The surface glycoprotein E2 of bovine viral diarrhoea virus contains an intracellular localization signal

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INTRODUCTION

Bovine viral diarrhoea virus (BVDV) is a member of the Pestivirus genus within the family Flaviviridae (Wengler et al., 1995). BVDV is the causative agent of bovine viral diarrhoea/mucosal disease (Baker, 1995). The virus genome consists of a single molecule of unsegmented positive-stranded RNA. It serves as a matrix for the synthesis of a polypeptide that is co- and post-translationally cleaved into the structural and non-structural proteins (Rümenapf et al., 1993). The structural proteins are the C (capsid) protein and three membrane glycoproteins, namely E(rns), E1 and E2 (Meyers & Thiel, 1996). The E2 protein is the major target of the protective immune response elicited after a BVDV infection (Donis et al., 1988; Magar et al., 1988).

Little information is available concerning the late stage of the BVDV replication cycle. Immunostaining of virus-infected cells indicated that glycoproteins E2 and E(rns) 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 most likely matures at intracellular membranes. So far, no signals that retain the BVDV envelope proteins in intracellular compartments have been determined.

The E2 protein, expressed by recombinant vesicular stomatitis virus (VSV), has been reported to be present on the plasma membrane (Grigera et al., 2000). Similarly, cells infected by recombinant baculovirus were also shown to contain BVDV E2 protein on the cell surface (Kweon et al., 1997); this is in contrast to the E2 proteins of members of other genera within the family Flaviviridae. The hepatitis C virus E2 protein has been shown to contain a retention signal within the membrane anchor that prevents transport to the cell surface (Cocquerel et al., 1998).

We have constructed chimeric E2 proteins that contain the membrane anchor (M) and/or the cytoplasmic tail (T) of other viral glycoproteins that are transported to the cell surface. Using different labelling procedures and detection methods we were able to show that the E2 protein M domain contains an intracellular localization signal and that an arginine residue within this hydrophobic domain is an essential element of the sorting signal. Interestingly, the export signal present in the cytoplasmic tail of the VSV...
G protein was found to overrule the retention signal within the E2 membrane anchor.

**METHODS**

**Cells and virus.** 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), were kindly provided by Karl-Klaus Conzelmann (Max-von-Pettenkofer-Institut, Munich, Germany). The cells were grown in Eagle’s minimal essential medium supplemented with non-essential amino acids, 0.5 mg geneticin ml⁻¹, and 5% fetal calf serum. Primary fetal calf kidney (FCK) cells were prepared as described (Orban et al., 1983) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The NADL strain of BVDV was kindly provided by Hans-Richard Frey (Institute for Virology, School of Veterinary Medicine Hannover). BVDV was twice plaque-purified on FCK cells.

**Construction of plasmids.** The G protein gene of VSV (strain Indiana) was amplified from the pVSV-XN2 vector (kindly provided by John K. Rose, New Haven, USA) by PCR using oligonucleotides a and b (Table 1). The two primers contained an EcoRI and a BamHI restriction site, respectively, which allowed us to clone the PCR product into the respective sites of the pTM1 vector (Moss et al., 1990) resulting in pTM1-G. The total open reading frame of G was sequenced and found to be identical with the published sequence (accession no. NC_001560).

For cloning of the BVDV E2 protein coding region, total RNA was prepared from FCK cells that had been infected with the NADL strain of BVDV. The RNA was reverse transcribed using the Expand reverse transcriptase (Roche Molecular Biochemicals) and random hexamers for priming. Nucleoetides 2462 to 3583 of the virus genome (accession no. AJ133738) were amplified from cDNA by PCR using oligonucleotides c and d. In a separate PCR reaction, the region of the VSV G gene encoding the signal peptide (amino acids 1–23) was amplified with oligonucleotides e and f. The product was purified (PCR Purification Kit, Qiagen), mixed with the purified E2 fragment of the former PCR reaction (molar ratio of 1:1), and heated for 2 min at 95°C for denaturation. The mixture was then incubated at 60°C for 30 s to allow the two fragments to anneal to each other. Hybridization was mediated by overlapping complementary sequences that were introduced into the PCR fragments by 5’ overhangs of the oligonucleotides c and d. A complete double-stranded DNA hybrid was obtained after incubating the mixture with Pfu polymerase (Promega) at 72°C for 3 min. The chimeric gene was subsequently amplified by PCR using oligonucleotides e and d. Taking advantage of SpeI and XhoI restriction sites included at the 5’ ends of these oligonucleotides, the PCR product was ligated into the pTM1 vector to obtain the pTM1-E2 plasmid. A similar overlapping PCR technique was applied for the construction of all other chimeric and mutant protein genes. The combined protein domains and the oligonucleotides used are indicated in Fig. 1. The cytoplasmic and transmembrane domains of BRSV (strain ATue51908) F protein were amplified from the pTM1-bF plasmid previously reported (Zimmer et al., 2001a).

**Immunofluorescence analysis.** BSR-T7/5 cells grown on 12-mm diameter coverslips were transfected with 1 μg of plasmid DNA and

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| w      | GTAGCCCTCTTGGGTTGCCGGCATATGTAAGTTGTACTG |
| x      | CAGTAACCAAGTCATATGGCCTACAAAAAGGGCTAC |

Table 1. Primers used for plasmid constructions
Western blot analysis of transfected cells. BSR-T7/5 cells grown in 35-mm diameter dishes were transfected with 3 μg of plasmid DNA and 6 μl Lipofectamine 2000 Reagent. At 24 h post-transfection, cells were scraped into 1 ml of PBS, pelleted by centrifugation, and lysed in 200 μl of NP40 lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40, protease inhibitors). Twofold-concentrated SDS sample buffer was added to the clarified lysate. The samples were run on a 10% SDS-polyacrylamide gel under non-reducing conditions and transferred to a nitrocellulose membrane. The blots were incubated with either a cocktail of BVD/CA1 and BVD/CA3 monoclonal antibodies (1:100 in PBS) or with a polyclonal rabbit anti-VSV serum (1:1000 in PBS). Following incubation with a streptavidin–peroxidase complex (Amersham; anti-rabbit immunoglobulin serum (Amersham; 1:1000). Following incubation with a cocktail of BVD/CA1 and BVD/CA3 monoclonal antibodies, BVD/CA1 and BVD/CA3, were used at a dilution of 1:50 each) of the BVD/CA1 and BVD/CA3 monoclonal antibodies (1:50 each) of the VSV G ectodomain was detected by chemiluminescence blotting substrate, Roche Diagnostics) (Zimmer et al., 2001b). Primary antibodies were detected with FITC-conjugated antibodies directed to either rabbit or mouse immunoglobulins (1:200 in PBS; Amersham Biosciences). If not otherwise indicated, antibodies were incubated with the cells for 60 min at room temperature followed by three washing steps with PBS. Conventional epifluorescence microscopy was performed with a Zeiss Axioplan 2 microscope and photographs were taken using a digital video camera (INTAS focus imager, INTAS, Göttingen, Germany).

Biotinylation and immunoprecipitation of surface proteins. At 24 h post-transfection (see above), BSR-T7/5 cells grown in 35-mm diameter dishes were labelled with an N-hydroxysuccinimide ester of biotin (sulfo-NHS-biotin; Pierce), and the virus antigens were immunoprecipitated from the cell lysates according to a published protocol (Zimmer et al., 2001a, b). For immunoprecipitation, a polyclonal rabbit anti-VSV serum (1:200) or a cocktail composed of the BVD/CA1 and BVD/CA3 monoclonal antibodies (1:50 each) was used.

Radiolabelling and treatment with endoglycosidas. At 24 h post-transfection (see above), BSR-T7/5 cells grown in 35-mm diameter dishes were metabolically labelled for 2 h with 100 μCi of [35S]methionine/[35S]cysteine (Tran35S-Label, ICN). The cells were lysed with 600 μl NP40 lysis buffer and the viral proteins were isolated by immunoprecipitation (see above) and eluted in 20 μl of 1% SDS. A 10 μl aliquot of the immunoprecipitate was diluted with 90 μl of 50 mM sodium acetate buffer, pH 5-5, containing 1% octylthioglucoside and a protease inhibitor cocktail. Endoglycosidase H (10 mU) (Calbiochem) were added to 30 μl of this dilution, and incubated for 2 h at 37°C. In parallel, a 10 μl aliquot of the immunoprecipitate was diluted with 90 μl of 50 mM phosphate buffer, pH 7-0, containing 1% octylthioglucoside and a protease inhibitor cocktail, and 30 μl of this dilution were treated with 1 U of N-glycosidase F (Roche) for 2 h at 37°C. The samples were run on a 12% SDS-polyacrylamide gel under reducing conditions and analysed by autoradiography (Zimmer et al., 2002).

RESULTS

Generation of chimeric proteins

Chimeric proteins were generated in order to determine whether the M and/or the T domain of the BVDV E2 protein affect the intracellular localization of this viral protein, (Fig. 1). The M, T, or MT domain of E2 was replaced by the corresponding domains of the envelope glycoprotein of another bovine virus, the fusion protein F of BRSV. This glycoprotein is known to be expressed on the surface of infected cells (Zimmer et al., 2001a, b, 2002). In addition, a chimeric protein was constructed that contained the VSV G protein ectodomain and the BVDV E2 protein MT domain. All constructs were cloned into the pTM1 vector under the control of the T7 promoter and transiently expressed in BSR-T7/5 cells that stably express the T7 RNA polymerase. Lysates of transfected cells were separated by SDS-PAGE under non-reducing conditions and the viral glycoproteins were detected by Western blotting (Fig. 2). With all constructs containing the E2 ectodomain, both the monomeric form and a dimeric form were detected (Fig. 2A). This finding indicates that replacement of the MT domain does not prevent the E2 ectodomain dimerization process. With respect to the band pattern, some chimeras differed from the authentic E2 protein. Whereas the monomeric form of both E2 and E2-F(T) appeared as a single band (lanes a and c), a doublet band was visible in the case of E2-F(M) and E2-F(M)–E2(T) (lanes b and d). As discussed later in more detail, the two bands most likely reflect differences in glycosylation. The only difference detectable between G and G-E2(MT) was in the electrophoretic mobility due to the different lengths of the MT domains (Fig. 2B).

Localization of E2 and chimeric proteins

Authentic and chimeric proteins were transiently expressed in BSR-T7/5 cells and analysed by immunofluorescence microscopy. E2 protein was detectable only after cell permeabilization, but not by surface staining, indicating an intracellular localization of the protein (Fig. 3A). Analysis of the chimeric proteins containing the M or the MT region from BRSV F protein revealed that both chimeras were efficiently transported to the plasma membrane. However, after transfection with the E2-F(T) gene, only a very low number of cells showed surface expression and staining of these cells was very weak. In contrast to E2, VSV G protein was transported to the cell surface very efficiently (Fig. 3B). However, the chimeric G protein containing the E2 MT domain was detected by immunofluorescence only after cell permeabilization. These results indicate that (i) the failure of E2 to be transported to the plasma membrane is a genuine property of this viral protein and not dependent on other BVDV proteins, (ii) the E2 membrane anchor is responsible for the intracellular distribution of this pestiviral glycoprotein.

To confirm the results obtained by immunofluorescence
analysis, transfected cells were analysed by surface biotinylation and immunoprecipitation (Fig. 4A). Biotinylated proteins were detected with the chimeric proteins E2-F(MT) (lane b) and E2-F(M)–E2(T) (lane d), but not with authentic E2 (lane a) and not with the chimeric protein E2-F(T) (lane c). Using the biotinylation approach, VSV G protein (Fig. 4B, lane b) but not the chimeric protein G-E2(MT) (lane a) was detected on the cell surface. These data are in agreement with the result obtained by immunofluorescence analysis.

**Effect of endoglycosidases**

To analyse whether the viral glycoproteins detected in transfected cells contain high-mannose or complex oligosaccharides, they were radioimmunoprecipitated from transfected cells and treated with endoglycosidase H or N-glycosidase F. Analysis by SDS-PAGE (Fig. 5) revealed two bands for parental E2 protein. The upper band disappeared after treatment with either of the two endoglycosidases indicating that it only contains high-mannose oligosaccharides. The lower band in the untreated samples probably represents the unglycosylated form of the corresponding protein. The presence of this band may indicate that the signal peptide used to express the E2 protein does not translocate the protein into the ER with 100% efficiency. However, it is also possible that some E2 molecules are not accessible to glycosylation, e.g. because of incorrect folding. The same protein pattern was obtained after endoglycosidase treatment of the chimeric protein E2-F(T) retained intracellularly (data not shown). The chimeric protein E2-F(MT) only shows a faint band at the position of the unglycosylated E2 proteins. Two bands are visible at the area where the glycosylated proteins are expected. The lower band most likely represents a form containing high-mannose oligosaccharides, because it was shifted to a position below the 46 kDa marker by treatment with endoglycosidase H. The same shift was observed with the upper band after treatment with N-glycosidase F indicating the presence of complex oligosaccharides. From this result, we conclude that at least a portion of the protein E2-F(MT) is transported to the Golgi where it acquires complex type sugar side-chains and becomes resistant to endoglycosidase H treatment. The VSV G protein was not susceptible to endoglycosidase H, but shifted to a lower position after N-glycosidase F treatment (Fig. 5). This result is expected, because the G protein is known to contain two complex N-linked oligosaccharides. By contrast, the G-E2(MT) protein was sensitive to endoglycosidase H, suggesting that this chimeric protein is located in an early compartment of the secretory pathway and, therefore, only contains high-mannose glycans.

**Fig. 1.** Schematic drawing of the chimeric BVDV E2 or VSV G proteins. BVDV E2 sequences (white boxes), VSV G sequences (light grey boxes) and BRSV F sequences (hatched boxes). (M) designates the membrane anchor, (T) the cytoplasmic tail or the carboxy-terminal domain and (MT) the membrane anchor plus cytoplasmic tail/carboxy-terminal domain, respectively. It should be noted that the size of boxes are not proportional to the length of the amino acid chain. The putative cytoplasmic tail of E2 comprises only seven amino acids, whereas the tails of the F and G proteins are 24 and 29 amino acids in length, respectively. The membrane anchors of E2, F and G are 24, 26, and 32 amino acids in length, respectively. The initial and terminal amino acids are shown once for each domain. The sequence of the membrane anchor of E2 with the crucial arginine residue (R) is shown at the bottom. For each construct, the signal peptide of the VSV G protein was used. Letters above arrows indicate the primers used for cloning (see Table 1).
Fig. 3. Surface and intracellular immunofluorescence analysis of parental and chimeric BVDV E2 (A), and parental and chimeric VSV G proteins (B). BSR-T7/5 cells were transfected with pTM1 plasmids containing the corresponding genes. For intracellular staining, cells were fixed 24 h post-transfection by methanol/acetone and incubated with antibodies directed against the E2 or G protein. For surface staining, cells were first incubated with antibodies directed against either of the two viral glycoproteins and then fixed by paraformaldehyde. FITC-conjugated anti-mouse or anti-rabbit immunoglobulins were used as secondary antibodies.

Fig. 4. Surface biotinylation of parental and chimeric BVDV E2 proteins (A) and parental and chimeric VSV G protein (B). BSR-T7/5 cells were transfected with pTM1 plasmids containing the corresponding genes. At 24 h post-transfection, cells were labelled with sulfo-NHS-biotin and proteins were immunoprecipitated from cell lysates using specific antibodies. The immunoprecipitates were separated by SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane and incubated with streptavidin–peroxidase. (A) lanes a: E2; b: E2–F(MT); c: E2–F(T); d: E2–F(M)–E2(T); e: untransfected cells. (B) lanes a: G–E2(MT); b: G; c: untransfected cells.

Fig. 5. Endoglycosidase sensitivity of parental and chimeric BVDV E2 and VSV G proteins. BSR-T7/5 cells were transfected with pTM1 plasmids containing the corresponding genes. At 24 h post-transfection, cells were metabolically labelled with [35S]methionine/[35S]cysteine and E2 and G proteins were immunoprecipitated from cell lysates. Immunoprecipitates were digested for 2 h with endoglycosidase H (H), N-glycosidase F (F) or left undigested (−) and separated by SDS-PAGE under reducing conditions. Radiolabelled proteins were visualized by exposing the dried gel to an autoradiography film.
Effect of deletions and point mutations in the E2 protein

To get more information about the sorting signal, E2 proteins were constructed in which the cytoplasmic tail or half of the membrane anchor and the tail were deleted resulting in E2(Tdel) or E2(M*Rdel), respectively (Fig. 1). Furthermore, a mutant was generated in which the central arginine residue within the membrane anchor was replaced by an alanine residue, E2(R/A). In surface proteins often one or more arginine residues separate the cytoplasmic tail from the membrane anchor (Dalbey, 1990). Therefore, two arginines were added to two E2 mutants resulting in E2(TdelRR) and E2(TdelRR+R/A) (Fig. 1).

Cells transfected with expression plasmids containing either of the different constructs, were subjected to SDS-PAGE under non-reducing conditions and analysed by Western blotting. As shown in Fig. 7, mutant E2(M*Rdel) was not detectable under these conditions (lane d). With the other mutants, both the monomeric and the dimeric forms of E2 were present. Expression of mutants containing a terminal RR motif (lane c) and/or an R/A mutation in the putative membrane anchor (lanes e and f) resulted in stronger signals than expression of the authentic E2 (lane a) or the E2 protein lacking the carboxy-terminal tail (lane b). These differences may reflect differences in the expression rate, in the protein stability, or in the capacity to adopt the native conformation under the Western blot conditions.

Intracellular staining by immunofluorescence analysis provided evidence for the presence of the mutant protein E2(M*Rdel) (Fig. 8). The failure to detect this protein by Western blot (Fig. 7, lane d) may be explained by difficulties of this mutant to retain antigenicity after the denaturing conditions of the SDS-PAGE. Among the different proteins, only mutants containing the R/A exchange in the membrane anchor, E2(R/A) and E2(TdelRR+R/A), were efficiently transported to the cell surface. Addition of two terminal arginine residues to the membrane anchor in the mutant E2(TdelRR) resulted in faint-spotted surface staining. The other mutants, E2(Tdel) and E2(M*Rdel), resembled the parental E2 protein, i.e. there was no evidence for transport to the cell surface. This result was confirmed when analysing transfected cells by surface biotinylation. As shown in Fig. 9 (lanes e and f), surface expression was detectable only with the E2(R/A) and E2(TdelRR+R/A) protein but not with the other mutants. The band visible above the E2 protein is present in all lanes and, therefore, not E2-specific. In this assay, the faint surface expression of the E2(TdelRR) protein detected by the immunofluorescence technique could not be demonstrated (Fig. 9, lane c). These results indicate that the central arginine residue within the membrane anchor is an important element of the intracellular localization signal of the E2 protein.

DISCUSSION

Previous studies on BVDV-infected cells reported that the BVDV E2 protein is not transported to the plasma membrane (Greiser-Wilke et al., 1991; Weiland et al., 1999; Grummer et al., 2001). In the present study, we show that intracellular retention of E2 is a genuine property of this
glycoprotein and does not require the interaction with other viral proteins. Analysis of transfected cells by both immuno-fluorescence microscopy and surface biotinylation failed to detect E2 on the cell surface. The results obtained with the deletion mutant E2(Tdel) and the chimeric proteins E2-F(MT), E2-F(M)-E2(T) and E2-F(T) indicated that the E2 membrane anchor is responsible for the intracellular localization of E2. Sensitivity to endoglycosidase H treatment revealed that the intracellularly retained proteins only contain high-mannose oligosaccharides and are therefore most probably located in an early compartment of the secretory pathway. Radiolabelling of the chimeric proteins that are transported to the cell surface showed that, after 2 h, at least half of the protein synthesized had acquired endoglycosidase H resistance. This indicated that a substantial portion had left the ER.

**Fig. 8.** Surface and intracellular immuno-fluorescence analysis of native and mutant E2 proteins. Experimental procedure as in Fig. 3.
Interestingly, the chimeric protein E2-G(T) was transported to the cell surface. The cytoplasmic tail of G is known to contain a specific export signal for transport out of the ER. A di-acidic motif (Asp–Xxx–Glu, Xxx being any amino acid), has been shown to efficiently recruit G and other proteins to vesicles mediating export from the ER (Nishimura & Balch, 1997). The extended motif Tyr–Thr–Asp–Ile–Glu–Met has been reported to further increase the export efficiency and also to be functional on proteins that otherwise only inefficiently exit the ER (Sevier et al., 2000). As the E2-G(T) protein contains an intracellular localization signal in the membrane anchor and an export signal in the carboxy-terminal domain, our data suggest that the export signal within the cytoplasmic tail of the VSV G protein overrules the ER localization signal within the M domain of the E2 protein.

Retention in the ER has been described for the E2 proteins of viruses belonging to other genera within the family Flaviviridae. The best characterized virus in this respect is the E2 protein of hepatitis C virus (HCV), where the retention signal has also been assigned to the transmembrane domain (Cocquerel et al., 1998). The E2 transmembrane domains of all flaviviruses have a characteristic structure. They consist of two short hydrophobic stretches of amino acids that are separated by one or several hydrophilic residues including at least one charged amino acid (Cocquerel et al., 2000). This bipartite organization reflects the dual function of this hydrophobic domain. As the E2 protein is synthesized as part of a polypeptide, it requires, at the carboxy terminus, not only a membrane anchor but also a signal peptide to allow signal peptidase to separate E2 in the lumen of the ER from the downstream p7 protein. Whereas the hydrophobic domain of HCV E2 protein is followed by only two terminal amino acids, the carboxy-terminal domain of the corresponding BVDV protein consists of a hydrophilic stretch of seven amino acids (Elbers et al., 1996; Harada et al., 2000). The importance of this tail is not known; it is dispensable for intracellular localization of E2 as indicated by the results obtained with the E2(Tdel) mutant. Another difference between the E2 proteins of HCV and BVDV is that the latter virus only contains a single charged residue in the membrane anchor compared to the Asp–Ala–Arg tripeptide in the former virus. Replacing the arginine residue in the membrane anchor of BVDV E2 by an alanine results in a protein that is efficiently transported to the cell surface. In the case of HCV, ER retention is abolished more efficiently when – in addition to the arginine – the aspartic acid is replaced by an alanine (Cocquerel et al., 2000). The arginine residue may allow the hydrophobic domain to adopt a hairpin-like structure that is required to function both as a membrane anchor and a signal peptide. In such a structure, both the aminoterminal and the carboxy-terminal end of the transmembrane domain are directed to the lumen of the ER. It has been pointed out that this organization may be transient, and that after processing in the ER the tail portion may reorientate in such a way that it faces the cytosolic side of the membrane (Allison et al., 1999). In the case of the HCV E2 protein, evidence for this reorientation process has been obtained by adding tags to either end and determining the location of the tags (Cocquerel et al., 2002). With the E2-G(T) mutant we have a function-based assay for the location of the carboxy terminus. The export signal in the tail domain of VSV G protein is functional, if it is exposed at the cytosolic side of the ER membrane. As our results suggest that the export signal in the E2-G(T) protein overrules the ER localization signal in the M domain, the tail of the chimeric protein is expected to face the cytosolic side. Therefore, our data are in agreement with the assumption that the membrane-spanning domain reorientates in such a way that the carboxy-terminal tail faces the cytosolic side of the membrane.

Transmembrane domains usually consist of 20–25 hydrophobic amino acids. At least 16 leucines are required to form a transmembrane α helix (Monne et al., 1999). The two hydrophobic stretches in the membrane anchor of BVDV E2 consist of 11 and 12 amino acids, respectively. Therefore, they are too short to form α helices by themselves. This may explain the failure to detect E2(M*Rdel) after Western blotting. The protein is synthesized, as seen by immunofluorescence analysis, but the conformation of this protein is not stable enough to be detected after blotting onto the membrane. The E2 protein may require the stable anchoring in the membrane to adopt the correct conformation. Whether the deletion mutants are secreted into the cell supernatant has to be determined in future studies. The two hydrophobic domains of the E2 proteins of hepatitis C virus are also too short to form α helices by themselves. Therefore, it has been proposed, for this protein, that the hairpin-like structure of the hydrophobic domain is maintained as long as it is associated with the translocon that transfers the nascent polypeptide from the cytosol to the lumen of the ER. Once the host signal peptidase has cleaved between E2 and p7, the hydrophobic domain may reorientate to an extended structure that allows integration...
into the lipid bilayer and exposes the carboxy terminus at the cytosolic side of the ER (Op de Beeck & Dubuisson, 2003). From our results and from the short length of the hydrophobic domain, we propose that the model described for hepatitis C virus E2 also applies to the BVDV E2 protein. On the other hand, the E2 protein of flaviviruses has a longer hydrophobic domain and may have a different topology (Zhang et al., 2003).

The arginine residue within the membrane anchor of E2 plays a role not only for the formation of a hairpin-like structure during association with the translocon, but is also important for the intracellular retention of E2. The E2(R/A) mutant was efficiently transported to the cell surface. A hydrophilic amino acid within the membrane anchor has been shown to be essential for intracellular localization not only of flavivirus proteins but also for cellular proteins retained in the ER (Bonifacino et al., 1991). Interestingly in Western blot analysis, the E2(R/A) mutant gave a stronger signal than the parental protein. It is not known whether the arginine residue affects cotranslational conformation detected by the antibody or whether it renders the protein more sensitive to proteolytic degradation. A different effect was observed when arginine residues were added to the membrane anchor. This modification only had a marginal effect on intracellular retention of E2. Analysis of the mutant E2(TdelRR) by Western blot resulted in a stronger signal than the parental protein. At this amino acid position, the presence of arginine residues is obviously favourable for optimal detection of E2. Many membrane proteins contain a charged amino acid at the transition of the membrane anchor to the cytoplasmic tail (Dalbey, 1990). The reason why a charged amino acid at this location is absent in the E2 protein may be related to the function of the transmembrane domain as a signal peptide. Possibly, arginine residues at the end of the membrane anchor have a negative effect on the cleavage by signal peptidase or on reorientation of the hydrophobic domain.

Our data on the intracellular retention of the E2 protein are in agreement with results reported for the homologous protein of other members of the family Flaviviridae. In the case of another pestivirus, classical swine fever virus, a chimeric E2 protein containing the membrane anchor/cytoplasmic tail of an influenza A virus haemagglutinin was incorporated into recombinant influenza virions, suggesting that this chimera was transported to the plasma membrane, the site of influenza virus maturation (Zhou et al., 1998). Our data are also consistent with the information available for BVDV-infected cells. However, they are in contrast with data on BVDV E2 protein expressed by recombinant VSV or baculovirus. Using these expression systems it has been reported that E2 protein is expressed on the cell surface (Kweon et al., 1997; Grigera et al., 2000). The reason for this discrepancy is not clear. One reason may be that upon overexpression of the E2 protein the cellular machinery for intracellular retention may be saturated and some E2 protein may be transported to the cell surface. This phenomenon has been reported also for E1 and E2 proteins of hepatitis C virus (Bartosch et al., 2003; Drummer et al., 2003; Hsu et al., 2003).

Retention of viral glycoproteins and virus morphogenesis at intracellular compartments may provide some advantage for pestiviruses. The absence of viral glycoproteins from the cell surface makes it more difficult for the immune system to detect foreign antigen and possibly prolongs the survival time of infected cells. Maybe this also contributes to the characteristic property of BVDV to cause intrauterine infection (Bolin et al., 1985). In future studies we plan to analyse mutant glycoproteins that are transported to the cell surface in the context of a BVDV infection. It will be interesting to see whether such mutations affect (i) the site of virus maturation, (ii) the incorporation into virus particles, (iii) the survival time of infected cells, and (iv) the capacity to cause intrauterine infections.

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REFERENCES


