The envelope glycoprotein E2 is a determinant of cell culture tropism in ruminant pestiviruses

Delin Liang, Ignacio Fernandez Sainz, Israrul H. Ansari, Laura H. V. G. Gil, Ventzislav Vassilev and Ruben O. Donis

INTRODUCTION

The genus Pestivirus of the family Flaviviridae comprises bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV) and ovine border disease virus (BDV) (Murphy et al., 1999). These viruses infect ruminants and related species in the order Artiodactyla, such as swine (Avalos-Ramirez et al., 2001; Carlsson & Belak, 1994; Nettleton & Entrican, 1995). CSFV is often responsible for fatal infections characterized primarily by vascular and immune alterations (Carbrey et al., 1980; Narita et al., 2000). Similarly, severe acute infections by BVDV in cattle are characterized by severe diarrhoea, sometimes with haemorrhagic complications (Perdrizet et al., 1987; Rebhun et al., 1989; Ridpath et al., 2000). BVDV, BDV and CSFV are thought to have diverged from a common ancestor by adaptation to various species, including cattle, sheep and swine. Pestiviruses can cross host species barriers and infect other animal species in the order Artiodactyla. For example, ruminant pestiviruses often infect swine (Liess & Moennig, 1990). However, pestivirus infection in the non-adapted host (e.g. BVDV in swine) appears to be inefficient (Carlsson & Belak, 1994). This scenario is in general recapitulated in vitro; pestiviruses show preference for cells of the species to which they are adapted: BVDV can replicate in swine cells, but only with greatly reduced efficiency (Flores et al., 1996; Roehe & Edwards, 1994).

METHODS

Cells. Bovine and ovine species were represented in vitro by Madin–Darby bovine kidney (MDBK) cells from ATCC and an SV-40 virus-immortalized cell line derived from lamb kidney, termed LG-lamb (L. Gil & R. Donis, unpublished data), respectively.

Creative of chimeric BVDV genomes. The chimeric BVDV genome with a substituted E2 glycoprotein gene was constructed by removing a segment of DNA encoding E2 in the BVDV infectious clone pNADLp15 (Vassilev et al., 1997) and inserting the homologous segment of BDV strain 31, giving rise to pN-E2bdv. To preserve E2 processing in the chimeras, the junctions were designed based on previously reported signalase processing sites at the termini of the bovine pestivirus E2 (Rumenapf et al., 1993). A similar approach was followed to engineer a control chimeric virus, pN-890E2, expressing the E2 of BVDV strain 890, an isolate that belongs to genotype II (Fig. 2) (Ridpath & Bolin, 1995). The pN-E2bdv chimeric genome was used as a template for in vitro RNA synthesis (Megascript, Ambion). RNA transcripts were electroporated into bovine cells and virus replication was quantified by plaque assay, as described previously (Vassilev et al., 1997).

Determination of yield of infectious progeny. MDBK cells or LG-lamb cells were infected with the wild-type (wt) parental viruses or the chimeric viruses at an m.o.i. of 3 and incubated for various intervals prior to harvesting by freeze-thawing. The clarified fluid was titrated by limiting dilution.

Normalized infection. The input multiplicity was adjusted to obtain the same percentage of MDBK and LG-lamb cells infected with wt and chimeric virus. The percentage of infected cells in each monolayer was determined by indirect immunofluorescence on wells treated in an identical fashion. The medium and cells were harvested at 12 h p.i. by freeze-thawing and the total yield of infectious progeny was determined by plaque assay on LG-lamb cells.

RESULTS

To determine whether similar restrictions exist for ovine and bovine pestiviruses, we evaluated the ability of BVDV...
and BDV strains to replicate and form plaques in cells of the homologous and heterologous species. We utilized strains with fewer than six passages in cell culture since their isolation from animal tissues. BDV isolates were grown in ovine cells. Bovine pestiviruses (either genotype I or II) were able to form plaques with similar efficiency in bovine and ovine kidney cell cultures (Table 1). In contrast, the plaquing efficiency of BDV isolates in MDBK cells was 15 to >100 times lower than in ovine cells (Table 1; Fig. 1). BVDV isolates of either biotype – cytopathic (CP) or non-cytopathic (NCP) – showed the same cell specificity. These results indicated a clear preference by the BDV isolates for replication in ovine cells. The greater replication efficiency of a pestivirus in cells of the homologous species is not surprising. Interestingly, the reverse was not true; the bovine pestivirus isolates plaqued with high efficiency in ovine cells (Table 1). The low efficiency of ovine pestivirus replication in MDBK cells could result from defects in any of the stages of the virus replication cycle. However, virus entry and assembly are obvious candidate restricting processes because of the obligatory intimate interaction of the viral envelope with the plasma membrane to gain entry and with the secretory pathway during assembly (Heil et al., 2001; Pancino et al., 1995; Schmidt et al., 2001). E2 in particular has been ascribed a key role in virus entry, because antibodies that bind to it are neutralizing and soluble E2 blocks virus entry (Donis et al., 1988; Hulst & Moormann, 1997; Weiland et al., 1990). Other studies have demonstrated that domain deletions within E2 render virions non-infectious (van Gennip et al., 2002). Thus, we hypothesized that the poor replication of BDV isolates in MDBK cells could be attributable to inadequate E2 function in these cells. To test this hypothesis, we engineered the transfer of the BDV E2 glycoprotein into the genome of BVDV (strain NADL) cloned as cDNA (Fig. 2). The specific infectivity of the chimeric RNA transcripts (pN-E2bdv and pN-890E2) electroporated into ovine cells was comparable with that of the parental genome: approximately 10^6 p.f.u. (μg RNA)^−1. The rescued virus, termed BVDV–E2bdv, was analysed by RT-PCR amplification followed by sequencing to corroborate the sequence of the chimeric E2 region and flanking regions from Erns and P7 (Fig. 2 and data not shown). A similar process was followed to rescue an infectious virus, termed BVDV–E2gII, from pN-890E2 RNA transcripts. The efficiency of the BVDV–E2gII rescue from RNA transfections was similar to that obtained with the BVDV–E2bdv chimera (not shown). The E2 replacement strategy that we employed is similar

<table>
<thead>
<tr>
<th>Pestivirus (species)</th>
<th>Isolate</th>
<th>P.f.u. ratio MDBK/LG-lamb*</th>
<th>Biotype</th>
<th>Genotype</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>BDV (ovine)</td>
<td>BD31</td>
<td>0</td>
<td>NCP</td>
<td>NA</td>
<td>Ridpath &amp; Bolin (1997)</td>
</tr>
<tr>
<td></td>
<td>Coos Bay</td>
<td>0</td>
<td>CP</td>
<td>NA</td>
<td>Ridpath et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>Idaho</td>
<td>0.08</td>
<td>NCP</td>
<td>NA</td>
<td>Niemi et al. (1982)</td>
</tr>
<tr>
<td></td>
<td>481</td>
<td>0</td>
<td>NCP</td>
<td>NA</td>
<td>Anderson et al. (1987)</td>
</tr>
<tr>
<td>BVDV (bovine)</td>
<td>NADL</td>
<td>1</td>
<td>CP</td>
<td>1</td>
<td>Gutekunst &amp; Malmquist (1963, 1964)</td>
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<tr>
<td></td>
<td>CP1</td>
<td>0.5</td>
<td>CP</td>
<td>1</td>
<td>Meyers et al. (1992)</td>
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<tr>
<td></td>
<td>Bossie</td>
<td>1</td>
<td>NCP</td>
<td>1</td>
<td>This paper</td>
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<tr>
<td></td>
<td>VS 201</td>
<td>0.5</td>
<td>NCP</td>
<td>1</td>
<td>This paper</td>
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<td></td>
<td>VS 269</td>
<td>1</td>
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<td>2</td>
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<tr>
<td></td>
<td>890</td>
<td>0.9</td>
<td>NCP</td>
<td>2</td>
<td>Ridpath &amp; Bolin (1995)</td>
</tr>
<tr>
<td></td>
<td>VS271</td>
<td>0.6</td>
<td>CP</td>
<td>2</td>
<td>This paper</td>
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</table>

*Monolayers were inoculated with identical aliquots of the viral isolates indicated and overlaid with solid media. P.f.u. ratio is the number of plaques in MDBK cells/number of plaques in LG-lamb cells. Data are mean values from two experiments performed in duplicate. NA, not applicable.

Fig. 1. BDV fails to form plaques in MDBK cells. Plaque and immunofocus assays in MDBK or sheep kidney (LG-lamb) cell monolayers inoculated with 25 p.f.u. BDV, strain 31 or Coos Bay. The monolayers infected with BDV strain 31 were fixed in acetone/methanol at 72 h p.i. and immunostained with a pestivirus-specific polyclonal antibody followed by peroxidase-conjugated antibody, as described (Vassilev & Donis, 2000). Because the Coos Bay strain of BDV is cytopathic, the cells were stained with crystal violet (Ridpath et al., 1992). No plaques were seen in MDBK cells infected with up to 1000 p.f.u. per well of the BDV strains (not shown).
to the one reported for the engineering of CSFV chimeras, expressing the E2 of BVDV (van Gennip et al., 2000). The exchange of E2 between different BVDV genotypes was well tolerated, as exemplified by BVDV–E2bdv chimeric virus. Our studies, as well as those previously reported by others, indicate that E2 exchanges among pestiviruses adapted to swine, cattle and sheep can consistently yield virus with adequate fitness for replication in cell culture (van Gennip et al., 2000).

To evaluate the replication efficiency of chimeric BVDV–E2bdv pestivirus in MDBK and ovine cells, we determined the yield of infectious progeny during a period of 24 h after infection (Fig. 3). The BVDV–E2bdv virus produced tenfold less infectious virus progeny than the BVDV parent after 12 h of infection in MDBK cells. This difference in progeny yield increased to 100-fold at 24 h post-infection (p.i.). In contrast, the MDBK cells were equally permissive for BVDV–E2bdv and BDV, due to the reduced fitness displayed by BVDV–E2bdv in these cells, caused by the E2 substitution (Fig. 3). E2 is thought to exist as a disulfide-linked heterodimer with E1 and to interact with E\textsuperscript{ns} non-covalently. In addition, the E2 precursor E2P7 may interact with the core protein before the release of P7 (Elbers et al., 1996). The failure of BVDV–E2bdv to replicate in bovine cells could be an artefact stemming from the incompatibility of the BDV E2 with the other viral proteins. To address this question, we engineered the replacement of the BVDV E2 with the E2 from BVDV strain 890, which is classified as genotype II. We used this strain because it has the same level of E2 amino acid divergence from BVDV strain NADL as BDV strain 31. The amino acid sequences of BDV 31 and BVDV strain 890 E2 are both ~58% identical to that of strain NADL (GenBank accession nos AAB37578, AAA82981 and NP_044403, data not shown). The growth curves of BVDV–E2gll in MDBK and ovine cells were very similar to that of the parental BVDV strain NADL (Fig. 3). These results indicated that the E2 from a divergent pestivirus, such as a genotype II strain, can establish the putative functional interaction(s) with the rest of the genotype I BVDV proteins to replicate efficiently in cell culture (Elbers et al., 1996). This finding is consistent with the hypothesis that the impaired growth of BVDV–E2bdv in MDBK cells is not caused by defective interactions among viral proteins, but rather results from inefficient E2–host interactions. Similarly, the efficient replication of BVDV–E2bdv in ovine cells, found to be comparable with that of BDV, suggests that the ovine E2 can function efficiently with the other viral structural or non-structural proteins of BVDV that may interact with it.

To assess the fitness of a virus to undergo multiple cycles of replication in cell cultures, we compared plaque formation by the BVDV–E2bdv with its parental virus. The results indicated that substitution of the authentic E2 in the bovine pestivirus with that of an ovine pestivirus caused a change in the cell culture host range of the virus; BVDV–E2bdv did not form visible plaques in MDBK cells, but formed plaques in sheep cells as efficiently as BDV (Fig. 4 and Table 2). Thus, the E2 substitution in the BVDV genome resulted in a chimeric virus whose growth properties in vitro resemble very closely those of the BDV that donated the E2 gene.

**Fig. 2.** Genomic structure of the BVDV–E2\textsubscript{bdv} and BVDV–E2\textsubscript{gll} chimeras. The nucleotide sequence encoding the E2 glycoprotein of genotype I BVDV strain NADL was replaced with the homologous sequence from BDV strain 31 resulting in the BVDV–E2\textsubscript{bdv} chimera expressing the polyprotein as shown in (A). An identical procedure was employed to construct BVDV–E2\textsubscript{gll}, a chimeric BVDV expressing the E2 from genotype II BVDV strain 890. The primary sequences of the regions of interest (the E2 N- and C-terminal junctions) from the parental BVDV strain NADL polyprotein are shown in (B) with those of BVDV strain 890 and the BDV strain 31, as well as the resulting chimeras (bold).
Comparing the relative replication efficiencies of the BVDV–E2bdv chimeric virus and the parent BVDV by plaque assay (Fig. 4) with those determined in the growth curve (Fig. 3), the plaque assay appears as a more dramatic difference. This is caused by the fact that the BVDV–E2bdv virus plaques were too small to be detected by the naked eye in crystal violet-stained monolayers. This was confirmed by immunofluorescent analysis of BVDV–E2bdv-infected MDBK cells; the percentage of BVDV–E2bdv-infected cells at 12 h p.i. was 10-fold lower than that of the wt BVDV-infected cells (Table 2).

Although the reduced infectivity of BVDV–E2bdv in bovine cells might explain its phenotype in MDBK cells, it was important to determine whether virion assembly is also affected. To address this issue, we compared the single-step yield of wt and BVDV–E2bdv following a normalized

Table 2. Differential infectivity of a BVDV–E2bdv chimeric pestivirus

<table>
<thead>
<tr>
<th>Pestivirus</th>
<th>Ratio MDBK/LG-lamb</th>
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<tbody>
<tr>
<td></td>
<td>IF*</td>
</tr>
<tr>
<td>BDV-31</td>
<td>0.10</td>
</tr>
<tr>
<td>BVDV–E2bdv</td>
<td>0.10</td>
</tr>
<tr>
<td>BVDV NADL</td>
<td>1.2</td>
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</table>

*The indicated viruses were used to infect MDBK or LG-lamb cells. At 12 h p.i., the cells were stained by indirect immunofluorescence (IF) and the % of infected cells in each culture used to calculate the ratio MDBK/LG-lamb. Data are mean values from two experiments performed in duplicate.
†A plaque assay performed in parallel was used to calculate the p.f.u. ratio (see Table 1).

Comparing the relative replication efficiencies of the BVDV–E2bdv chimeric virus and the parent BVDV by plaque assay (Fig. 4) with those determined in the growth curve (Fig. 3), the plaque assay appears as a more dramatic difference. This is caused by the fact that the BVDV–E2bdv virus plaques were too small to be detected by the naked eye in crystal violet-stained monolayers. This was confirmed by immunofluorescent analysis of BVDV–E2bdv-infected MDBK cells; the percentage of BVDV–E2bdv-infected cells at 12 h p.i. was 10-fold lower than that of the wt BVDV-infected cells (Table 2).

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infection of MDBK and LG-lamb cells. The BVDV–E2bdv progeny yield was 4-7 times lower than that of wt BVDV-NADL in MDBK cells (data not shown). In contrast, wt and chimeric virus yields from LG-lamb cells were not significantly different. Three possibilities should be considered to explain the yield reduction of BVDV–E2bdv in bovine but not ovine cells: reduced genome replication efficiency, reduced virion assembly or production of poorly infectious virions. Since no cis-acting RNA element in the E2-coding region is required for pestivirus genome replication, the observed bovine cell restriction phenotype of BVDV–E2bdv appears to be caused by E2 and its interactions with host factors during virion assembly and subsequent entry into a new cell (Behrens et al., 1998).

Taken together, these studies demonstrate that BDV E2 functions efficiently in the chimeric virus to infect cells derived from its natural sheep host but fails to mediate efficient infection of MDBK cells. E2 forms a heterodimer with E1, whose function is unknown (Weiland et al., 1990). The extensive sequence divergence between BDV and BVDV E2 is not accompanied by equally profound changes in E1 (Becher et al., 1999; Deng & Brock, 1992). However, our results suggest that the putative interactions of BDV E1 with a heterologous E2 from BDV or BVDV type II remain functional in the chimeras.

The relative contribution of entry or assembly defects to the inefficient replication of BVDV–E2bdv in MDBK as compared with LG-lamb cells cannot be derived from this study. Further research will be necessary to quantify the efficiency of each of these processes in BVDV–E2bdv infections. These findings have potentially significant implications for the understanding of host factor involvement in the pestivirus replication cycle. Further mapping of the E2 domain that determines efficient infection of MDBK cells using a homologue-scanning strategy may shed light on BVDV assembly and infectivity in ruminant cell cultures (Morrison et al., 1994).

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REFERENCES


