Characterization and purification of recombinant bovine viral diarrhea virus particles with epitope-tagged envelope proteins

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Bovine viral diarrhea virus (BVDV) belongs to the genus Pestivirus within the family Flaviviridae. The lipid membrane of the virions is supposed to contain the three glycosylated envelope proteins E\textsuperscript{ms}, E1 and E2, but detailed studies of virus assembly are complicated because no efficient purification method for pestviruses has been described so far. In this study, we generated infectious BVDV with N-terminally FLAG-tagged E\textsuperscript{ms} or E2 proteins, respectively. The expression of the epitope-tagged E\textsuperscript{ms} and E2 proteins could be shown by immunofluorescence and Western blot experiments. Furthermore, an affinity tag purification protocol for the isolation and concentration of infectious BVDV was established. In the preparation with a titre of \(10^{8.75}\) TCID\textsubscript{50} ml\(^{-1}\), spherical particles with a diameter of 43–58 nm (mean diameter: 48 nm) could be detected by negative staining electron microscopy, and immunogold labelling located both E\textsuperscript{ms} and E2 proteins at the virus membrane.

Today, it is possible to fuse viral proteins with epitopes such as FLAG or STREP to study the virus life cycle in detail or to get a closer view on the protein structure. For example, a FLAG-epitope was successfully introduced into the variable loop 1 of gp120 of simian immunodeficiency virus (Laird & Desrosiers, 2007). FLAG-epitopes were also inserted in the C-terminal part of the CSFV-E1 protein, in the N-terminal part or the hypervariable region 1 of the E2 protein of hepatitis C virus (HCV) (Holinka \textit{et al.}, 2009; Merz \textit{et al.}, 2011; Takahashi \textit{et al.}, 2010)

In this study, we report for the first time the construction, characterization and purification of recombinant BVDV particles with a FLAG-epitope-tagged envelope. To this end, the FLAG-epitope (DYKDDDDK) and a linker sequence (SG) were inserted into the infectious cDNA clone pA/BVDV (Meyers \textit{et al.}, 1996) encoding the BVDV type 1 origin (Chu & Zee, 1984; Coria \textit{et al.}, 1983; Fetzer \textit{et al.}, 2005). For E\textsuperscript{ms} and E2 homodimers and for E1 heterodimers with E2 can be detected in infected cells (Rümenapf \textit{et al.}, 1993; Weiland \textit{et al.}, 1990). The envelope protein E\textsuperscript{ms} lacks a typical membrane anchor and seems to be important for a first cell contact by binding to glycosaminoglycans (Fetzer \textit{et al.}, 2005; Iqbal \textit{et al.}, 2000). E1–E2 heterodimers are essential for virus entry by binding to the bovine CD46 molecule (Maurer \textit{et al.}, 2004; Ronecker \textit{et al.}, 2008).

A supplementary table is available with the online version of this paper.
strain CP7 either upstream of the E<sup>ens</sup> or E2 gene by restriction-free cloning (Unger et al., 2010). In a first step, PCR fragments were amplified from the pA/BVDV by using the Phusion HF DNA polymerase (Finnzymes) with the primers cp7_<sup>E<sup>ens</sup></sup>_flag_F and cp7_1930_R, or cp7_E2_flag_F and cp7_2958_R (Supplementary Table S1, available in JGV Online). In the next step, the generated PCR fragments were used as megaprimers in a fusion PCR with the plasmid pA/BVDV as a template. The resulting plasmids CP7_<sup>E<sup>ens</sup></sup>_flag and CP7_E2_flag are shown in Fig. 1(a).

![Diagram](http://vir.sgmjournals.org)

**Fig. 1.** (a) Schematic representation of the genome structure of BVDV type I strain CP7 and of the generated constructs. The sequence for a FLAG-epitope (underlined) and a linker sequence for two amino acids (serine and glycine) was inserted upstream of the E<sup>ens</sup> or E2 genes. The construct CP7_<sup>E<sup>ens</sup></sup>_flagII contains an additional codon for a cysteine upstream of the FLAG-epitope and a mutation (T<sub>102</sub>-A) in the E<sup>ens</sup> sequence (*). (b) IF staining of KOP-R cells inoculated with the viruses CP7_<sup>E<sup>ens</sup></sup>_flagII or CP7_E2_flag, respectively. Cells were inoculated with 0.5 ml of the transfection supernatants or the supernatant of the first passage and stained with the E2-specific mAb mix at 48–72 h p.i. (c) IF staining of KOP-R cells inoculated with the viruses CP7_<sup>E<sup>ens</sup></sup>_flagII or CP7_E2_flag, respectively. Cells were inoculated with 0.1 ml of the supernatant of the second passage and stained with the anti-FLAG M2 mAb at 48–72 h p.i. (d) Western blots of KOP-R cells inoculated with the parental virus CP7 and the viruses CP7_<sup>E<sup>ens</sup></sup>_flagII and CP7_E2_flag. Staining was done with the E2-specific mAb WB214 and the anti-FLAG M2 mAb. (e) Growth kinetic experiment for comparison of the viruses CP7_<sup>E<sup>ens</sup></sup>_flagII (√), CP7_E2_flag (●) and the parental virus CP7 (◆). KOP-R cells were inoculated with an m.o.i. of 0.01.

The cDNA CP7_<sup>E<sup>ens</sup></sup>_flag was linearized and *in vitro* transcribed by using the T7 RiboMax Large Scale RNA Production System (Promega). KOP-R cells, a diploid bovine oesophageal cell line, [RIE244, Collection of Cell Lines in Veterinary Medicine (CCLV), Friedrich-Loeffler-Institut, Insel Riems] were electroporated with recombinant RNA using a Gene Pulser Xcell Electroporation System (Bio-Rad) and the supernatant was passaged several times on bovine cells. Immunofluorescence (IF) staining with a mix of the BVDV type 1 E2-specific mAbs CA1/2,
CA34/1/2 and CA3/2/22 kindly provided by Irene Greiser-Wilke (Tierärztliche Hochschule, Hannover, Germany) revealed that the construct was able to produce infectious virus progeny, but virus propagation was highly inefficient during the first passages (data not shown). We could not detect any expression of the FLAG-epitope (data not shown) in IF staining experiments with the FLAG-epitope-specific mAb M2 (Sigma). Sequence analysis revealed two adaptive mutations in the RNA of the virus CP7_Erms_flag. We detected one amino acid substitution in the FLAG-epitope (Y→C) which was probably the reason for the failing of IF staining of the FLAG-epitope, and one amino acid substitution within the E rms sequence (T102→A). Therefore, a new construct with the observed T102→A substitution within the E rms sequence was generated. Furthermore, an additional C was introduced upstream of the FLAG-epitope to compensate the Y→C substitution within the FLAG-epitope, which was observed after amplification of the virus CP7_Erms_flag. In brief, a megaprimer was amplified from the plasmid CP7_Erms_flag by using the primers cp7_Erms_C_F and cp7_Erms_T102A_R (Supplementary Table S1). Afterwards, this new megaprimer and the plasmid pA/BVDV as a template were used for a fusion PCR to generate the plasmid CP7_Erms_flagII (Fig. 1a).

The cDNAs CP7_Erms_flagII and CP7_E2_flag were linearized and in vitro-transcribed. KOP-R cells were electroporated with recombinant RNAs and the transfection supernatants were subsequently passaged on bovine cells. Both constructs were able to replicate autonomously in KOP-R cells, but IF staining at 48–72 h post-infection (p.i.) with a mix of E2-specific mAbs revealed a different efficiency in the production of infectious virus progeny during the first passages. While the propagation of the virus with the FLAG-tagged E2 protein was efficient from the first passage on, the first passage of the virus with the FLAG-tagged E2 protein was efficient from the first passages. While the propagation of the virus CP7_E2_flag-infected cells was still high (Fig. 2a, b). It needed at least one passage for adapting the sequence for efficient virus growth. IF staining with the FLAG-epitope-specific mAb M2 demonstrated the expression of the FLAG-epitope at 48–72 h p.i. (Fig. 1c).

The expression of the FLAG-tagged E rms and E2 proteins could also be demonstrated by Western blot analysis of KOP-R cells infected with the viruses CP7_Erms_flagII and CP7_E2_flag. To this end, the cells were lysed at 48 h p.i., proteins were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The blot was incubated with the anti-FLAG M2 mAb [diluted 1:1000 in Tris-buffered saline with 0.1 % Tween (TBS-T) with 3 % low-fat milk] or the BVDV-E2-specific mAb WB214 (CVL Weybridge) (diluted 1:400 in TBS-T). As secondary antibody, an HRP-conjugated anti-mouse antibody (Dianova) (diluted 1:20000 in TBS-T) was used. KOP-R cells, infected with the parental virus CP7, were used as controls. Nearly equal amounts of the E2 protein could be detected in cells infected with CP7, CP7_Erms_flagII or CP7_E2_flag (Fig. 1d). Furthermore, in cells infected with the virus CP7_Erms_flagII, a FLAG-tagged E rms monomer with a size of about 50 kDa as well as a FLAG-tagged E rms-E1 protein with a size of about 60–65 kDa that was first described by Wegelt et al. (2009) could be detected (Fig. 1d). The expression of a FLAG-tagged E2-protein with a size of about 55 kDa could be shown for cells infected with the virus CP7_E2_flag (Fig. 1d).

In order to compare the envelope-tagged viruses and the parental virus CP7 in their growth characteristics, growth kinetic experiments were performed. Therefore, KOP-R cells were inoculated with the parental virus CP7, the virus CP7_Erms_flagII or the virus CP7_E2_flag at an m.o.i. of 0.01. Titres were examined as TCID50 ml−1 by IF staining with mAb C16 (Edwards et al., 1988; Peters et al., 1986) kindly provided by Irene Greiser-Wilke (Tierärztliche Hochschule, Hannover, Germany). Both envelope-tagged viruses CP7_Erms_flagII and CP7_E2_flag showed nearly the same growth characteristics as the parental virus CP7 (Fig. 1e). To investigate if adaptive mutations have led to similar growth, RNA was isolated from the virus stocks of CP7_Erms_flagII or CP7_E2_flag and RT-PCR fragments of the E rms or E2 region were sequenced. A substitution of the previously introduced C upstream of the FLAG-epitope (C-Y) and an amino acid substitution within the E rms sequence (L135→S) in the genome of the virus CP7_Erms_flagII could be observed. In the RNA of the virus CP7_E2_flag we could not detect any amino acid substitution.

Furthermore, the FLAG-epitope-tags were used to purify the recombinant viruses. To this end, we propagated the virus CP7_Erms_flagII and the virus CP7_E2_flag in KOP-R cell cultures without FCS. The supernatants were harvested and filtered at 48–72 h p.i. The parental virus CP7, propagated and harvested in the same way, served as negative control during the purification experiments. The titres were examined as TCID50 ml−1 by IF staining with mAb C16. The parental virus reached an original titre of 104.75 TCID50 ml−1. For the epitope-tagged viruses titres of 104.75 TCID50 ml−1 (CP7_Erms_flagII) and 105.375 TCID50 ml−1 (CP7_E2_flag) were examined. The viruses were then purified by using either the anti-FLAG M2 agarose affinity gel (Sigma) or the anti-FLAG M2 magnetic beads (Sigma). To this end, 1 ml of the infectious cell culture supernatant was incubated with the equilibrated agarose affinity gel or the magnetic beads for 1 h at room temperature. After several washing steps with PBS, the elution of the virions from the agarose or magnetic beads was performed with 1 ml of a synthetic FLAG-peptide solution (Sigma) with a concentration of 100 µg ml−1. The eluates were filtered and tested for infectivity. The titres of the eluates were examined as TCID50 ml−1 by IF staining with mAb C16. While there was nearly no infectivity after purification of the parental virus CP7, the infectivity of the eluates from the supernatants of CP7_Erms_flagII- and CP7_E2_flag-infected cells was still high (Fig. 2a, b). It
seemed that the purification with the anti-FLAG M2 magnetic beads was slightly more efficient than the purification with the anti-FLAG M2 agarose beads. This result may be due to the easier handling procedures for magnetic beads. Most interestingly, the purification efficiency of either virions with a FLAG-tagged Erns protein or virions with a FLAG-tagged E2 protein was similar.

For electron microscopical studies, 330 ml culture supernatant from CP7_E2_flag-infected KOP-R cells was harvested at 72 h p.i., filtered and precipitated with 8% polyethylene glycol 6000 and 0.7 M NaCl overnight at 4 °C. After low-speed centrifugation the sediment was resuspended in Tris-EDTA-NaCl buffer and purified by using the anti-FLAG M2 magnetic beads. By elution of the virions from the magnetic beads with 100 μl of a synthetic FLAG-peptide solution a virus preparation with a titre of 10^8.75 TCID50 ml^-1 was obtained. The BVDV particles were adsorbed to Formvar-coated nickel grids and stained with uranyl acetate. Spherical particle structures with 43–58 nm (mean diameter: 48 nm) could be observed (Fig. 3a). For immunoelectron microscopy, the grids with the adsorbed particles were incubated with the BVDV-E2-specific mAb WB214 or the BVDV-E^rms^-specific mAb WB210 (CVL Weybridge). Incubation with 10 nm goat anti-mouse colloidal gold (Biocell International) was followed by negative staining with phosphotungstic acid (pH 7.4). The grids were examined with the transmission electron microscope Tecnai 12 (Philips) at 80 kV. E2 and Erns proteins could be located at the membrane of the purified BVDV particles (Fig. 3b, c).

Taken together, we generated and characterized two recombinant BVDV with a FLAG-epitope in their envelope proteins Erns and E2. After introduction of two mutations...

**Fig. 2.** Affinity tag purification results using supernatants of CP7-, CP7_E^rms_flagII- and CP7_E2_flag-infected KOP-R cells and the anti-FLAG M2 agarose affinity gel (a) or the anti-FLAG M2 magnetic beads (b).

**Fig. 3.** (a) Negative staining electron microscopy of purified BVDV particles CP7_E2_flag. (b) Immunogold labelling of purified BVDV particles CP7_E2_flag with the E2-specific mAb WB214. (c) Immunogold labelling of purified BVDV particles CP7_E2_flag with the E^rms^-specific mAb WB210. Bars, 300 and 100 nm (inset).
into the genome of the virus CP7_E2\textsuperscript{rm}-flag, both viruses could be efficiently propagated in bovine cells and replicated in a similar way as the parental virus CP7. Obviously, the N-terminal introduction of the FLAG-epitope severely impaired the folding of the glycoprotein E\textsuperscript{rm} in a way that adaptive mutations were necessary to obtain a functional protein. The N-terminal fusion of the FLAG-epitope with the E2 protein seemed to have no negative influence on the functionality of the protein \textit{in vitro}. Similar results are also published for the N-terminal fusion of a FLAG-epitope with the HCV-E2 protein (Merz et al., 2011). In contrast, an internal introduction of a FLAG-epitope in the HCV-E2 protein resulted in a mutation of a potential FLAG-epitope in the HCV-E2 protein (Merz \textit{et al.}, 2011). The expression of the epitope-tag could be shown \textit{in vitro} and \textit{in vivo} experiments. The molecular masses for E\textsuperscript{rm}, E\textsuperscript{rm}-E1 and E2 estimated in the Western blots with the anti-FLAG M2 mAb are in agreement with previous studies where protein-specific mAbs were used. Furthermore, we developed a system for purification of envelope-tagged BVDV by using anti-FLAG M2 agarose and anti-FLAG M2 magnetic beads that allowed detection of BVDV particles with 43–58 nm in diameter by electron microscopy. The observed virion sizes are in agreement with former descriptions of purified BVDV particles with 40–60 nm in diameter (Ohmann, 1990). Because of the similar purification efficiencies of virions with a FLAG-tagged E\textsuperscript{rm} or E2 protein we assumed that the E\textsuperscript{rm} protein, although lacking a typical membrane anchor, is like the E2 protein closely associated with the viral membrane of infectious virions. Indeed, by immunogold labelling we were able to locate both E\textsuperscript{rm} and E2 proteins at the virus membrane. However, the development of FLAG-epitope-tagged virions now allows further studies to gain new insights into BVDV assembly and morphogenesis.

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**References**


essential for virus entry and depends on charged residues in the transmembrane domains. J Gen Virol 89, 2114–2121.


