS3-specific MAb BVD/C16 were used (Bolin *et al.*, 1988; Cay *et al.*, 1989; Greiser-Wilke

et al., 1991*b*). As a positive control, MAb BVD/CA27 was included. This MAb is directed against the putative cellular receptor for BVDV (Schelp *et al.*, 1995), which is, presumably, the bovine CD46 (boCD46) (Rumenapf *et al.*, 2000). As an additional control for the immunoprecipitation of biotinylated viral surface proteins, the SFV transmembrane protein p62 was precipitated using MAb SFV/C8 and the nucleocapsid protein was precipitated using MAb SFV/C2 (Greiser-Wilke *et al.*, 1991*a*). As a negative control for the immunoprecipitation of radioactively labelled proteins, lysates from BVDV-infected cells were precipitated using the unrelated MAb BM/40, which is specific for *Brucella melitensis* lipopolysaccharide (Greiser-Wilke & Moennig, 1987).

■ Surface and intracellular indirect immunofluorescence (IF) staining

CFM analysis. For intracellular staining, cells were grown on coverslips and infected with virus at an m.o.i. of 1. After 48 h, the cells were fixed for 5 min in ice-cold 80% acetone. After washing with Ca²⁺- and Mg²⁺-deficient PBS (PBSM⁻), the coverslips (infected cells and mock-infected controls) were incubated for 15 min with MAbs (cell culture supernatants diluted 1:10 in PBSM⁻). After two additional washing steps with PBSM⁻, the coverslips were incubated for 15 min with the ALEXA488 conjugate (Molecular Probes) diluted 1:500 in PBSM⁻, washed and analysed by CFM (Zeiss LM510).

To stain the cell surface, coverslips with viable cells were incubated for 30 min on ice with the appropriate MAbs. After two washing steps, the coverslips were fixed with 1% paraformaldehyde (PFA) (Sigma) in PBSM⁻ and incubated with the ALEXA488 conjugate for 30 min on ice. The coverslips were then washed and analysed by CFM. Cell viability was determined using 10⁻⁵ M propidium iodide (PI) in PBSM⁻ (Sigma). From preparations with surface-stained cells, only samples with greater than 90% viable cells were evaluated.

Fluorescence-activated cell sorter (FACS) analysis. Virus-infected cells (25 cm² flasks, m.o.i. of 1) were detached with a trypsin solution and transferred to a U-bottomed 96-well microtitre plate containing 4 × 10⁴ cells per well.

For intracellular staining, cells were washed twice with PBSM⁻ and fixed with 1% PFA for 15 min on ice. After washing with PBSM⁻, the cells were permeabilized for 5 min at room temperature with 0.0025% digitonin (Sigma) in PBSM⁻ and washed once with PBSM⁻. The BVDV-specific MAbs (diluted cell culture supernatants) were added for 15 min and, subsequently, the cells were washed twice with PBSM⁻. The ALEXA488 conjugate was then added and incubated for 15 min. After two additional washes with PBS, the cells were resuspended in 100 µl PI solution. Only PI-positive cells were evaluated by flow cytometry.

To stain the cell surface (Beer & Wolf, 1999), detached cells were washed twice with PBSM⁻ and incubated with BVDV-specific MAbs for 30 min on ice. After two washes, the ALEXA488 conjugate was added for 30 min on ice. After two additional washes with PBS, cells were resuspended in 100 µl PI solution and IF was measured by flow cytometry. Only PI-negative cells were analysed for surface expression of BVDV proteins (5000 cells per sample).

FACS analysis was performed with a FACScan cytofluorometer (Becton Dickinson) using the Cell Quest (Becton Dickinson) and WinMDI (Salk Institute) software.

■ **Isotopic labelling and subcellular fractionation.** Twenty-four h after seeding 3 × 10⁶ cells per tissue culture dish (10 cm diameter, 3 dishes per experiment), the cells were infected with BVDV or mock-infected. After 48 h, the monolayers were rinsed three times with PBSM⁻ and overlaid with methionine- and cysteine-free medium (Gibco BRL) supplemented with 10% dialysed foetal calf serum (dFCS). Incubation

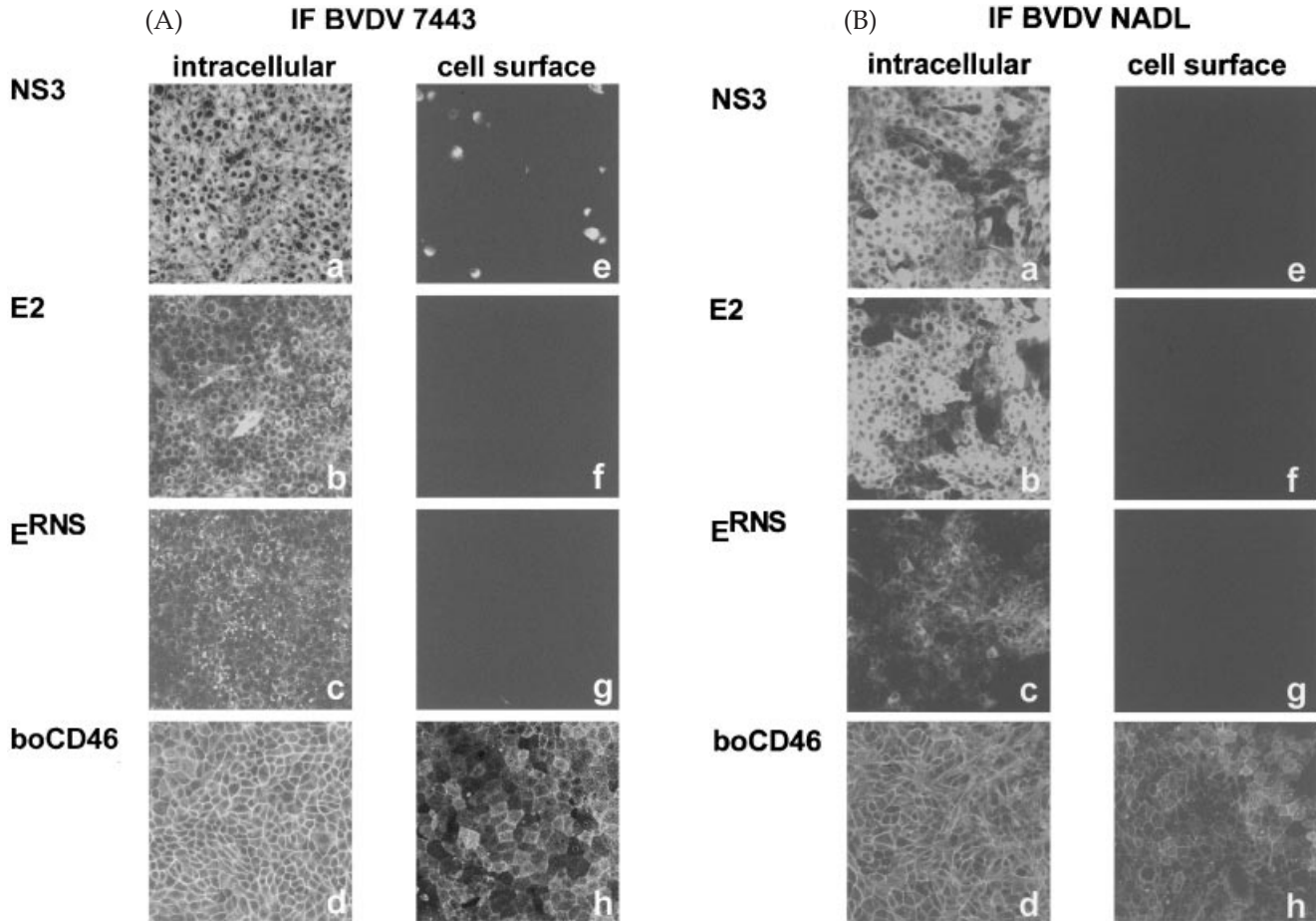


Fig. 1. MDBK cells were infected with (A) BVDV strain 7443 or (B) BVDV strain NADL and IF-stained using MAbs against the NS3 (MAb BVD/C16), E^{RNS} (MAb BVD/C42), E2 (MAb BVD/CA3) and boCD46 (MAb BVD/CA27) proteins. The IF signals were analysed by CFM. Strong intracellular NS3-, E^{RNS}- and E2-specific IF signals were detectable in the cytoplasm of permeabilized cells (panels a–c). In contrast, no specific IF signals were detectable on the surface of BVDV-infected cells. BoCD46 was detected in both preparations (panels d and h).

was for 2 h at 37 °C. Labelling was performed by the addition of 5.5×10^6 Bq *trans* [³⁵S]methionine (ICN) in 2 ml methionine- and cysteine-free medium with 10% dFCS. The cultures were then incubated for 6 h at 37 °C. The cells were rinsed twice with ice-cold PBSM⁻ and once with bidistilled water (Aqua bidist). The cell monolayers were removed using a rubber policeman, resuspended in 1 ml ice-cold Aqua bidist and centrifuged at 4 °C for 6 min at 800 *g*. The sedimented cells were pre-swollen at 4 °C for 60 min in 1 ml Aqua bidist containing Complete protease inhibitors (Boehringer). After microscopic examination, the remaining cells were lysed by ultrasonic waves at 35 kHz for 5–10 s, without damaging the nuclei. Lysed cells were centrifuged at 4 °C for 10 min at 1000 *g* to remove the nuclei. The resulting supernatants were ultracentrifuged at 4 °C for 60 min at 100 000 *g* in order to separate cellular membranes and soluble components from the cytosol (De Duve, 1971). The supernatants (800 μ l) were carefully decanted into a new reaction tube, mixed with 200 μ l $5 \times$ RIPA buffer (1 \times RIPA comprises 150 mM NaCl, 1.0% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8) and stored for 10 min on ice. The sediments were resuspended by adding 1 ml $1 \times$ RIPA buffer and were also incubated for 10 min on ice. For RIP analysis, samples were

divided into two equal aliquots and incubated with a mixture of MAbs BVD/C16, C42 and CA3 (immunoaffinity purified) and protein G-Sepharose beads (Pharmacia) overnight. After washing the beads three times with RIPA buffer and eluting the proteins by boiling in sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris-HCl, pH 6.8), SDS-PAGE was performed in 7.5% acrylamide stacking gels with 15% acrylamide resolving gels. After electrophoresis, the gels were fixed in 30/12.5% (v/v) methanol-acetic acid and dried under vacuum. Dried gels were analysed using a PhosphorImager (Molecular Dynamics).

Duplicate cultures were prepared as described above, but without adding the radioactive label. These samples were used to assess the level of contamination in the fractions containing soluble and membrane-associated cell components, respectively, by measuring the activity of the tracking enzymes.

■ **Determination of enzyme activity.** The activity of the following enzymes was determined for each of the fractions: (a) the mitochondrial enzymes succinate dehydrogenase (SDH) and glutamate dehydrogenase (GluDH), which are specific for the membrane fraction (Singer, 1974; Ludtke *et al.*, 1975) and (b) glycerol-3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH), which are specific for the

cytosolic fraction (Gonzalez-Cerezo & Dalziel, 1975; Lee *et al.*, 1982). The activity of these enzymes was determined using the Ultrospec 2000 photometer (Pharmacia). The enzyme kinetics were evaluated using the Swift Reaction Kinetics 1.01 software (Pharmacia).

■ **Biotinylation of proteins in cell lysates.** Intracellular biotinylation of viral proteins was performed using the Cellular Labelling and Immunoprecipitation kit (Roche), as instructed by the manufacturer.

■ **Biotinylation of cell surfaces.** MDBK cells were infected 24 h after seeding into 6-well plates with BVDV or mock-infected with medium. As a control, BHK-21 cells were infected with SFV or mock-infected. After 24 h, the cell monolayers were rinsed three times with ice-cold PBS and overlaid with 0.5 ml biotin solution (0.5 mg sulpho-*N*-hydroxysuccinimide-biotin (Pierce) per ml of PBS, pH 7.2). The cells were then labelled for 1 h at 4 °C. The biotin solution was removed and the cells were rinsed once with ice-cold 0.1 M glycine solution to stop the reaction. After that, the cells were washed for 15 min with 0.1 M glycine at 4 °C. The last washing step was performed with ice-cold PBS. Proteins were solubilized by the addition of 1 ml RIPA buffer and virus proteins were immunoprecipitated as described above. For this, 500 µl aliquots were incubated overnight with a pool of MAbs (BVD/C16, CA3 and C42 or SFV/C2 and C8, respectively).

After immunoprecipitation and SDS-PAGE (7.5% acrylamide stacking gels with 15% acrylamide resolving gels), the proteins were transferred to PVDF membranes (Millipore). The membranes were incubated for 1 h at room temperature with a streptavidin peroxidase-conjugated antibody (Amersham) diluted 1:1000. Proteins were detected using the BM chemiluminescence blotting substrate POD (Roche).

Results

Surface and intracellular indirect IF staining

The presence of the BVDV proteins NS2-3/NS3, E2 and E^{RNS}, as detected by indirect IF using MAbs BVD/C16, CA3 and C42, was compared between fixed (permeabilized) and viable (fixed after incubation with MAbs) MDBK cells infected with ncp BVDV strain 7443 (Fig. 1A) and cp BVDV strain NADL (Fig. 1B). Mock-infected cells served as controls (data not shown). Analysis by CFM demonstrated that in permeabilized ncp and cp BVDV-infected MDBK cells, strong NS2-3/NS3-, E2- and E^{RNS}-specific IF signals were detectable in the cytoplasm of more than 90% of the cells. In contrast, there was no NS2-3/NS3-, E2- or E^{RNS}-specific IF signal either in the nucleus or on the surface of virus-infected cells. Single positive cells visible after staining with the NS3-specific MAb BVD/C16 were attributed to cells that were damaged during the staining procedure (Fig. 1B, panel c). As a positive control, binding of BVD/CA27 (boCD46) to the cell surface was detected in both preparations (Fig. 1, panels d and h). Mock-infected cells showed no signal either in the cytoplasm or on the cell surface.

Similar results were obtained by FACS analysis. Fluorescence histograms of MDBK cells infected with BVDV strain 7443 and mock-infected cells after intracellular or surface IF staining using MAbs BVD/C16, CA3 and C42 are shown in Fig. 2. As the histograms demonstrate, nearly 100% of the infected MDBK cells show a strong intracellular NS2-3- and

E2-specific signal and a weaker E^{RNS}-specific signal (Fig. 2, panels a–c). No significant NS2-3-, E2- or E^{RNS}-specific IF signals were detected on the cell surfaces of the viable virus-infected cells (Fig. 2, panels e–g) when compared to stained mock-infected cells. Comparable results were obtained with cells infected with cp BVDV strain NADL (data not shown).

Detection of viral proteins after biotinylation of the surface of virus-infected cells by immunoblotting

To analyse the expression of viral proteins on the surface of infected cells, the cells were labelled *in vivo* with sulpho-*N*-hydroxysuccinimide-biotin, a chemical compound that is unable to penetrate biological membranes (Grumbach & Veh, 1991). In a preceding experiment, it was shown that the viral proteins in lysates of infected cells were successfully labelled with biotin and that biotinylation did not affect immunoprecipitation by the MAbs (data not shown). After lysis and immunoprecipitation of biotinylated virus-infected MDBK cells, neither the envelope glycoproteins E^{RNS} and E2 nor the non-structural proteins NS2-3 and NS3 were detected (Fig. 3, lanes 7 and 9). Only MAb BVD/CA27 (boCD46) precipitated the putative BVDV receptor protein boCD46, which has a molecular mass of about 56 kDa (Fig. 3, lanes 4, 6 and 8) (Schelp *et al.*, 2000).

In contrast, in lysates of BHK cells infected with SFV, the envelope protein p62 was visible after precipitation with MAb SFV/C8 (Fig. 3, lane 3). The nucleocapsid protein was not detected by surface biotinylation (Fig. 3). This is in agreement with the finding that the SFV envelope glycoproteins are transported to the cell surface where virus budding occurs (Zhao & Garoff, 1992).

Localization of viral proteins in subcellular fractions

Subcellular fractionation was performed to assess if, and to what extent, the envelope glycoproteins E^{RNS} and E2 and the non-structural proteins NS2-3 (ncp BVDV) and NS3 (cp BVDV) are membrane-associated. For this, it was found to be sufficient to analyse the membrane and cytosolic fractions, since this would be enough to establish whether the viral proteins of interest are soluble or not. As demonstrated by IF analysis, BVDV replicates in the cytoplasm of infected cells only; therefore, the nuclear fraction was discarded. The cytoplasmic components were fractionated by differential centrifugation to yield one fraction containing organelles with membrane-bound or membrane-associated proteins and one fraction with the soluble components of the cytoplasm. An additional step to separate the mitochondria was not performed, since these organelles are not involved in viral protein synthesis. To estimate the degree of contamination of each of the fractions, the specific activity of the tracking enzymes GAPDH and LDH (cytosolic fraction) and SDH and GluDH (membrane fraction) was determined. As expected, only a low

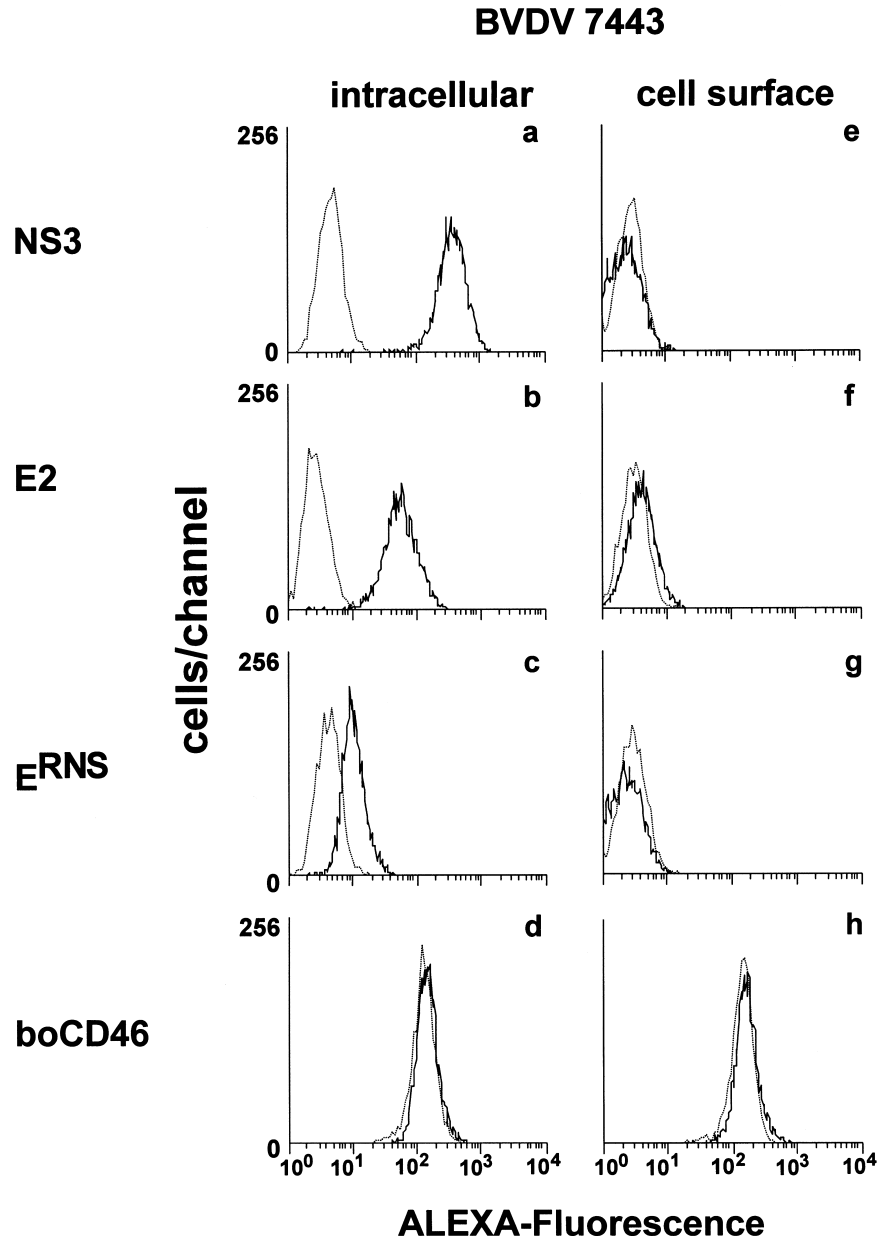


Fig. 2. Fluorescence histograms of BVDV strain 7443-infected (black line) and mock-infected (dotted line) MDBK cells at 48 h post-infection (m.o.i of 1) after intracellular (panels a–d) or surface (panels e–h) IF staining using the NS3-, E^{RNS}-, E2- and boCD46-specific MAbs. In permeabilized BVDV-infected cells, strong intracellular NS3- and E2-specific IF signals were detected. In contrast, no (NS3, panel e), or only marginal (E2, panel f), IF signals were detectable on the surface of BVDV-infected cells as compared to the mock-infected controls. A weaker E^{RNS}-specific IF signal was detected after intracellular staining with MAb BVD/C42 (panel c), but not on the cell surface (panel g). Staining of boCD46 with MAb BVD/CA27 served as a positive control (panels d and h). Only PI-negative cells were evaluated in the viable cell preparations after surface staining.

level of contamination between the two fractions was detected, comprising about 3–15% of the total enzyme activity only (Table 1). To detect the BVDV proteins in each of the two fractions, equal amounts of radioactive counts (d.p.m.) were incubated with pools of the three MAbs BVD/C42, CA3 and C16 (Fig. 4). In the fractions of mock-infected cells, no specific bands corresponding to BVDV proteins could be detected. In

contrast, in the membrane fraction of cells infected with ncp BVDV, prominent bands with molecular masses of about 48 (E^{RNS}), 56 (E2) and 125 kDa (NS2-3) were seen; a minor band with a molecular mass of about 20 kDa was also detected. In the corresponding cytosolic fraction, no bands corresponding to the NS2-3 protein or the E2 glycoprotein were detectable, while a weak band corresponding to the E^{RNS} protein was

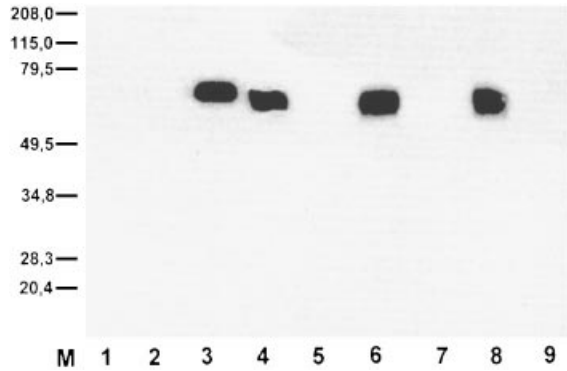


Fig. 3. Immunoprecipitation with specific MAbs of biotinylated cell surface proteins from lysates of BHK cells infected with SFV and MDBK cells infected with BVDV, respectively. Lane 1, negative control (mock-infected BHK cells immunoprecipitated with SFV/C8, a p62-specific MAb); lane 2, negative control (BHK cells infected with SFV and immunoprecipitated with SFV/C2, a nucleocapsid protein-specific MAb); lane 3, positive control (BHK cells infected with SFV and immunoprecipitated with MAb SFV/C8); lane 4, positive control (mock-infected MDBK cells immunoprecipitated with MAb BVD/CA27); lane 5, mock-infected MDBK cells immunoprecipitated with MAbs BVD/CA3, C46 and C16; lanes 6 and 7, MDBK cells infected with ncp BVDV strain 7443 and immunoprecipitated with MAbs BVD/CA27 and CA3, and C46 and C16, respectively; lanes 8 and 9, MDBK cells infected with cp BVDV strain NADL and immunoprecipitated with MAbs BVD/CA27 and CA3, and C46 and C16, respectively.

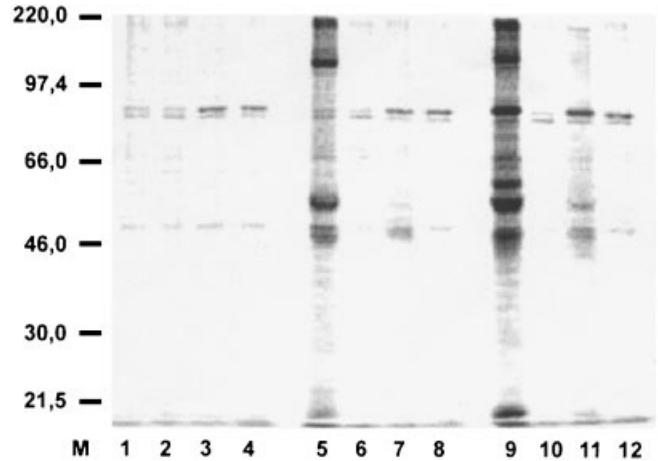


Fig. 4. Localization of BVDV-specific proteins in subcellular fractions of virus-infected cells. Cells were labelled with [³⁵S]methionine and subcellular fractions were prepared as described in Methods. Proteins from both the soluble cytosolic fractions (lanes 3, 4, 7, 8, 11 and 12) and the membranous fractions (lanes 1, 2, 5, 6, 9 and 10) were immunoprecipitated using MAbs BVD/CA3, C42 and C16 (uneven lanes) and BM/40 (negative controls, even lanes). Lanes 1–4, mock-infected MDBK cells; lanes 5–8, MDBK cells infected with ncp BVDV strain 7443; lanes 9–12, MDBK cells infected with cp BVDV strain NADL.

clearly visible. Similar results were found for the subcellular fractions of cells infected with cp BVDV. Here, both the NS2-3 and the NS3 proteins characteristic for cp BVDV were detected in the membrane fraction only. In this fraction, the 20 kDa protein and an additional protein with a molecular mass of about 60 kDa were also visible. In the cytosolic fraction, only weak bands corresponding to the E2 and E^{RNS} proteins could be precipitated.

Discussion

Many enveloped viruses acquire their envelope by budding through the plasma membrane of the host cell. Using the compartmentalization mechanisms of the cells, these viruses direct the insertion of their surface glycoproteins into the cell membrane (Birdwell & Strauss, 1974; Woodman & Edwardson, 1986). Another possibility for leaving the cell is budding into the cisternae of the ER, maturing in the Golgi apparatus and exocytosis or cell lysis (McNulty *et al.*, 1976). Regarding

Table 1. Total activity of the tracking enzymes

Total enzyme activity was defined as that recorded in the membrane and cytosol fractions of mock-infected cells and cells infected with ncp BVDV 7443 or cp BVDV NADL, respectively.

Enzyme	Fraction	Enzyme activity (%)		
		Mock-infected	ncp BVDV	cp BVDV
SDH	Membrane	91	92	91
	Cytosol	9	8	9
GluDH	Membrane	85	90	90
	Cytosol	15	10	10
GAPDH	Membrane	3	4	4
	Cytosol	97	96	96
LDH	Membrane	1	3	3
	Cytosol	99	97	97

pestiviruses, the available information is modest and partly inconsistent. Ultrastructural electron microscopy studies of bovine embryonic testis cells infected with BVDV demonstrated that virus replication took place totally within the cytoplasm, in association with structures formed from modified ER. Budding from the cell surface was not observed (Gray & Nettleton, 1987). Thus, the mechanism of release of mature virus particles from the host cell as well as the molecular pathways induced by cp BVDV are still unclear. In this study, a detailed localization of single viral proteins in and on BVDV-infected cells was performed in order to obtain information relating to the possible mechanisms of BVDV particle release.

In our experiments using CFM and FACS analysis, we obtained only strong intracellular but no extracellular IF signals. In addition, immunoprecipitation of biotinylated cell surface proteins from virus-infected cells with BVDV-specific MAbs showed that neither the glycoproteins E^{RNS} and E2 proteins nor the NS3 and NS2-3 proteins were detectable on the cell surface. Although this is in agreement with earlier experiments using the same MAbs to detect the antigens on the surface of viable BVDV-infected cells by IF assay (Greiser-Wilke *et al.*, 1991b), it contradicts more recent reports (Weiland *et al.*, 1999). Here, IF microscopy on live adherent cells as well as fluorescent cytometry analysis of viable cells led to the detection of the BVDV E^{RNS} and E2 glycoproteins on the cell surface. However, in further investigations by immune electron microscopy, Weiland *et al.* (1999) showed that the plasma membrane remained completely unlabelled and concluded that the positive IF signal was probably mediated by the binding of MAbs to virions adhering to the extracellular membrane and not to proteins integrated in the plasma membrane. Moreover, Weiland *et al.* (1999) did not use the very specific PI exclusion as a marker for cell viability during their FACS analysis, making it likely that the cells were damaged and thus permeabilized for the MAbs and, therefore, generating false-positive signals.

Localization of viral proteins in the soluble and membranous fractions of BVDV-infected cells showed that the proteins analysed (NS3, NS2-3, E^{RNS} and E2) appeared to be mainly membrane-bound, or at least membrane-associated, in cells infected with both cp and ncp BVDV biotypes. Interestingly, two additional proteins of about 20 and 60 kDa were precipitated from BVDV-infected cell lysates using the E2-specific MAb BVD/CA3. These proteins had been observed before in lysates of infected cells labelled under hypertonic initiation block (HIB) conditions (Donis & Dubovi, 1987). In RIP analysis using hyperimmune serum, the 20 kDa protein was visible after a prolonged exposure, whereas the 60 kDa protein was only detectable in lysates of cells labelled under HIB. The latter protein is probably the uncleaved E2p7 protein (Elbers *et al.*, 1996), whereas the origin of the 20 kDa protein remains unclear.

In the cytosolic fractions, no NS2-3, but small amounts of E2 and E^{RNS}, could be detected. This may be due to the contamination of the soluble fraction with membranous

components. On the other hand, it is conceivable that at least E^{RNS} is also present in a soluble form, as large amounts of the protein are shed into the medium by BVDV-infected cells (Rumenapf *et al.*, 1993). It remains to be determined whether the BVDV proteins analysed are membrane-bound or merely membrane-associated.

These results show similarities to the distribution of hepatitis C virus (HCV) proteins. The HCV structural proteins E1 and E2 and the core protein accumulate in the ER (Kim *et al.*, 1999). The NS2 protein derived from processing at the NS2-3 site is a transmembrane polypeptide, with the C terminus translocated in the lumen of the ER and the N terminus located in the cytosol (Santolini *et al.*, 1995). The remaining non-structural proteins are accumulated in the ER and the Golgi region (Kim *et al.*, 1999). In contrast to BVDV NS3, HCV NS3 was detected in the nucleus as well as in the cytoplasm (Muramatsu *et al.*, 1997). Ultrastructural studies of cells infected with HCV showed that, in some instances, the outer membranes of the particles were connected to the ER membrane, indicating possible virus budding into the cisternae (Serafino *et al.*, 1997).

In summary, we conclude that mature BVDV particles are not released by budding from the plasma membrane but by budding at the membranes of the ER, similar to HCV. Since none of the viral proteins analysed was detected on the cell surface, this is not in agreement with the concept that the cell association of pestiviruses is due to firm adhesion of virions to the cell surface (Weiland *et al.*, 1999). In addition, as the only currently known marker protein for cytopathogenicity (Donis & Dubovi, 1987), namely the NS3 protein, was found to be membrane-bound, or at least membrane-associated, to cells infected with both virus biotypes, there seems to be no correlation between the location of this protein and its function in the cell.

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