Expression of the surface glycoprotein E2 of 
**Bovine viral diarrhea virus** by recombinant 
vesicular stomatitis virus

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This study analysed the transport behaviour of the glycoprotein E2 of **Bovine viral diarrhea virus** (BVDV) expressed from recombinant vesicular stomatitis virus (rVSV). E2 protein was found to be retained at an intracellular compartment. A chimeric protein containing the membrane anchor and cytoplasmic tail of the VSV G protein, E2–G(MT), was transported to the cell surface. Only the latter protein was incorporated into rVSV particles in significant amounts. A soluble form of E2 lacking the membrane anchor, E2(MTdel), appeared to be affected in conformational stability. In contrast to both membrane-anchored forms of E2, expression of the soluble form was detectable only by immunofluorescence microscopy but not by Western blotting. These results are in agreement with reports of intracellular retention of the E2 protein due to a retention signal in the membrane anchor. However, in another analysis of E2 expressed from rVSV, E2 protein was reported to be transported to the cell surface and incorporated into VSV particles [Grigera, P. R., Marzocca, M. P., Capozzo, A. V. E., Buonocore, L., Donis, R. O. & Rose, J. K. (2000). *Virus Res* **69**, 3–15]. Reasons for these contradictory results are discussed.

**INTRODUCTION**

*Bovine viral diarrhea virus* (BVDV) is a member of the genus *Pestivirus* within the family *Flaviviridae* (Wengler et al., 1995). BVDV is the causative agent of bovine viral diarrhoea/mucosal disease, which has a high economic impact (Baker, 1995). The viral genome consists of a single molecule of unsegmented, positive-stranded RNA of about 12 300–16 500 nt (Tautz et al., 1994). The RNA serves as a template for synthesis of a polyprotein, which is co- and post-translationally cleaved into the structural and non-structural proteins (Rümenapf et al., 1993). The structural proteins comprise the capsid (C) protein and three membrane glycoproteins, namely E\(\text{ms}\), E1 and E2 (Meyers & Thiel, 1996).

The E2 protein is the major target of the protective immune response elicited against BVDV infection. E2 is a main component of the virions and, together with E\(\text{ms}\) and E1, forms the surface projections protruding from the viral envelope (Thiel et al., 1991). In infected cells as well as in virions, it can be found as a homodimer of about 100 kDa or, together with E1, as a heterodimer of about 75 kDa (Thiel et al., 1991). Immunostaining of infected cells indicates that E2 and E\(\text{ms}\) are absent from the plasma membrane (Greiser-Wilke et al., 1991; Grummer et al., 2001; Weiland et al., 1999). Viral envelope proteins occasionally detected by surface staining have been attributed to virus particles accumulating on the cell surface (Weiland et al., 1999). The absence of BVDV glycoproteins from the plasma membrane is consistent with the failure to detect virus budding from the cell surface (Gray & Nettleton, 1987), suggesting that BVDV probably matures at intracellular membranes. Recently, we have shown that the E2 protein of BVDV contains an intracellular localization signal that is responsible for retention in the endoplasmic reticulum (Köhl et al., 2004).

The E2 protein has been expressed by different plasmids (Harpin et al., 1997, 1999; Nobiron et al., 2001, 2003) and viral vectors. The latter include baculoviruses (Bolin & Ridpath, 1996) and vesicular stomatitis virus (VSV) (Grigera et al., 2000). The E2 protein of the related *Classical swine fever virus* (CSFV) has been expressed by influenza virus (Zhou et al., 1998) and lentiviral pseudotypes (Wang et al., 2004).

VSV can be used as a high-level expression vector by introducing an extra transcription unit into the DNA copy of the VSV genome. This vector allows recovery of VSV recombinants expressing an additional gene between the G and L genes (Schnell et al., 1996a, b; Kretzschmar et al., 1997). Recombinant VSV (rVSV) has been used for the expression of many foreign proteins, for example CD4
(Schnell et al., 1996b), the haemagglutinin and the neuraminidase of influenza viruses (Kretzschmar et al., 1997; Roberts et al., 1998), the Gag and Env proteins of human immunodeficiency virus (Johnson et al., 1997; Rose et al., 2000, 2001; Haglund et al., 2000, 2002), the haemagglutinin and fusion proteins of Measles virus (Schnell et al., 1996b; Schlereth et al., 2000), the fusion and attachment proteins of Human respiratory syncytial virus (Kahn et al., 1997), the E2 protein of BVDV (Grigera et al., 2000), the structural proteins C, E1 and E2 of Hepatitis C virus (HCV) (Ezelle et al., 2002), or the glycoproteins of Marburg virus, Ebola virus and Lassa virus (Garbutt et al., 2004).

The advantage of the rVSV vector system is the great tolerance of VSV to genetic manipulations and the simple genetic organization with only five genes. In addition, VSV does not undergo reassembly or integrate into the host genome (Ezelle et al., 2002; Wagner & Rose, 1996).

rVSV has also been used as a live-virus vaccine candidate (Ezelle et al., 2002; Grigera et al., 2000; Haglund et al., 2002; Roberts et al., 1998; Rose et al., 2000, 2001; Schlereth et al., 2000). An attractive feature of rVSV as a tool for vaccination studies is that naturally occurring VSV infections in humans are rare and relatively harmless. The seroprevalence of antibodies specific for VSV is generally low within the human population (Ezelle et al., 2002; Wagner & Rose, 1996). VSV induces a strong humoral and cellular immune response (Fehr et al., 1996; Roberts et al., 1998).

Grigera et al. (2000) constructed rVSV containing the E2 protein of BVDV (VSV–E2). In those experiments, E2 was reported to be expressed at the cell surface of infected cells and incorporated into VSV particles. This transport behaviour of E2 is in contrast to our results obtained with expression plasmids (Köhl et al., 2004) and to results reported about the homologous proteins of other flaviviruses. The E2 protein of HCV and the E protein of Tick-borne encephalitis virus (TBEV) also contain a retention signal within the membrane anchor that prevents transport to the cell surface (Allison et al., 1999; Cocquerel et al., 1998).

We analysed the expression and localization of E2, a chimeric E2 and an anchorless form of E2. We found that E2 protein is retained intracellularly. Chimeric E2 protein containing the cytoplasmic tail and the membrane anchor of the VSV G protein was transported to the cell surface. Only the latter protein could be incorporated into VSV particles in significant amounts.

METHODS

Cells and viruses. BSR-T7/5 cells, a subline of BHK-21 cells stably expressing T7 RNA polymerase under the control of the cytomegalovirus promoter (Buchholz et al., 1999; Sutter et al., 1995), were kindly provided by Dr Conzelmann (Max-von-Pettenkofer-Institut, Munich, Germany). The cells were grown in Eagle’s minimal essential medium (EMEM) supplemented with non-essential amino acids, 0.5 mg geneticin ml⁻¹ and 5 % fetal calf serum (FCS). BHK-21 cells were obtained from DSMZ and grown in EMEM supplemented with non-essential amino acids and 4 % FCS. Primary fetal calf kidney (FCK) cells were prepared as described previously (Orban et al., 1983) and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % FCS. The NADL strain of BVDV was kindly provided by Dr Frey (Institute for Virology, University of Veterinary Medicine Hannover, Germany). Recombinant Vaccinia virus (VV), MVA-T7, was provided by Dr Sutter (Technische Universität, München, Germany) and propagated in primary chicken fibroblasts.

Plasmids. The plasmid pVSV-XN2 consisted of pBluescript SK(+) containing the cDNA of VSV. The plasmids pBS-N, pBS-P and pBS-L encoded the N, P and L proteins of the VSV polymerase complex (Lawson et al., 1995; Schnell et al., 1996a, b). All plasmids were kindly provided by Dr J. K. Rose (Yale University School of Medicine, CT, USA) and Dr M. J. Schnell (Thomas Jefferson University, PA, USA).

Cloning of E2 genes and integration into VSV cDNA. The open reading frames of E2, E2–G(MT) and E2(MTdel) (Fig. 1) were constructed according to a published protocol (Köhl et al., 2004). The signal peptide portion (aa 1–23) of the VSV G gene was fused to the 5’ end of E2, E2–G(MT) and E2(MTdel). All E2 genes contain a Xhol restriction site at the 5’ end and an Nhel restriction site at the 3’ end, which were used for cleavage by restriction enzymes. The cDNA of VSV (pVSV-XN2) was also cleaved by the restriction enzymes Xhol and Nhel, which are situated between the G and L genes of VSV. Taking advantage of the Xhol and Nhel restriction sites, the E2 open reading frames were ligated into the cDNA of VSV (Fig. 1) and sequenced.

Recovery of rVSV. BSR-T7/5 cells (1.5 × 10⁵ ml⁻¹) were seeded in 35 mm diameter dishes and incubated for 24 h at 37 °C in 5 % CO₂. BSR-T7/5 cells (~ 95 % confluent) were infected with MVA-T7, a recombinant VV encoding the T7 RNA polymerase, and transfected after 1 h with 5 μg parental VSV cDNA (pVSV-XN2), VSV-E2, VSV-E2–G(MT) or VSV-E2(MTdel). 1.5 μg pBS-N, 2.5 μg pBS-P and 1 μg pBS-L using the Lipofectamine 2000 transfection reagent (Invitrogen). After 48 h of incubation at 37 °C in 5 % CO₂, supernatants of transfected cells were collected and centrifuged at 2000 g for 10 min to remove cell debris. Clarified supernatants were passed through a 0.2 μm pore-size filter to remove VV. Two millilitres of this filtrate was used to infect confluent BHK-21 cells. After 20 h, virus was harvested and stored at ~ 80 °C.

Growth analysis. Confluent BHK-21 cells were infected with VSV-E2, VSV-E2–G(MT) and VSV-E2(MTdel) or parental VSV at an m.o.i. of 1. After an adsorption time of 1 h, the inoculum was removed, the cells were washed three times with PBS, and EEM containing 1 % and 5 % fetal calf serum (FCS) were obtained from DSMZ and grown in EMEM supplemented with non-essential amino acids and 4 % FCS. Samples were taken at 0, 3, 6, 9, 12, 15 and 24 h post-infection (p.i.) and frozen at ~ 80 °C. The experiment was performed in duplicate and each sample was analysed twice by plaque assay.

Immunofluorescence analysis. Confluent BHK-21 cells grown on 12 mm diameter coverslips were infected with VSV-E2, VSV-E2–G(MT), VSV-E2(MTdel) or parental VSV at an m.o.i. of 5. At 6 h after infection, cells were fixed and stained with antibodies specific for VSV or E2 according to a published protocol (Köhl et al., 2004).

Western blot analysis of infected cells. Confluent BHK-21 cells grown on 35 mm diameter dishes were infected with VSV-E2, VSV-E2–G(MT), VSV-E2(MTdel) or parental VSV at an m.o.i. of 5. At 6 h after infection, cell lysates were prepared and analysed as described previously (Köhl et al., 2004).
**Expression of BVDV E2 protein by recombinant VSV**

**Biotinylation and immunoprecipitation of surface proteins.** Confluent BHK-21 cells grown on 35 mm diameter dishes were infected with VSV-E2, VSV-E2–G(MT), VSV-E2(MTdel) or parental VSV at an m.o.i. of 5. At 6 h after infection, cells were incubated for 20 min at 4°C with 0.5 ml PBS containing 0.5 mg Sulfo-NHS-biotin (Pierce) ml⁻¹. Cells were washed once with 0.1 M glycine in PBS and incubated in the same solution for 15 min at 4°C. Cells were solubilized in NP-40 lysis buffer and centrifuged to remove cell debris. Supernatants were collected and incubated with 50 μl of a 50% slurry of protein A–Sepharose (Sigma) and 20 μl of the monoclonal antibodies (mAbs) BVD/CA1 and BVD/CA3 (Bolin et al., 1988) to immunoprecipitate the E2 proteins or 5 μl of a polyclonal anti-VSV serum to immunoprecipitate the G protein. These mixtures were incubated overnight at 4°C by shaking. Immunoprecipitates were collected by centrifugation, washed three times with NP-40 lysis buffer and eluted by boiling the beads in twofold-concentrated SDS-sample buffer. The precipitated proteins were run on a 10% SDS-polyacrylamide gel under non-reducing conditions and transferred to nitrocellulose using a semi-dry blotting technique (Kyhe-Anderson, 1984). The membrane was incubated with blocking reagent (Roche Molecular Biochemicals) overnight at 4°C and incubated with streptavidin–peroxidase (diluted 1:1000; Amersham Pharmacia Biotech) for 1 h at room temperature. The membrane was incubated for 1 min with a chemiluminescent peroxidase substrate (BM chemiluminescence blotting substrate; Roche Molecular Biochemicals). The resulting light emission was detected and documented using a supercooled CCD camera (Chemi-Doc System; Bio-Rad).

**Immunoprecipitation of soluble E2 proteins.** Supernatants of BHK-21 cells infected with VSV-E2, VSV-E2–G(MT), VSV-E2(MTdel) or parental VSV were collected at 6 h p.i. and cell debris was pelleted. One millilitre of each supernatant was incubated with 100 μl of a 50% slurry of protein A–Sepharose and 50 μl of the antibodies BVD/CA1 and BVD/CA3 and incubated overnight at 4°C with shaking. Samples were prepared and analysed as described above.

**Purification of recombinant viruses.** Confluent BHK-21 cells grown in 145 mm diameter dishes were infected with VSV-E2, VSV-E2–G(MT), VSV-E2(MTdel) or parental VSV at an m.o.i. of 5. For purification of the virions, four dishes were taken for each virus. After 20 h, supernatants were centrifuged at 2000 g for 15 min at 4°C to remove cell debris. Sucrose gradient centrifugation was performed as described previously (Krempl & Herrler, 2001). The pellets of the purified virions were resuspended in 100 μl PBS lacking Ca²⁺ and Mg²⁺ (PBSM). For Western blot analysis of purified virions, samples were diluted 1:100 in PBS prior to mixing with two-fold-concentrated SDS-sample buffer. Aliquots of 20 μl were run on a 10% SDS-polyacrylamide gel under non-reducing conditions and transferred to nitrocellulose using a semi-dry blotting technique. The blots were blocked overnight at 4°C with blocking reagent (Roche) and incubated either with a cocktail of the BVD/CA1 and BVD/CA3 mAbs (each diluted 1:60 in PBS) or with a polyclonal rabbit anti-VSV serum (1:10 000 in PBS) followed by incubation with biotinylated anti-mouse or anti-rabbit immunoglobulin serum (1:1000; Amersham). After incubation with a streptavidin–peroxidase complex (1:2000; Amersham), the membrane was incubated for 1 min with a chemiluminescent peroxidase substrate. The resulting light emission was detected and documented using a supercooled CCD camera.

**Neutralization assays.** Neutralization assays were performed on confluent FCK cells grown on 24 mm diameter dishes. A serial two-fold dilution was prepared from a polyclonal anti-VSV serum (1:4000 to 1:1 024 000) and a polyclonal anti-BVDV serum (1:160 to 1:10 024) in DMEM. Each antibody dilution was incubated with 30 p.f.u. rVSV or BVDV for 45 min at 37°C, 5% CO². The mixtures of virus and antibodies were used to infect FCK cells for 1 h at 37°C. Following adsorption, cells were overlaid with 0.9% seaplague agarose (Biozym) in DMEM. VSV-infected cells were incubated for 24 h and BVDV-infected cells for 72 h at 37°C. Viable cells were...
fixed and stained with crystal violet. Plaques of three independent experiments were counted to determine the neutralizing activity for each antibody dilution.

**Electron microscopy and immunogold labelling.** Two 14.5 cm diameter dishes seeded with BHK-21 cells were infected with VSV-E2, VSV-E2–G(MT), VSV-E2(MTdel) and parental VSV at an m.o.i. of 2. Virus was harvested 16 h p.i. and cell debris was removed by centrifugation of the supernatant at 2000 g for 15 min. Virions were pelleted through a 20% sucrose cushion by centrifugation at 120,000 g for 90 min. Virus pellets were resuspended in 200 µl PBSM, adsorbed to nickel-coated grids for 60 min and blocked for 30 min in 1% BSA in PBS. Grids were placed on a 50 µl drop of the antibodies BVD/CA1 and BVD/CA3 (1:1) for 2 h. After six washing steps for 5 min with PBS, 0.5% BSA and 0.1% gelatin, grids were placed on a 50 µl drop of anti-mouse IgG labelled with 6 nm gold particles (Aurion). Grids were washed as described above, followed by six additional washing steps with PBS. Samples were fixed for 5 min with 2.5% glutaraldehyde, washed with water and subjected to negative staining by incubation of the grids on a 50 µl drop of anti-mouse IgG labelled with 6 nm gold particles (Aurion). Grids were washed as described above, followed by six additional washing steps with PBS. Images of viruses were obtained using a Zeiss EM10C/EM10CR microscope.

**RESULTS**

**Generation and propagation of rVSV**

In this study, three forms of the E2 protein were analysed: (i) unmodified E2, (ii) a chimeric protein in which the ectodomain of E2 was connected to the membrane anchor and cytoplasmic tail of the VSV G protein [E2–G(MT)] and (iii) a truncated E2 lacking a membrane anchor/cytoplasmic tail (E2-MTdel) (Fig. 1). Translocation of the three proteins into the lumen of the endoplasmic reticulum was mediated by the signal peptide of the VSV G protein. cDNAs encoding the open reading frames of one of these proteins were inserted into a transcription unit located between the G and L genes of the genomic VSV cDNA (Fig. 1). Viruses were rescued by transfection of BSR-T7/5 cells with plasmids containing the cDNA of VSV-E2, VSV-E2–G(MT), VSV-E2(MTdel) or parental VSV and expression plasmids encoding the N, P and L proteins. To increase T7 expression, cells were infected with MVA-T7 prior to transfection. At 48 h post-transfection, supernatants were used to infect BHK-21 cells for virus propagation. In this way, VSV-E2, VSV-E2–G(MT), VSV-E2(MTdel) and parental VSV were rescued successfully.

The growth kinetics of VSV-E2, VSV-E2–G(MT), VSV-E2(MTdel) and parental VSV were analysed with BHK-21 cells. As shown in Fig. 2, VSV-E2, VSV-E2–G(MT) and VSV-E2(MTdel) showed growth characteristics similar to those of parental VSV. The addition of any of the different E2 open reading frames did not affect the growth behaviour of the recombinant viruses in cell culture.

**Expression and localization of E2, E2–G(MT) and E2(MTdel)**

In order to compare expression of the different forms of the E2 protein, cell lysates of infected BHK-21 cells were separated by SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis. Using an antiserum
specific for VSV membrane proteins, all rVSVs showed the same band pattern for the G (60 kDa) and M (30 kDa) proteins of VSV (Fig. 3a). As shown in Fig. 3(b), all analysed E2 proteins were expressed by the viral vector VSV. E2 was detected in a monomeric and a dimeric form with molecular masses of about 50 and 100 kDa, respectively. E2–G(MT) was also detected in a monomeric and a dimeric form with molecular masses of about 60–66 and 130 kDa. The larger size of the chimeric protein is explained only to a minor degree by the longer membrane anchor/cytoplasmic tail portion derived from the VSV G protein. The major size difference is accounted for by fact that E2 is retained intracellularly and only contains mannose-rich oligosaccharides, whereas the N-glycans of E2–G(MT) are processed on their way to the cell surface into complex-type oligosaccharides (Köhler et al., 2004). E2(MTdel) was identified only as a monomer with a molecular mass of about 40–46 kDa. There was a difference in the intensity of the E2 bands. The signals observed with E2–G(MT) were stronger than those of E2. E2(MTdel) was detected only as a faint band on Western blots.

To localize the different E2 proteins in cells, immunofluorescence and cell-surface biotinylation experiments were performed using BHK-21 cells infected with VSV-E2, VSV-E2–G(MT), VSV-E2(MTdel) or parental VSV. As shown in Fig. 4(a), immunofluorescence microscopy of permeabilized cells revealed bright signals for all three forms of E2. This finding indicated that the weak detection of E2(MTdel) in Fig. 3 was not due to low expression of this truncated E2 but due to the experimental setting of the Western blot (see Discussion). When non-permeabilized cells were analysed, chimeric protein E2–G(MT) was the only protein detectable at the cell surface. To confirm this result, surface biotinylation experiments were performed (Fig. 4b). Again, E2–G(MT), but not E2 or E2(MTdel), was detected on the cell surface.

**Incorporation into virus particles**

Next, we addressed the question of whether E2, E2–G(MT) and E2(MTdel) were incorporated into the envelope of rVSVs. Virions were purified by sucrose gradient centrifugation. Viral proteins were separated by SDS-PAGE and analysed by Western blotting using a rabbit antiserum specific for VSV or mAbs specific for E2 protein. SDS-PAGE was performed under non-reducing conditions to preserve the dimeric forms of E2. Fig. 5 shows that all rVSVs showed the same band pattern of VSV proteins. Using E2-specific mAbs, only E2–G(MT) and E2 were identified in the virus fraction. Immunostaining indicated that the chimeric E2–G(MT) protein was present in a larger amount than the unmodified E2 protein. The latter protein required a threefold-longer development time for visualization. Therefore, the quantitative difference between E2 and E2–G(MT) levels in the purified virus fractions (Fig. 5) was greater than that observed in infected cells (Fig. 3).

To get more information about incorporation of E2 and E2–G(MT) into the VSV envelope, we investigated VSV particles by electron microscopy. Purified virions were stained using E2-specific mAbs, followed by secondary gold-labelled antibodies. Fig. 6 shows gold particles bound to VSV-E2–G(MT). Only a few gold particles were observed in the VSV-E2 sample and they were not clearly associated with the viral surface (see arrow). No gold-labelled secondary antibodies were bound to VSV-E2(MTdel) and parental VSV. Morphological differences between the different viruses were not observed.

**Neutralization of rVSVs by different antibodies**

To analyse whether the E2 proteins contributed to the infection process of rVSV, we performed neutralization assays with antibodies specific for VSV or BVDV (Table 1). Using an anti-VSV antiserum, all rVSVs were neutralized.

![Fig. 4. Analysis of cell-surface expression of E2 proteins in BHK-21 cells infected with VSV-E2, VSV-E2–G(MT), VSV-E2(MTdel) or parental VSV. (a) Immunofluorescence analysis of infected BHK-21 cells. Cells in the upper panel were fixed and permeabilized with methanol/acetone and immunostained to detect intracellular antigen. Cells in the lower panel were fixed with paraformaldehyde and immunostained to detect cell-surface expression of E2 protein. (b) Surface biotinylation of infected BHK-21 cells. Infected cells were labelled with Sulfo-NHS-biotin and E2 proteins were immunoprecipitated using specific mAbs. The immunoprecipitates were separated by SDS-PAGE under non-reducing conditions, transferred to nitrocellulose membrane and incubated with streptavidin–peroxidase. Molecular mass markers (kDa) are indicated.](http://vir.sgmjournals.org)
with no significant differences compared with parental VSV. As a negative control, we used BVDV, which was not neutralized by this serum. When an antiserum specific for BVDV was analysed, BVDV was neutralized efficiently. VSV-E2, VSV-E2–G(MT), VSV-E2(MTdel) and parental VSV were not neutralized by this serum. These results showed that there was no significant difference in neutralization behaviour between parental VSV and rVSVs expressing any of the E2 proteins.

**DISCUSSION**

We have shown that the E2 protein of BVDV expressed from rVSV was incorporated into virus particles in substantial amounts, whereas incorporation of type of retention signal has been reported for the E2 proteins of other flaviviruses, e.g. HCV and TBEV (Allison et al., 1999; Cocquerel et al., 1998). Our data demonstrate that the retention signal of the BVDV-E2 protein is also functional in the context of a VSV infection. In contrast to our data, Grigera et al. (2000) reported that the E2 protein of BVDV expressed from rVSV was present on the cell surface. An explanation of these contradictory results may be that, upon strong expression of E2, the cellular retention machinery may become saturated and excess protein will be transported to the cell surface. A similar situation has been reported for the surface protein S of coronaviruses. The S protein of *Transmissible gastroenteritis virus* has been shown to contain a tyrosine-based retention signal in the cytoplasmic tail (Schwegmann-Wessels et al., 2004). When the coronavirus surface protein is expressed from VV or from baculovirus vectors rather than from expression plasmids, some protein is transported to the cell surface, albeit with low efficiency. It would be interesting to know the transport kinetics of the E2 protein under the expression conditions applied by Grigera and co-workers.

Replacement of the membrane anchor of E2 by the anchor domain of a surface protein results in a protein that is transported to the cell surface (Köhl et al., 2004). Recognition of the chimeric protein E2–G(MT) by mAbs suggested that there were no major differences in the conformation between the chimeric protein and the unmodified protein. In contrast, E2(MTdel) was recognized readily in immunofluorescence experiments whereas only a weak signal was observed on Western blots for the monomeric form of E2. No dimeric form was detectable. From this result, we concluded that E2(MTdel), which is expected to be a soluble form of E2, is less stable than the two membrane-anchored forms of E2. In our Western blot analysis, the proteins were subjected to electrophoretic separation in the absence of reducing agents; under these conditions, the membrane-anchored E2 proteins are retained in a dimeric form that is recognized by mAbs after immobilization of the proteins on membranes. The soluble E2 protein was not able to retain the dimeric form and the immobilized monomeric form renatured only inefficiently, as indicated by the weak interaction with mAbs. Therefore, the membrane anchor appears to play an important role in stabilizing the conformation of E2. This interpretation does not exclude the possibility that, with plasmid expression systems, soluble E2 dimers are detectable by Western blotting at varying intensity (Grigera et al., 2000; Köhl et al., 2004).

The chimeric protein E2–G(MT) should be helpful in future studies to analyse the importance of intracellular retention of E2 in BVDV infection. As the E2 protein is the major target of neutralizing antibodies, the absence of this protein from the cell surface may prolong the survival time of infected cells. This question needs to be addressed in future experiments in the context of a BVDV infection.

rVSV was found to incorporate E2–G(MT) into virus particles in substantial amounts, whereas incorporation of
E2 (with unmodified membrane anchor/cytoplasmic tail) was very inefficient. A higher expression level of the chimeric protein (Fig. 3) may contribute to this finding. However, the difference is explained more convincingly by the transport behaviour of the two proteins. E2–G(MT) is transported to the cell surface where VSV matures by a budding process from the plasma membrane. As the E2 protein is retained intracellularly, it is not expected to be packed into VSV particles. Grigera et al. (2000) reported that the E2 protein is incorporated into VSV virions. This difference is probably due to the fact that, under their conditions, some E2 protein was transported to the cell surface for reasons that have been discussed above. In this context, it is interesting to note that incorporation of the E2 protein of a related pestivirus, CSFV, also required exchange of the membrane anchor and cytoplasmic tail for efficient incorporation into recombinant influenza virions (Zhou et al., 1998). The unmodified E1 and E2 proteins of the same virus have been shown to be incorporated into lentiviral pseudotypes (Wang et al., 2004). However, the amount of protein detected was very low and infectivity was detectable only by use of the sensitive luciferase detection system. It would be interesting to know whether chimeric E1/E2 proteins are incorporated more efficiently into these viral pseudotype particles.

As no difference was detected between E2 and E2–G(MT) in reactivity with mAbs, rVSV expressing E2–G(MT) may be an interesting tool for immunization studies. It would be interesting to know whether rVSV-E2 or rVSV-E2–G(MT) is more potent in inducing a protective immune response. At present, such experiments are hampered because rVSVs expressing foreign viral surface proteins are classified in Germany as agents to be handled under Biosafety Level 3 conditions. One concern about enveloped viruses expressing foreign viral surface proteins is an altered tropism due to the biological activities of the foreign protein. In the case of E2 protein, our neutralization assays indicated that the recombinant virus fully depends on the G protein for virus entry and that E2 was not functional in this process to a measurable extent. Entry of flaviviruses requires both the E1 and E2 proteins and in the case of BVDV presumably also the Erns protein. Therefore, rVSV expressing only the E2 protein of BVDV may not differ in its biological activities from unmodified VSV.

**Table 1. Neutralization of rVSVs**

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<th>VSV</th>
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<tr>
<td>VSV-E2</td>
<td>1 : 16 000</td>
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<tr>
<td>VSV-E2–G(MT)</td>
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<tr>
<td>VSV-E2(MTdel)</td>
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<td>VSV</td>
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<td>BVDV</td>
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*Neutralization titres were expressed as antibody dilutions that caused a 50 % plaque reduction compared with the control.
†The dilution of 1 : 4000 was the highest concentration tested in this assay and did not contain any neutralizing activity.

**REFERENCES**


t and E2 at the cell surface and on isolated particles. *J Gen Virol* 80, 1157–1165.
