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Unique Localization of Bovine Viral Diarrhea Virus Non-Structural NS4B Protein in Infected Cells

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ABSTRACT

Bovine viral diarrhea virus (BVDV), an important pathogen infecting ruminants, has 2 biotypes: cytopathic (cp) and noncytopathic (ncp), which are related to the onset of disease. The viral replication complex is composed of viral non-structural (NS) proteins, raising the possibility that NS protein(s) play a role in viral biotypes. To gain insight into this possible role, we analysed the intracellular localization of each NS protein in both cp and ncp virus-infected cells, and found that NS4B protein, a possible anchor protein of the viral replication complex, showed a unique dotted localization pattern that markedly merged with an endoplasmic reticulum marker, unlike other NS proteins, although there was no difference in the localization of NS4B protein between the 2 biotypes.

Indexing terms/Keywords

Bovine viral diarrhea virus; Non-structural protein; Localization

Academic Discipline And Sub-Disciplines

Veterinary Science

SUBJECT CLASSIFICATION

Virology

TYPE (METHOD/APPROACH)

Molecular Analysis

INTRODUCTION

Bovine viral diarrhea virus (BVDV), a pestivirus belonging to the family *Flaviviridae,* is a major pathogen infecting ruminants such as cattle. Both viral genotypes, BVDV-1 and -2, are classified into 2 biotypes, noncytopathic (ncp) and cytopathic (cp), based on their ability to induce a cytopathic effect in cell culture.

Flaviviral non-structural (NS) proteins play important roles in viral genome replication and virus assembly (Hughes et al., 2009; Li and McNally 2001; Ma et al., 2011; Popescu et al., 2011; Stapleford and Lindenbach, 2011). The BVDV proteins NS3, NS4A, NS4B, NS5A, and NS5B form the viral replication complex (Behrens et al., 1998; Tomassini et al., 2003). NS4B is an integral membrane protein associated with virus-induced membrane alteration (Weiskircher et al., 2009), and is presumed to serve as a scaffold for viral replication complex formation. NS3 and NS5A proteins co-localize in the endoplasmic reticulum (ER) of BVDV-infected cells (Yamane et al., 2009; Zahoor et al., 2009) and NS3, NS4B, and NS5A have been shown to physically associate with these proteins (Qu et al., 2001).

Cellular membrane rearrangement is commonly observed in flavivirus-infected cells (Egger et al., 2002; Mackenzie et al., 2005; Miller and Krijnse-Locker 2008). The NS proteins of flaviviruses, such as West Nile virus (WNV) and dengue virus (DENV), associate with the virus replication complex, inducing membrane alteration of the virus-infected cells (Egger et al., 2002; Gillespie et al., 2010; Welsch et al., 2009). The expression of the hepatitis C virus (HCV) NS4B protein alone is sufficient to induce membrane rearrangement, by its lipid modification and polymerization activities (Yu et al., 2006). Altered membrane structure is able to exclude cellular nucleases and proteases, ultimately increasing the efficiency of viral genome replication and virus assembly.

These observations suggest that NS proteins may play a role in determining BVDV biotypes. A previous study indicated that uncleaved NS2-3 protein is abundant in ncp virus-infected cells, while cleaved NS3 protein, possessing full protease/replicase activity, is observed in cp virus-infected cells. These data suggest that NS3 protein is a factor in viral cytopathogenicity, potentially increasing viral RNA replication (Lackner et al., 2004). Another possible determinant of BVDV biotypes is the amount of double-stranded RNA (dsRNA) produced during viral genome replication. dsRNA induces

interferon (IFN) synthesis and apoptosis of the infected cells, which can be attributed to the innate immune response against viral infection. IFN induces expression of a dsRNA-dependent protein kinase (PKR) (Meurs et al., 1990), which phosphorylates eukaryotic translation initiation factor 2 (eIF2α) (Williams 1999), leading to the suppression of translation and causing apoptosis. PKR also regulates the activities of apoptosis-related proteins such as Fas (Balachandran et al., 1998; Takizawa et al., 1996), and activates the caspase pathway (Gil and Esteban 2000). Infection with cp BVDV readily activates PKR and the caspase pathway (Gil et al., 2006; Yamane et al., 2005). The amount of dsRNA in cp BVDV-infected cells is 100 times more than in ncp BVDV-infected cells, indicating that dsRNA-induced apoptosis is involved in viral cytopathogenicity (Yamane et al., 2006).

In this study, to gain insight into BVDV biotypes, we examined and compared the localization of NS proteins in cp and ncp virus-infected cells.

MATERIALS AND MOTHODS

Cells and viruses

MDBK cells and LB9.K cells were obtained from the American Type Culture Collection (ATCC). Primary bovine foetal muscle (BFM) cells were described previously (Yamane et al., 2005). MDBK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum (FCS) at 37°C in a humidified 5% CO_2 atmosphere. LB9.K cells and BFM cells were grown in DMEM supplemented with 10% FCS at 37°C in a humidified 5% CO_2 atmosphere. Prior to use, cells and FCS were confirmed to be free of BVDV by reverse transcriptase-polymerase chain reaction (RT-PCR). BVDV Nose, KS86-1cp, and KS86-1ncp strains were described previously (Nagai et al., 2003).

Plasmid construction

The NS4B gene sequence derived from BVDV cp Nose strain was amplified by RT-PCR using primers with *Eco*RI and *Xho*I restriction sites at the 5′ and 3′ ends, respectively, and cloned into the pGEX-5X-1 plasmid (GE Healthcare Life Sciences), named pGEX-NS4B. A partial NS4B gene sequence, encoding amino acids 292–411, was cloned into *Eco*RI and *Sal*I sites of the plasmid, named pGEX-partial NS4B. The NS4B gene sequence was also cloned into pGBKT7 between *Eco*RI and *Xho*I sites. Myc-tagged NS4B gene was subcloned into the pCAGGS mammalian expression plasmid, named pCAG-NS4B, between *Xho*I and *Bgl*II sites. The plasmids pME-NS3 and pCAG-NS5A were described previously (Yamane et al., 2009; Zahoor et al., 2010). The NS4A gene sequence derived from Nose strain was generated using primers with *Nde*I and *Pst*I sites at the 5′ and 3′ ends, respectively, and cloned into the plasmid pME18S-myc, named pME-NS4A. Myc-tagged NS4A gene was subcloned into a pCAGGS vector, named pCAG-NS4A, between *Xho*I and *Bgl*II sites.

Expression and purification of the GST fusion protein

E. coli BL21 strain was transformed with either pGEX-NS4B or pGEX-partial NS4B and grown in Luria-Bertani (LB) broth containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol to the exponential phase. The expression of the glutathione S-transferase (GST)-fusion proteins was induced by incubating with 0.1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) for an additional 3 h after reaching the exponential growth phase at 37°C. After centrifugation at 6,000 × *g* for 30 min at 4°C, the bacterial pellets were suspended in GST soluble buffer (40 mM Tris-HCl pH 7.5, 5 mM EDTA pH 8.0, 0.5% Triton X-100) supplemented with the cOmplete Protease Inhibitor Cocktail (Roche Diagnostics). The bacterial suspension was disrupted by ultra-sonication and was centrifuged at 14,000 × *g* for 15 min at 4°C. The supernatants were incubated with a 50% slurry GST-Sepharose 4B beads (GE Healthcare Life Sciences) overnight at 4°C with end-over-end rotating, and then the beads were washed with GST soluble buffer 5 times. The GST-fusion protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Bio-Safe Coomassie (Bio-Rad) according to the manufacturer's protocol. To elute the fusion protein, the beads were incubated with elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) for 30 min and centrifuged to collect the supernatants. This procedure was repeated 3 times. Eluted recombinant proteins were quantified using the Protein Quantification Kit-Rapid according to the manufacturer's protocol (Dojindo Molecular Technologies).

Antibodies

The antisera against NS4B protein were generated as follows: BALB/c mice (8 weeks old; female) were intraperitoneally injected with GST-partial NS4B protein 3 times at 2-week intervals. The experiment was conducted according to the Guidelines for the Care and Use of Laboratory Animals, Graduate School of Agriculture and Life Sciences, the University of Tokyo. Rabbit anti-NS3 pAb and mouse anti-NS5A monoclonal antibody (mAb) were described previously (Yamane et al., 2009; Zahoor et al., 2009). Rabbit anti-NS5A pAb (WU170) was kindly provided by Charles M. Rice (Rockefeller University) (Qu et al., 2001). Mouse anti-actin mAb was purchased from Millipore. Rabbit anti-calnexin pAb was from Enzo Life Sciences and rabbit anti-golgin97 pAb was from Abcam. Mouse anti-dsRNA mAb J2 was from English & Scientific Consulting. Horseradish peroxidase (HRP)-conjugated rabbit anti-GST pAb was from GE Healthcare Life Sciences. HRP-conjugated anti-myc mAb was from Santa Cruz Biotechnology. HRP-conjugated sheep anti-mouse IgG pAb was from

Amersham. FITC-conjugated anti-myc mAb, Alexa 488-conjugated goat anti-mouse pAb, and Alexa 633-conjugated goat anti-rabbit pAb were from Invitrogen.

Immunoblot analysis

SDS-PAGE and electro-transfer of proteins were performed as previously described (Yamane et al., 2006; 2009). BVDV-infected cell lysates were resolved by 10% SDS-PAGE and electro-transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). After blocking with 3% nonfat milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) at 4°C for 30 min, the membranes were incubated with primary antibodies at room temperature (RT) for 1 h. After washing, the membranes were incubated at RT for 1 h with HRP-conjugated secondary antibodies. The protein bands were detected by enhanced chemiluminescence (ECL; GE Healthcare Life Sciences). The images were taken with the LAS-4000 mini image analyser system (Fujifilm).

Transfection

LB9.K cells were transfected with the mammalian expression plasmid using Lipofectamine 2000 (Invitrogen) as described in the manufacturer's protocol. The cells were seeded 24 h before transfection. At 24 h post transfection, cells were washed with PBS and lysed using lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM NaF, 1% Triton X-100, 0.15% SDS, 1% NP40, 1% deoxycholic acid sodium salt) supplemented with cOmplete Protease Inhibitor Cocktail.

Immunofluorescence and confocal microscopy

The cells were grown on 8-well chamber slides (Nunc), and infected with BVDV or transfected with the mammalian expression plasmids. The slides were washed with PBS and fixed with 4% paraformaldehyde for 15 min. Fixed cells were permeabilised with 0.5% Triton X-100 for 15 min at RT. The cells were blocked with PBS containing 10% BSA and probed with the primary antibodies for 1 h. After washing with PBS-T, the cells were incubated with their respective secondary antibodies conjugated with either Alexa 488 or Alexa 633. After washing with PBS-T, the slides were treated with mounting medium (Dako), and the images were visualized using a laser-scanning confocal microscope (LSM-510; Carl Zeiss).

Co-localization analysis

Pearson's correlation coefficients were obtained from WCIF ImageJ (Tony Collins and Wayne Rasban, Wright Cell Imaging Facility, Toronto, Canada).

RESULTS

Detection of NS proteins in virus-infected cells

To analyse the localization of BVDV NS proteins, the LB9.K cells infected with KS86-1cp or KS86-1ncp at a multiplicity of infection (MOI) of 75 were double-stained with anti-NS4B and anti-NS5A polyclonal antibodies (pAbs) at 12 h, 18 h, and 24 h post infection (p.i.) (Figure 1). NS4B protein showed a dotted staining pattern at all time-points, while NS5A protein showed a homogenous staining pattern in the cytoplasm. NS4B protein was partially co-localized with NS5A protein at all time-points in the infected cells. We also examined the expression and localization of NS3 using anti-NS3 pAb. Similarly to NS5A protein, NS3 protein showed a homogenous staining pattern in the cytoplasm and was partially co-localized with NS4B protein in the infected cells (Figure 1). We observed similar results in other cells, including MDBK and BFM cells (data not shown). Together, these findings suggest that the NS proteins are expressed at similar levels in cp and ncp strain-infected cells, and there is no difference in their localization between the 2 biotypes.

Detection of NS proteins in transfected cells

It is possible that localization of the NS proteins (Figure 1) is determined by interaction with other viral factors in virus-infected cells. To assess this possibility, LB9.K cells were transfected with each NS-expression plasmid prepared from cp virus, and each NS protein containing a myc-tag was detected by immunostaining with anti-myc antibody at 24 h post transfection. NS4B protein exhibited dot-like fluorescent signals or a punctate staining pattern, while NS3 and NS5A proteins exhibited nearly homogenous staining patterns in the cytoplasm (Figure 2), indicating results equivalent to those seen in virus-infected cells. We also detected NS4A protein in transfected cells, showing localization similar to that of NS3 and NS5A proteins. These results demonstrate that the unique localization of NS4B observed in virus-infected cells is likely determined by a distinct property of the protein.

Immunoblot detection of NS4B protein in virus-infected cells

It is interesting to note that the localization of NS4B protein was different from that of NS4A (Figure 2). In BVDV-infected cells, NS4A protein also showed a homogenous staining pattern (Mohamed et al., 2014). Since anti-NS4B antibody can also detect the uncleaved NS4A-4B protein form in virus-infected cells (Lindenbach et al., 2013), the dot-like fluorescence signals observed in Figure 1 may show the localization of this uncleaved form. To clarify this possibility, we examined the kinetics of

NS4B protein expression in virus-infected cells. NS4B protein was detected in the cp virus-infected cell lysate by an immunoblot assay with anti-NS4B pAb at several time-points (Figure 3). At 24 h p.i., a weak signal at approximately 45 kDa was detected, suggesting expression of the uncleaved NS4A-4B protein form. These results indicated that NS4 protein is rapidly processed into NS4A and NS4B proteins in virus-infected cells, authenticating the dot-like localization of the NS4B protein.

Subcellular localization of NS4B protein in virus-infected cells

Our data suggest that the BVDV NS4B protein could be associated with NS3 and NS5A proteins (Figure 1). We previously showed that NS3 and NS5A proteins localize to the ER (Yamane et al., 2009; Zahoor et al., 2009), suggesting that NS4B protein may also localize to this compartment. To assess this possibility, we analysed the subcellular localization of NS4B protein in the virus-infected LB9.K cells. The KS86-1cp or KS86-1ncp-infected cells were immunostained with anti-NS4B pAb, anti-calnexin pAb as a marker for the ER, and anti-golgin97 pAb as a marker for the Golgi at 24 h p.i. NS4B protein signals were partially merged with the ER marker in the cells infected with either biotype (Figure 4A), but not with the Golgi marker (Figure 4B). Pearson's coefficients supported the localization of NS4B protein in the ER, with a Pearson's r of 0.78 for NS4B/calnexin, 0.23 for NS4B/golgin97 in cp-virus infected cells, 0.54 for NS4B/calnexin, and 0.26 for NS4B/golgin97 in ncp-virus infected cells. Similar results were obtained in LB9.K cells at 12 h p.i., as well as in MDBK cells at 24 p.i. (data not shown). Taken together, we conclude that NS4B protein might be localized in the ER and that there is no obvious difference in its localization between the 2 biotypes in BVDV-infected cells.

Detection and subcellular localization of dsRNA in virus-infected cells

To detect dsRNA in the virus-infected cells, the MDBK cells were infected with KS86-1cp or KS86-1ncp at an MOI of 10 and were fixed and stained using anti-dsRNA mAb at 12, 18, and 24 h p.i. (Figure 5). The positive dot-like fluorescence signals in the cp virus-infected cells were detected at all 3 time-points, albeit with a very weak signal at 12 h p.i., whereas dsRNA was not detected in the ncp-infected cells at any time-points. These data demonstrated different levels of dsRNA accumulation between BVDV biotypes, supporting our previous results that showed the amount of dsRNA in cp virus-infected cells is 100 times more than the amount in ncp-infected cells (Yamane et al., 2006).

To assess the subcellular localization of dsRNA, we infected the cells with KS86-1cp at an MOI of 75 and immunostained the cells with either anti-dsRNA mAb and anti-calnexin pAb or anti-golgin97 pAb at 24 h p.i. The dotted fluorescence signal of the dsRNA was merged with that of the ER (Figure 6A), but not the Golgi apparatus (Figure 6B), with a Pearson's r of 0.49 for dsRNA/calnexin and 0.10 for dsRNA/golgin97 in cp strain-infected cells. These results suggest that the virus replication complex, where dsRNA is produced, is associated with the ER.

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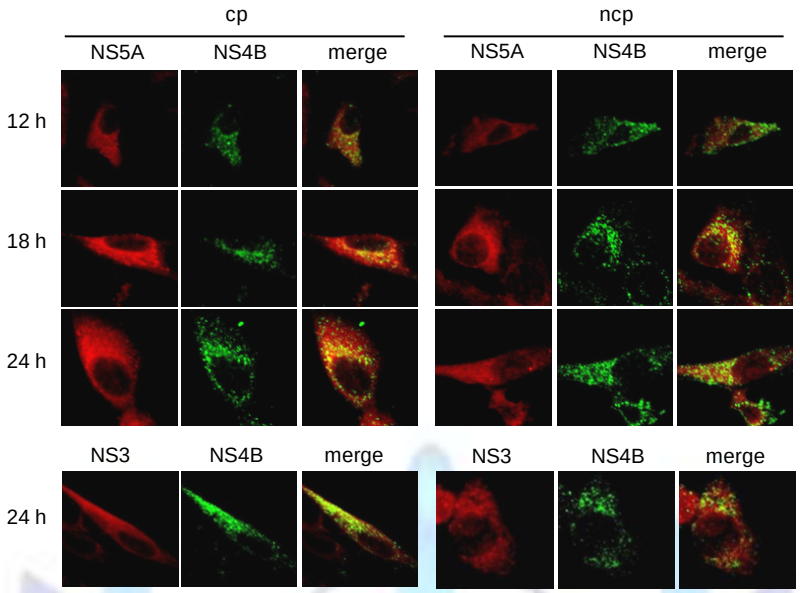
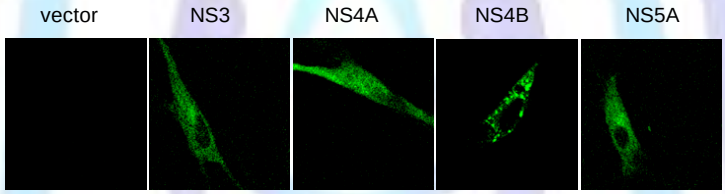
Figure 1. Expression of NS proteins in virus-infected cells. LB9.K cells were infected with cp or ncp virus, fixed, and stained



with anti-NS4B pAb or anti-NS5A mAb at 12 h, 18 h, and 24 h p.i. or with anti-NS3 pAb at 24 h p.i. Green fluorescence indicates NS4B protein, and red fluorescence indicates NS5A or NS3 protein. Merged images are also shown.

Figure 2. Expression of NS proteins in plasmid-transfected cells. LB9.K cells were transfected with the plasmid expressing



each myc-tagged NS protein from the cp virus (pME-NS3, pCAG-NS4A, pCAG-NS4B, or pCAG-NS5A) or empty vector (pCAG-myc). At 24 h p.t., the cells were fixed and stained with anti-myc mAb. Green fluorescence indicates NS proteins.

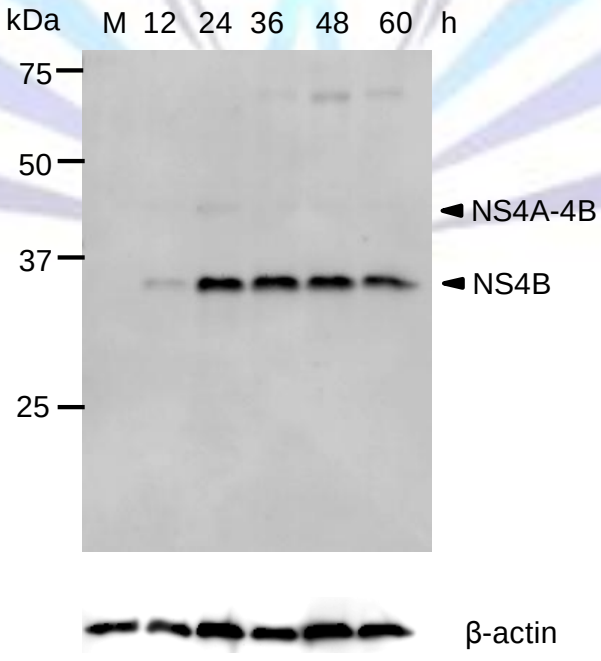


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Figure 3. Immunoblot detection of NS4B protein in virus-infected cells. MDBK cells were infected with the cp virus at an MOI of 3.5. The cell lysates were prepared at 12 h, 24 h, 36 h, 48 h, and 60 h p.i., and analysed by immunodetection of anti-NS4B pAb. The lysate of mock-infected cells was also analysed at 12 h p.i. and is presented in lane M. β-actin was used as an internal loading control.

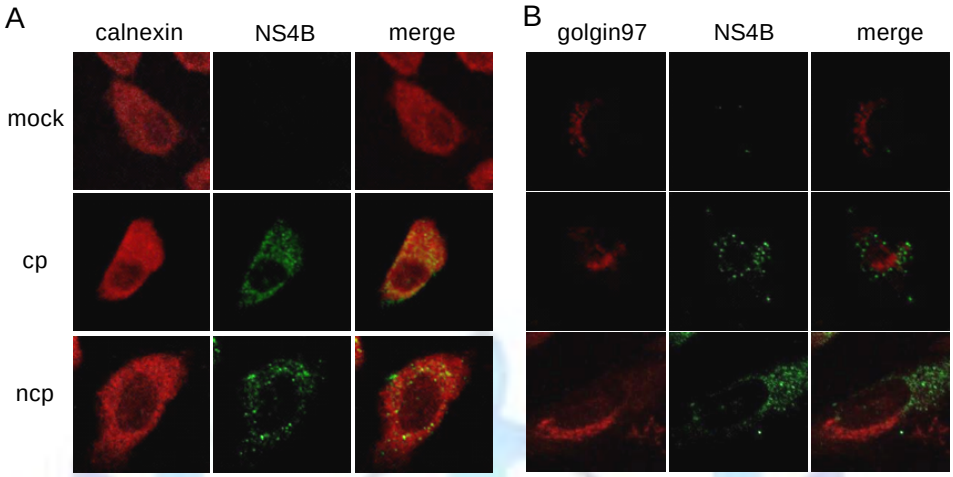

Figure 4. Subcellular localization of the NS4B protein in virus-infected cells. LB9.K cells were infected with cp or ncp virus, fixed, and stained with anti-NS4B pAb and anti-calnexin pAb (A) or with anti-golgin97 pAb (B) at 24 h p.i. Green fluorescence indicates NS4B, and red fluorescence indicates calnexin or golgin97. Merged images are also shown.

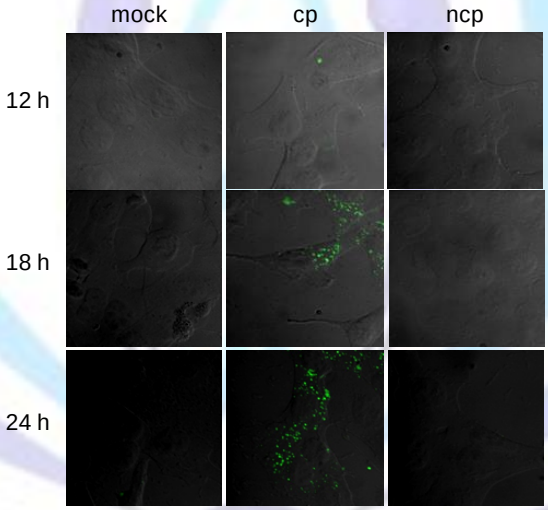

Figure 5. Expression of dsRNA in virus-infected cells. MDBK cells were infected with cp or ncp virus or mock-infected, fixed, and stained with anti-dsRNA mAb at 12 h, 18 h, and 24 h p.i. Green fluorescence indicates dsRNA.

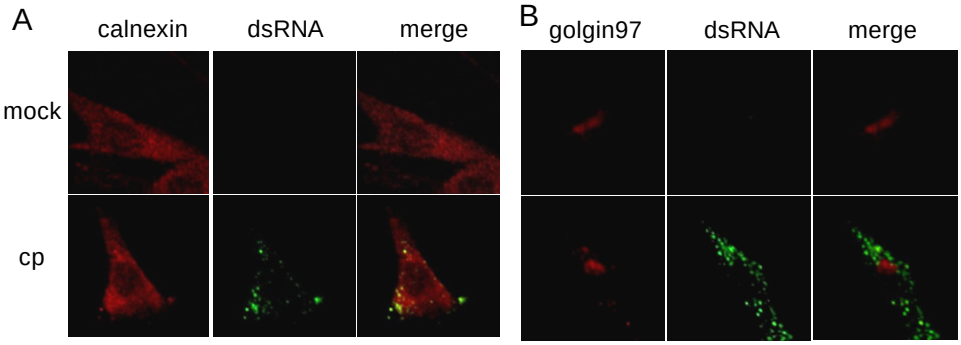

Figure 6. Subcellular localization of dsRNA in virus-infected cells. LB9.K cells were infected with cp or ncp virus, fixed, and stained at 24 h p.i. with anti-dsRNA mAb and anti-calnexin pAb (A) or with anti-golgin97 pAb (B). Green fluorescence indicates dsRNA, and red fluorescence indicates calnexin or golgin97. Merged images are also shown.

DISCUSSION

BVDV has 2 biotypes, cp and ncp, which are related to the onset of disease. The mechanisms of viral cytopathogenicity have yet to be elucidated in detail. One possibility is that the difference in cytopathogenicity is due to different levels of dsRNA accumulation, which regulates apoptosis of virus-infected cells, between the 2 biotypes. Since dsRNA is produced in the NS protein-composed replication complex, biological differences in the NS proteins of each biotype may also contribute to virus cytopathogenicity. Here, we found similar expression levels and localization of the NS proteins between the 2 biotypes, raising the possibility that localization of the replication complex should be unrelated to BVDV cytopathogenicity.

The NS4B protein showed a unique dot-like localization that differed from other NS proteins and that partially merged with the ER marker in virus-infected cells. As an integral membrane protein, the NS4B protein is believed to be an anchor protein in the replication complex (Weiskircher et al., 2009). Supporting this view, the replication complex must be present in a dot-like distribution like the NS4B protein. In addition, we found that dsRNA likely contained in the replication complex was also detected as dot-like fluorescence signals, which were merged with the ER marker. Taken together, these results strongly suggest that BVDV replication complex might be associated with the ER, although we did not directly confirm co-localization of NS4B protein with dsRNA. This supports the notion that BVDV may induce rearrangement of the ER membrane, as reported for other *Flaviviridae* viruses such as WNV, DENV, and HCV (Gillespie et al., 2010; Welsch et al., 2009; Miyanari et al., 2007).

Notably, our finding that NS4B co-localizes in the ER of BVDV-infected cells was different from a previous report showing that NS4B localizes to the Golgi complex of infected cells (Weiskircher et al., 2009). This discrepancy may be due to different virus strains, the cells used, or other different experimental factors.

We conclude that similar localization of the NS proteins between the 2 biotypes revealed that intracellular trafficking of NS proteins is biotype-independent. It is still unknown why the levels of dsRNA accumulation differ between cp and ncp BVDV. Further investigation is required to explain this intriguing observation.

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