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 Ems, E1 and E2, but detailed studies of virus assembly are complicated because no efficient purification method for pestiviruses has been described so far. In this study, we generated infectious BVDV with N-terminally FLAG-tagged Ems or E2 proteins, respectively. The expression of the epitope-tagged Ems 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 108.75 TCID50 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 Ems 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 et al., 2009; Merz et al., 2011; Takahashi 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 et al., 1996) encoding the BVDV type 1 origin (Chu & Zee, 1984; Coria et al., 1983; Fetzer et al., 2005). For Ems and E2 homodimers and for E1 heterodimers with E2 can be detected in infected cells (Rümenapf et al., 1993; Weiland et al., 1990). The envelope protein Ems lacks a typical membrane anchor and seems to be important for a first cell contact by binding to glycosaminoglycans (Fetzer et al., 2005; Iqbal et al., 2000). E1–E2 heterodimers are essential for virus entry by binding to the bovine CD46 molecule (Maurer et al., 2004; Ronecker et al., 2008). Until now, many steps during pestivirus morphogenesis are inconclusive and detailed structural analysis is complicated because a simple method for purification of pestivirus particles is not available.
strain CP7 either upstream of the E"ms 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_E"ms_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_E"ms_flag and CP7_E2_flag are shown in Fig. 1(a).

The cDNA CP7_E"ms_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,

![Fig. 1.](http://vir.sgmjournals.org)
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_Erns_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<sup>ms</sup> sequence (T<sub>102</sub>A). Therefore, a new construct with the observed T<sub>102</sub>A substitution within the E<sup>ms</sup> 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_Erns_flag. In brief, a megaprimer was amplified from the plasmid CP7_Erns_flag by using the primers cp7_Erns_C_F and cp7_Erns_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_Erns_flagII (Fig. 1a).

The cDNAs CP7_Erns_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 during the first passages, we could not detect any expression of the FLAG-epitope (data not shown). We could not detect any amino acid substitution in the FLAG-epitope Y–C substitution during the first passages (data not shown). We could not detect any amino acid substitution in the FLAG-epitope Y–C substitution during the first passages (data not shown). We could not detect any amino acid substitution in the FLAG-epitope Y–C substitution during the first passages (data not shown). We could not detect any amino acid substitution in the FLAG-epitope Y–C substitution during the first passages (data not shown).

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_Erns_flagII or the virus CP7_E2_flag at an m.o.i. of 0.01. Titres were examined as TCID<sub>50</sub> ml<sup>–1</sup> 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_Erns_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_Erns_flagII or CP7_E2_flag and RT-PCR fragments of the E<sup>ms</sup> 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<sup>ms</sup> sequence (L<sub>135</sub>S) in the genome of the virus CP7_Erns_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_Erns_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 TCID<sub>50</sub> ml<sup>–1</sup> by IF staining with mAb C16. The parental virus reached an original titre of 10<sup>5.125</sup> TCID<sub>50</sub> ml<sup>–1</sup>. For the epitope-tagged viruses titres of 10<sup>4.75</sup> TCID<sub>50</sub> ml<sup>–1</sup> (CP7_Erns_flagII) and 10<sup>5.375</sup> TCID<sub>50</sub> ml<sup>–1</sup> (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<sup>–1</sup>. The eluates were filtered and tested for infectivity. The titres of the eluates were examined as TCID<sub>50</sub> ml<sup>–1</sup> 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_Erns_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 phosphtungstic acid (pH 7.4). The grids were examined with the transmission electron microscope Tecnai 12 (Philips) at 80 kV. E2 and E^rms 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 E^rms and E2. After introduction of two mutations
into the genome of the virus CP7_E\textsuperscript{rns}_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{rns} 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 E2 protein (Takahashi et al., 2010). The expression of the epitope-tag could be shown in IF and Western blot experiments. The molecular masses for E\textsuperscript{rns}, E\textsuperscript{rns}-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{rns} or E2 protein we assumed that the E\textsuperscript{rns} 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{rns} 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


