Formation of bovine viral diarrhea virus E1–E2 heterodimers is essential for virus entry and depends on charged residues in the transmembrane domains

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The envelope of bovine viral diarrhea virus (BVDV) contains the glycoproteins Erns, E1 and E2. Complementation of a recombinant vesicular stomatitis virus (VSV) with BVDV glycoproteins resulted in infectious pseudotyped viruses. To elucidate the specific role of each of the single envelope glycoproteins during viral entry, pseudotypes were generated bearing the BVDV envelope proteins in different combinations. Pseudoviruses that contained E1 and E2 but not Erns were infectious, indicating that Erns is dispensable for virus entry. VSV/BVDV pseudotypes with chimeric proteins (the ectodomain of the BVDV glycoprotein and the transmembrane domain of the VSV-G protein) were not infectious. The fact that E1–E2 heterodimers were not detected if one of the proteins was chimeric indicated that the heterodimers are crucial for BVDV entry. It was shown by site-directed mutagenesis that the charged amino acids in the transmembrane domains of BVDV E1 (lysine and arginine) and the charged amino acid in the transmembrane domain of E2 (arginine) play a key role in heterodimer formation. Pseudoviruses bearing the mutation E2-R/A, where the charged amino acid was substituted by alanine, were not infectious, supporting the hypothesis that E1–E2 heterodimers are essential for BVDV entry.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an enveloped, positive-stranded RNA virus that belongs to the genus Pestivirus in the family Flaviviridae. Other species within this genus are classical swine fever virus (CSFV) and border disease virus of sheep. The genome consists of a single open reading frame, which is translated into a polyprotein that is co- and post-translationally cleaved into structural and non-structural proteins (Rümenapf et al., 1993). The structural proteins comprise the nucleocapsid C protein and three envelope glycoproteins, Erns, E1 and E2, of which at least Erns and E2 are postulated to be responsible for virus attachment and/or cell entry (Donis & Dubovi, 1987; Hulst & Moormann, 1997). E1 is assumed to function as a membrane anchor for E2 (Rümenapf et al., 1993). Erns and E2 form disulphide-linked homodimers, whereas E1 is found as heterodimers in association with E2 (Rümenapf et al., 1993; Weiland et al., 1990). Neither Erns nor E2 is detectable on the cell surface of infected cells, but they are associated with intracellular membranes (Greiser-Wilke et al., 1991; Grummer et al., 2001). Recently, an endoplasmic reticulum (ER) retention signal within the membrane anchor of the E2 protein was identified (Köhletal., 2004). It is well known that the Erns protein is secreted from infected cells due to the lack of a typical transmembrane anchor (Fetzer et al., 2005; Rümenapf et al., 1993).

The exact function and interaction of the three envelope proteins in virus entry is as yet inconclusive. For CSFV, it has been shown using lentiviral pseudotypes that E1 and E2 are sufficient to mediate virus entry, whilst Erns is dispensable in this process (Wang et al., 2004). On the other hand, it was shown that pestiviral Erns has the ability to bind to glycosaminoglycans, which implies a possible role in initial binding to the surface of permissive cells (Hulst et al., 2001; Iqbal et al., 2000).

To analyse further the essential interactions of the BVDV glycoproteins and their role in virus entry, vesicular stomatitis virus (VSV) pseudotypes containing BVDV glycoproteins were constructed. Because VSV gets its envelope by budding at the plasma membrane, chimeric Erns, E1 and E2 containing the membrane anchor and cytoplasmic tail of glycoprotein G of VSV (VSV-G) were generated to redirect the BVDV envelope proteins...
efficiently to the plasma membrane. In contrast to infected cells, in transiently transfected cells, BVDV envelope proteins were found in the cytoplasm but also at the plasma membrane. This enabled the generation of pseudotypes containing native as well as chimeric BVDV glycoproteins. Protein interactions in transfected cells were analysed by site-directed mutagenesis. Infection assays with variants of VSV/BVDV pseudotypes were carried out to explore the prerequisites for successful BVDV entry.

METHODS

**Cells.** HeLa and BHK-21 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (FCS). Fetal bovine kidney (FBK) cells were grown in DMEM with 5% bovine serum that was free of BVDV and corresponding antibodies. BHK-G43 cells, a stable cell line conditionally expressing the VSV-G protein (Hanika et al., 2005), were grown in selection medium (Eagle’s minimal essential medium supplemented with 250 µl hygromycin B ml⁻¹, 1 mg zeocin ml⁻¹ and 5% FCS). All cell cultures were maintained at 37 °C and 5% CO₂.

**Virus.** ΔG-VSV-G is a recombinant VSV with the gene for enhanced green fluorescent protein (EGFP) replacing the glycoprotein G gene (Hanika et al., 2005). For propagation of ΔG-VSV-G, BHK-G43 cells were treated with mifepristone (diluted 1:1000) and infected with the recombinant VSV 24 h later. Cell culture supernatants were harvested and cell debris removed by centrifugation at 24 h post-infection (p.i.). The culture supernatants were aliquotted and stored at −80 °C.

**Construction of plasmids.** Chimeric E⁹⁹, E₁ and E₂ (E⁹⁹-MAT, E₁-MAT and E₂-MAT) were generated by replacing the putative transmembrane domains of the BVDV envelope proteins with the membrane anchor (MA) and cytoplasmic tail (T) of the VSV-G protein. To generate plasmids containing E⁹⁹-MAT, E₁, E₂-MAT, an overlapping PCR technique was performed as described previously (Köhler et al., 2004). The BVDV E⁹⁹ and E₁ genes were amplified from a full-length clone, pACNR/NADL (Mendez et al., 1998), by PCR. The gene fragments for the relevant parts of the VSV-G protein were amplified using the plasmid pTM1-E2-G(MT) (Köhler et al., 2004). Purified PCR products were mixed in a molar ratio of 1:1:1 and hybridization was carried out as reported previously (Köhler et al., 2004). After amplifying the hybrid genes, the PCR products were digested with BamHI and Xhol and the fragments ligated into the vector pCG1 (Cathomen et al., 1995). For site-directed mutagenesis for generation of E₁-C/A, E₁-G/S, Flag–E₁-K, Flag–E₁-R and Flag–E₁-KR, modified sense and antisense primers were used. To generate E₂-MAT, E₂ and E₂-R/A, the genes were amplified from pTM1-E2-G(MT), pTM1-E2 or pTM1-E2(R/A) (Köhler et al., 2004), respectively. BamHI and Xhol restriction sites were added to the 5' ends of the respective sense and antisense primers. After cutting with BamHI and Xhol, the PCR products were ligated into vector pCG1 with T4 ligase (Fermentas). The combined protein domains used are shown in Fig. 1. Oligonucleotide sequences are available in Supplementary Table S1 (available in JGV Online).

**Transfection.** Transfections were carried out using Lipofectamine 2000 (Invitrogen Life Technologies) as indicated by the manufacturer. For co-transfections, the recommended amount of plasmid DNA was divided equally (1:1:1) among the different gene constructs.

**Antibodies.** The E-specific monoclonal antibody (mAb) BVD/CA3 (Bolin et al., 1988) and the E⁹⁹-specific mAb BVD/C12 (Greiser-Wilke et al., 1991) were used. Flag–E₁ was detected using an antibody recognizing the Flag epitope (anti-Flag M2; Sigma-Aldrich). For detection of E₁-MAT, the anti-VSV-G mAb P5D4 (Sigma-Aldrich) directed against the cytoplasmic tail of the VSV-G protein was used. mAb I1 was directed against VSV (anti-VSV-I1; Lefrancois & Lyles, 1982). A fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (Sigma-Aldrich) was used for immunofluorescence analysis and a peroxidase-conjugated anti-mouse antibody (Dako Cytomation) was used for Western blotting.

**Immunofluorescence.** Transfected cells were fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100 at 20 h post-transfection. Cells were incubated with the mAbs for 1 h at 37 °C, followed by FITC-conjugated anti-mouse IgG (Sigma). Cells were analysed by fluorescence microscopy using a Zeiss Axioptot 2 microscope with 450–490 nm band-pass filters.
Flow cytometry analysis. Transfected BHK-21 cells were detached with 200 μl Accutase (Sigma-Aldrich) at room temperature for 15 min. The cells were resuspended in 800 μl PBS, transferred to a 1.5 ml reaction tube and centrifuged at 200 g for 3 min at 4 °C. The pellets were resuspended in 100 μl of the appropriate antibodies, transferred to a microtitre plate and incubated for 1 h at 4 °C. Cells were washed three times with 100 μl MIF buffer (1 g BSA and 10 mg sodium azide in 100 ml PBS) and centrifuged at 200 g at 4 °C for 3 min. Subsequently, the cells were incubated with an FITC-conjugated secondary antibody, diluted 1:500 in MIF buffer, for 1 h at 4 °C. After additional washing steps, the cells were resuspended in 100 μl MIF buffer and immediately analysed by flow cytometry. As a negative control, BHK-21 cells were stained with the secondary antibody only.

Western blotting. BHK-21 cells were lysed at 20 h post-transfection in 200 μl NP-40 lysis buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.5 % sodium deoxycholate, 1 % NP-40, Complete protease inhibitor (Roche)]. Proteins were separated by SDS-PAGE using 5 % acrylamide stacking gels and 10–12.5 % acrylamide resolving gels and transferred to PVDF membranes (Millipore). The blots were incubated with the appropriate mAb (diluted 1:100 in PBS), followed by incubation with horseradish peroxidase-conjugated anti-mouse antibody (diluted 1:1000; DakoCytomation). Proteins were visualized using BM Chemiluminescence Western Blotting Substrate (POD; Roche) or an ECL Advance Western Blotting Detection kit (GE Healthcare).

Pseudotypes. BHK-21 cells grown in 35 mm diameter dishes were transfected with a total amount of 4 μg plasmid DNA. At 20 h post-transfection, the cells were infected with ΔG-VSV-G (m.o.i. of 10) for 1 h at 37 °C. After appropriate washing with DMEM, the cells were inoculated with mAb anti-VSV-I1 for 1 h at 37 °C to neutralize unabsorbed virus. The cells were washed again and 2 ml culture medium was added. After 20 h, the cell culture supernatants were harvested, clarified by centrifugation and inoculated directly onto cells.

Infectivity of pseudotypes. FBK, HeLa and BHK-21 cells were infected with the pseudotype viruses (100 μl cell culture supernatant per well of an eight-well chamber slide; Nunc) and incubated for 24 h at 37 °C. The cells were fixed with 3 % paraformaldehyde and the number of infectious units (IU) of virus was determined by counting the number of EGFP-positive cells.

Neutralization assays. Neutralization assays were performed with a bovine antiserum to BVDV, with mAb BVD/CA3 or with a neutralizing anti-VSV antibody prior to inoculation of BVDV-susceptible cells. As expected, no fluorescence was detected after pre-incubation with the bovine antiserum and mAb BVD/CA3, indicating that the viruses ΔG-VSV-E1-E2 and ΔG-VSV-E1-E2 were neutralized, whilst the anti-VSV antibody could not prevent infection of bovine cells. In contrast, the BVDV-specific antibodies were not able to inhibit infection of the positive-control ΔG-VSV-G, but the infectivity of this pseudovirus was eliminated by pre-incubation with the anti-VSV antibody (data not shown).

Infectivity of VSV pseudotypes with chimeric and native BVDV envelope proteins

To analyse which envelope glycoproteins are needed for BVDV entry, VSV pseudotypes bearing all possible combinations of the native (E\textsuperscript{ns}, E1 and E2) and chimeric (E\textsuperscript{ns}-MAT, E1–MAT and E2–MAT) proteins were generated. As a positive control, cells were transfected with a plasmid encoding VSV-G; cells were mock-transfected as a negative control. At 20 h p.i., cell culture supernatants were harvested and analysed for infectivity by fluorescence microscopy. Only the pseudoviruses ΔG-VSV-E\textsuperscript{ns}-E1-E2 and ΔG-VSV-E1-E2 were able to infect BVDV-permissive cells (Fig. 3). In contrast, none of the VSV/BVDV pseudoviruses containing chimeric E\textsuperscript{ns}, E1 and/or E2 could infect susceptible cells.

Infection of HeLa and BHK-21 cells – which are permissive for wild-type VSV but not for BVDV – failed (data not shown), indicating that the VSV/BVDV pseudoviruses had the same cell tropism as wild-type BVDV.

Neutralization of pseudoviruses

Neutralization assays were carried out to confirm that infectivity of the pseudoviruses was mediated by the BVDV envelope proteins. The pseudotyped viruses were incubated with bovine antiserum to BVDV, with mAb BVD/CA3 or with a neutralizing anti-VSV antibody prior to inoculation of BVDV-susceptible cells. As expected, no fluorescence was detected after pre-incubation with the bovine antiserum and mAb BVD/CA3, indicating that the viruses ΔG-VSV-E\textsuperscript{ns}-E1-E2 and ΔG-VSV-E1-E2 were neutralized, whilst the anti-VSV antibody could not prevent infection of bovine cells. In contrast, the BVDV-specific antibodies were not able to inhibit infection of the positive-control ΔG-VSV-G, but the infectivity of this pseudovirus was eliminated by pre-incubation with the anti-VSV antibody (data not shown).

Influences on heterodimer formation

To clarify the non-infectious nature of pseudoviruses with chimeric BVDV envelope proteins, cells were co-transfected with different combinations of native and chimeric E1 and E2 and analysed by Western blotting. E1–E2 heterodimers could be detected, as well as E2 monomers and dimers (Fig. 4a). There was no heterodimer formation if one of the proteins was chimeric (Fig. 4b). In contrast to the monomeric form of E2 represented by a single band,
Fig. 2. (a) Immunofluorescence analysis of cells expressing native or chimeric envelope proteins. BHK-21 cells were transfected with pCG1 plasmids containing the genes indicated. For intracellular staining, cells were fixed with 3% paraformaldehyde, permeabilized with 0.2% Triton X-100 and incubated with the appropriate mAbs. An FITC-conjugated anti-mouse antibody was used as secondary antibody. Strong intracellular protein-specific immunofluorescence signals were detectable for the native and chimeric E\textsuperscript{Mx} and E2 proteins. The chimeric E1–MAT showed a bright fluorescence with perinuclear accumulation. (b) Flow cytometry analysis of cells expressing native or chimeric envelope proteins. Transfected BHK-21 cells were detached with Accutase and incubated with the appropriate primary antibodies. Subsequently, the cells were incubated with an FITC-conjugated secondary antibody and analysed immediately by flow cytometry (shaded curve). As a negative control, cells were mock infected and incubated with the FITC-conjugated antibody only (open curve).
E2–MAT monomers appeared as a double band of approximately 50 kDa. These most likely represent different glycosylation forms of the protein, due to the efficient transport of E2–MAT to the plasma membrane.

The presence of Flag–E1 or E1–MAT was confirmed by Western blot analysis under reducing conditions (Fig. 4c, d).

**Analysis of amino acids involved in heterodimer formation**

To analyse the conditions for heterodimer formation, the cysteine residue at position 668 located in the hypothesized membrane anchor of E1 was substituted for alanine (E1-C/A) and serine (E1-C/S). The charged amino acids located at positions 671 and 674 within the transmembrane domains of E1 were replaced by alanine, respectively. Replacement by alanine and linkage with a Flag epitope resulted in the constructs Flag–E1-K, Flag–E1-R and Flag–E1-KR (Fig. 1). Furthermore, mutant E2-R/A, where the central arginine within the membrane anchor was replaced by alanine (Köhl *et al.*, 2004), was included in the study. Subsequently, the mutated proteins were analysed in cotransfection studies for their ability to form heterodimers. Regardless of the combination, the monomeric as well as the dimeric forms of E2 and E2-R/A were found (Figs 5a and 6a). Similar to E2–MAT, E2-R/A monomers were represented by a double band of about 50 kDa, most likely reflecting different glycosylation forms of the protein. This also resulted in a higher molecular mass for E2-R/A homodimers compared with homodimers of the native E2. Heterodimers were present in cells co-transfected with E2 and E1-C/A, E2 and E1-C/S (data not shown), E2 and Flag–E1, E2 and Flag–E1-K, E2 and Flag–E1-R, and E2 and Flag–E1-KR (Fig. 5a). However, in cells co-expressing E2 and Flag–E1, heterodimer formation was the dominating event. In contrast, in cells co-expressing the mutated Flag–E1 and E2 proteins, the monomeric and dimeric form of E2 were mainly detectable. In particular, in cells co-expressing the Flag–E1-KR protein, heterodimer formation was clearly reduced (Fig. 5a). In cells co-expressing E2-R/A and Flag–E1, no heterodimer formation was detectable (Fig. 6a).

The expression of Flag–E1, Flag–E1-K, Flag–E1-R and Flag–E1-KR was confirmed by detection with an anti-Flag antibody (Fig. 5b and Fig. 6b).
Role of E1–E2 heterodimers during pseudovirus entry

To confirm the requirement of heterodimers for BVDV entry, VSV pseudotypes with E2-R/A and E1 were generated. Focus was put on the E2-R/A protein because it was completely unable to form heterodimers (Fig. 6a). In contrast, the mutated E1 proteins displayed reduced heterodimer formation (Fig. 5a). As a positive control, cells were transfected with a plasmid encoding VSV-G, whilst mock-transfected cells were used as a negative control. Cell culture supernatants were harvested at 20 h p.i. and incubated with BVDV-permissive cells. Specific fluorescence signals could only be observed with VSV/BVDV pseudotypes bearing the native proteins, whereas pseudoviruses with E2-R/A failed to infect the cells (data not shown).

DISCUSSION

To elucidate the functional role of the BVDV envelope glycoproteins during virus entry, a pseudotype system based on a recombinant VSV was developed. VSV pseudotypes have been widely used to study the early steps of infection of various viruses such as hepatitis C virus (HCV) (Lagging et al., 1998) and severe acute respiratory syndrome-associated coronavirus (Ren et al., 2006). For efficient redirection of the BVDV envelope proteins to the plasma membrane, chimeric E^rms^, E1 and E2 proteins were generated containing the membrane anchor and cytoplasmic tail of the VSV-G protein. In addition, the native BVDV envelope proteins were also included in this study.

Immunofluorescence and FACS analyses of transiently transfected cells revealed that native E^rms^ and E2, as well as E^rms^-MAT, E1-MAT and E2-MAT, were localized intracellularly. Additionally, all proteins could be detected on the cell surface except for E1-MAT. This was totally unexpected for the native proteins, as in cells infected with the wild-type virus all proteins are exclusively localized intracellularly (Greiser-Wilke et al., 1991; Grummer et al., 2001). However, a similar phenomenon has been reported for HCV E1 and E2 proteins in transfected cells, as well as for proteins of plant cells, and is most likely due to saturation/leakiness of ER retention following overexpression (Bartosch et al., 2003; Crofts et al., 1999; Hsu et al., 2003).

Interestingly, all VSV pseudotypes containing the chimeric envelope proteins failed to infect permissive cells, whereas pseudotypes with the native BVDV envelope proteins were infectious. By neutralizing the infectivity of these pseudoviruses with anti-E2 antibodies or sera from BVDV-infected animals, it was clearly demonstrated that infectivity was indeed mediated by the BVDV envelope proteins. As observed for the closely related porcine pestivirus CSFV, E^rms^ seems to be dispensable for the virus entry process (Wang et al., 2004). This could not have been anticipated for BVDV, as porcine and ruminant pestiviruses might have completely different mechanisms in entering cells and show different tropisms: whilst BVDV infects cell cultures and animals of different species, CSFV is strictly restricted...
to porcine cell lines and animals of the family Suidae. Despite these differences, our results obtained with the bovine pestivirus BVDV confirmed the observations of Wang et al. (2004).

The non-infectious nature of VSV/BVDV pseudotypes with chimeric envelope proteins led to the conclusion that the correct function of E1–MAT and E2–MAT was altered – most probably due to the modified transmembrane domains. Indeed, for most membrane proteins, the transmembrane domain is more than just an anchor to the membrane (Cocquerel et al., 2000). The transmembrane domains of HCV envelope proteins E1 and E2 possess a signal sequence function in their C-terminal half, play a major role in ER localization of E1 and E2, and are potentially involved in the assembly of these envelope proteins (Cocquerel et al., 2000). This may explain why human immunodeficiency virus pseudotypes expressing HCV E1–E2 envelope proteins that lack these multi-functional transmembrane domains are also non-infectious (Dubuisson, 2000). HCV and BVDV as related members of the family Flaviviridae both have anchor domains that are composed of two stretches of hydrophobic residues separated by a short segment containing at least one fully conserved charged residue (Cocquerel et al., 2000). These parallels in amino acid sequence may also reflect similar functions. The charged residues within the transmembrane domains of HCV E1 and E2 have been identified to play a major role in biogenesis of the non-covalently linked E1–E2 heterodimers (Ciccozola et al., 2005, 2006; Cocquerel et al., 2000). In contrast to the non-covalent interaction of HCV E1 and E2, it has been postulated that the glycoproteins of pestiviruses such as CSFV and BVDV form disulfide-linked heterodimers and homodimers, respectively (Thiel et al., 1991; van Rijn et al., 1994; Weiland et al., 1990). As disulfide bonds are based on cysteine residues, this could be the reason for the inability of E1–MAT to form heterodimers. By generating the chimeric protein E1–MAT, a cysteine residue at position 668 was deleted together with the hypothesized membrane anchor. To explore whether this cysteine is essential for heterodimer formation, it was substituted with alanine and serine, respectively. As the mutations had no effect on heterodimerization, we concluded that other amino acids within the transmembrane domain of E1 must be responsible for this process. This hypothesis was additionally supported by the fact that, although the generation of E2–MAT was not linked to the loss of a cysteine residue, the protein was not able to form heterodimers. We therefore focused on the charged amino acids in the transmembrane domains and replaced them with alanine. Because both residues in the transmembrane domain of E1 (lysine and arginine) and the amino acid in the transmembrane domain of E2 (arginine) are positively charged, a direct interaction through an ion pair can be excluded. For BVDV E2, it has been supposed that the transmembrane domain initially forms a hairpin-like structure in which both the N-terminal and C-terminal ends are oriented towards the lumen of the ER and the charged residue faces the cytosol (Köhl et al., 2004). Signal sequence cleavage at the C terminus of the proteins leads to reorientation of the C-terminal end, which is subsequently directed towards the cytosolic face of the membrane with the charged residue in the middle of the membrane spanning sequence (Cocquerel et al., 2002; Köhl et al., 2004). Thus, it is likely that the lack of interaction is due to conformational changes of the transmembrane domains of the mutated BVDV proteins. These results led us to the conclusion that these domains are needed for heterodimer formation. Beyond this, we demonstrated that pseudoviruses bearing mutated BVDV envelope proteins, which were unable to form heterodimers, failed to infect BVDV-permissive cells, indicating that E1–E2 heterodimers play a key role in BVDV entry.

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