SII-3

Superinfection exclusion of BVDV occurs not only at the level of structural protein-dispensable viral replication but also at the level of structural protein-required viral entry

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ABSTRACT

For a variety of viruses, the primary virus infection has been shown to prevent superinfection with a homologous secondary virus; however, the mechanism of exclusion has not been clearly understood. In this work, we demonstrated that BVDV-infected MDBK cells were protected from superinfection with a homologous superinfecting BVDV, one of the positive-sense RNA pestiviruses, but not with an unrelated rhabdovirus, such as vesicular stomatitis virus. Once superinfection exclusion was established by a primary infection with BVDV, the transfected infectious BVD viral RNA genome was shown to be competent for viral translation, but not viral replication. In addition, our results also demonstrated that upon superinfection, the viral RNA genome of viral particles was not transferred into the cytoplasm of BVDV-infected cells. Using newly developed system involving rapid generation of the MDBK cells expressing BVD viral proteins, we subsequently found that expression of the viral structural proteins was dispensable for the block occurring at the level of viral RNA replication, but required for the exclusion at the level of viral entry step. Altogether, these findings provide evidence that the superinfection exclusion of BVDV occurs not only at the level of viral replication in which the viral replicase are involved, but also at the level of viral entry with which the viral structural proteins are associated, and that a cellular factor(s) play an essential role in this process.

INTRODUCTION

The Flaviviridae family currently consists of three genera, the classical flaviviruses, the pestiviruses, and hepatitis C viruses. Members of the pestiviruses include bovine viral diarrhea virus (BVDV), classical swine fever virus, and border disease virus, which are important animal pathogens. Infections of cattle with BVDV can result in a wide range of clinical signs from acute and self-limiting outcomes as the result of host immune response to a sporadic fatal mucosal disease (MD). In animals which have died of MD, two biotypes, cytopathic (cp) and noncytopathic (ncp), have been recognized based on cytopathogenicity when grown in tissue culture cells. Infection during pregnancy can lead to abortion, fetal defects, or birth of calves persistently infected with ncp BVDV, which are immunotolerant to the infecting virus. Interestingly, it has been suggested that persistently infected animals may subsequently develop MD by occurrence of a cp virus in addition to the ncp virus by RNA recombination. This idea is supported by the fact that in animals which have died of MD, an antigenically closely related virus pair of ncp and cp viruses can be isolated. Furthermore, superinfection of persistently infected animals with a closely related cp virus can result in MD.

The pestiviruses are enveloped, single-stranded positive-sense RNA viruses.

The RNA genome of BVDV is approximately 12.5 kb in size and encodes a single long open reading frame, which is flanked by 5′ nontranslated region (NTR) and unpolyadenylated 3′ NTR. The large nascent polyprotein of approximately 4,000 amino acids is translated via an internal ribosome entry site (IRES) mechanism. The polyprotein is co- and post-translationally processed by cellular and viral proteases into mature viral proteins, designated from the N-terminus as Npro, C, Erns, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Major viral structural proteins are the capsid (C) and three associated glycoproteins at the surface of the virion (Erns, E1, and E2). Among the nonstructural proteins of BVDV, NS3 contains sequence motifs for serine protease, NTPase, and helicase activity. The NS3 serine protease activity with a cofactor NS4A has been shown to be responsible for cleavage at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B sites. However, the cleavage at NS2/NS3 site can occur by various mechanisms, and NS2-3 cleavage has been associated with viral cytopathogenicity in tissue culture cells. Sequence analysis of NS5B reveals the presence of a characteristic sequence motif (Gly-Asp-Asp) for the viral RNA-dependent RNA polymerase, and it has been shown to have this activity using the purified protein. Recently, viral structural proteins (C, Erns, E1, E2) and Npro, p7, and NS2 are directly shown to be dispensable for autonomous viral RNA replication in transfected cells.

The ability of an established virus infection to interfere with a homologous superinfecting virus is known as superinfection exclusion or homologous interference. In many viruses, but not all, the mechanism of superinfection exclusion is not yet known, however, it has been suggested to take place at various stage of viral life cycle, including receptor-mediated attachment and penetration of viral core into the infected cells. For VSV, it has been reported that endocytic vesicle formation and internalization of receptor-bound ligands are decreased upon primary infection, which may be a mechanism for superinfection exclusion. Other viruses including HIV, down-regulation of the CD4 primary cellular receptor by three viral proteins (Env, Nef, and Vpu) has been shown to be a mechanism to prevent superinfection. In addition, temperature-sensitive mutants of Sindbis virus have also been used to investigate superinfection exclusion. Following infection of cells with Sindbis virus, superinfection exclusion can be observed by inability of the superinfecting viral genome to be replicated in the non-permissive temperature, although the factors involved remain to be elucidated.

In this report, we examined molecular mechanisms of superinfection exclusion using BVDV as a model system, and involvement of a cellular factor(s) in this process.

RESULTS

ncp BVDV-infected MDBK cells are protected from CPE when superinfected with cp BVDV, but not with VSV. To examine whether ncp NADLcIns- BVDV-infected MDBK cells are protected from cytopathic effect (CPE) when superinfected with the isogenic cp NADL BVDV, naive MDBK cells were first infected with ncp NADLcIns- virus at a multiplicity of 8-10 FFU/cell to ensure all cells were infected. At 12-hr post-infection, the ncp NADLcIns--infected cells were superinfected with isogenic cp NADL virus as shown in Figure 1A. In parallel, the uninfected MDBK cells were infected with only cp NADL virus without exposing the cells to ncp NADLcIns- virus as a control. As illustrated in Figure 1A, the CPE caused by cp NADL viral replication was monitored quantitatively by plaque assay or qualitatively by culture assay.

In the MDBK cells infected with only cp NADL virus, a CPE with morphological changes clearly visible by light microscopy was displayed after 1-day postinfection in culture assay (data not shown) or after 2-day postinfection in plaque assay (data not shown). At 5 day postinfection, clear plaques were formed as a result of the cp viral replication in plaque assays (Figure 1B, dish 4), and no viable cells were observed in culture assays (Figure 1D, dish 4). In contrast, neither plaques (Figure 1B, dish 3) nor any recognizable CPE (Figure 1D, dish 3) was found upon superinfection of the previously ncp NADLcIns--infected MDBK cells with cp NADL virus, even after 5-day postsuperinfection. The cells were continuously incubated until 5 days of infection when naive MDBK cells started to die due to overgrowth in our experimental conditions. As a control, the primary ncp NADLcIns--infected MDBK cells (Figure 1B and D, dish 2) did not show any signs of CPE after 5-day postinfection as seen with naive MDBK cells (Figure 1B and D, dish 1).

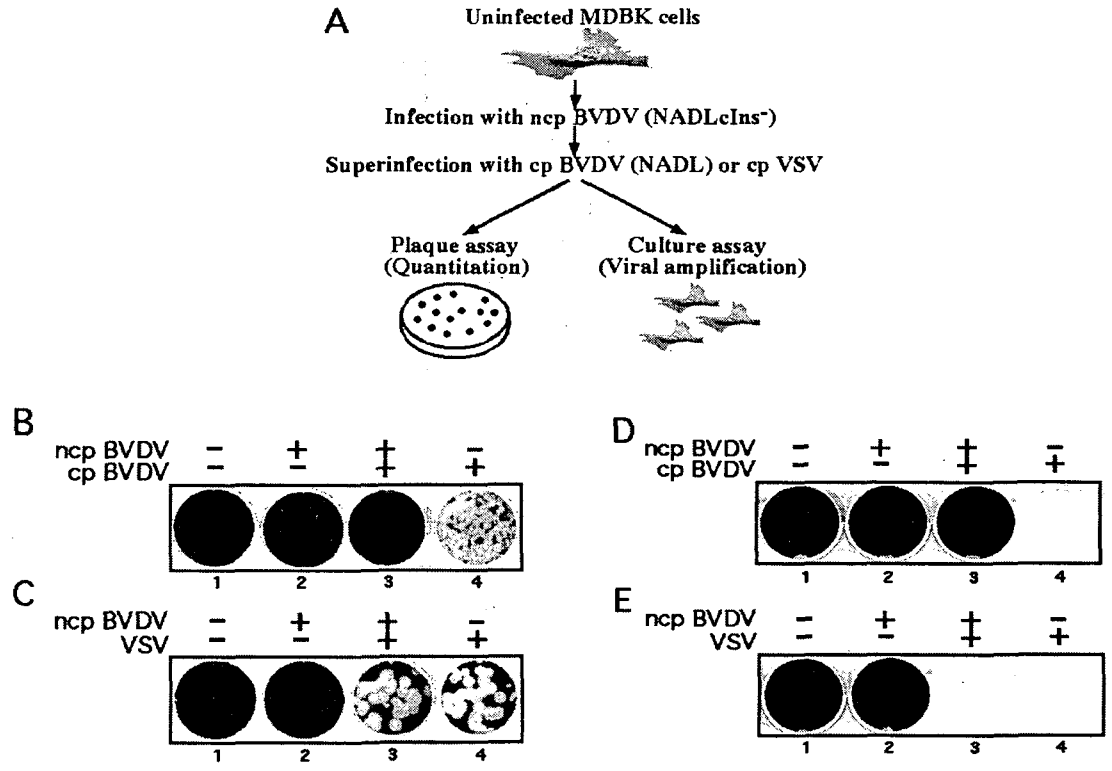

Fig. 1. Acutely ncp BVDV-infected MDBK cells are protected from CPE when superinfected with homologous cp BVDV, but not with VSV. (A) Schematic diagram outlining the identification of superinfection exclusion in ncp BVDV-infected MDBK cells. (B, C, D, and E) Naive MDBK cells were first mock-infected (-) or infected with ncp NADLcIns- BVDV for 1 hr at an MOI of 8-10 FFU. In parallel, naive MDBK cells were either mock-infected or infected with only cp NADL BVDV (B and D) or cp VSV (C and E) without preexposing to ncp NADLcIns- BVDV. At 12 hr post-infection, the infected cells were washed three times with DMEM, and either mock-superinfected (-) or superinfected with cp NADL BVDV (+) or cp VSV (bottom panel) for 1 hr. The cells were subsequently overlaid with agarose (B and C) or incubated in DMEM containing 10% horse serum (D and E). After 5 days (B and D) or 3 days (C and E) incubation, the cells were fixed and stained with crystal violet to visualize CPE.

We tested the possibility that the ncp NADLcIns--infected MDBK cells are generally protected from CPE caused by infection of other non-pestiviruses. To do so, the unrelated VSV (one of the negative-sense RNA rhabdoviruses) was used to challenge the ncp NADLcIns--infected MDBK cells as mentioned above (Figure 1A). The cells infected with only VSV (Figure 1C, dish 4) showed the characteristic large plaque formation at 3-day postinfection, and both size and number of plaques in the ncp NADLcIns--infected and VSV-superinfected MDBK cells were indistinguishable to those in the naive cells infected with only VSV (Figure 1C, compare dishes 3 and 4). This is also confirmed by culture assays as shown in Figure 1E, compare dishes 3 and 4. Therefore, our results have demonstrated that MDBK cells infected with either ncp NADLcIns- or ncp SD-1 virus (data not shown) were specifically protected from CPE when superinfected with cp NADL BVDV, but not with VSV.

Homologous superinfecting BVDV fails to replicate in the acutely BVDV-infected MDBK cells. The fact that ncp BVDV-infected cells were protected from CPE upon superinfection with cp BVDV raised two possibilities. First, in contrast to uninfected MDBK cells, the failure to support viral replication of the superinfecting cp virus in the ncp virus-infected cells may result in the protection from CPE. Alternatively, homologous superinfecting cp virus may be able to replicate in the ncp virus-infected cells. If cellular alteration caused by the primary ncp virus infection allows the infected cells to inhibit CPE, then the primary ncp virus-infected cells may appear to be protected from CPE in the presence of superinfecting cp viral replication. For this reason, we examined the question whether homologous superinfecting ncp BVDV can replicate in the primary ncp BVDV-infected MDBK cells.

Instead of using cp NADL virus for superinfection, the ncp NADLcIns--infected MDBK cells were superinfected with the homologous ncp NADLcIns- pac virus which encodes a dominant selective marker, a pac gene in its genome (Figure 2A). Expression of the pac gene upon superinfection allows us to determine viral replication by selecting the cells in the presence of puromycin (Figure 2A).

When naive MDBK cells were infected with only ncp NADLcIns-pac virus, as a positive control, the infected cells formed foci under overlaid agar in the presence of puromycin (Figure 2B) and also continued to grow in the culture media with the antibiotic (Figure 2C). As the result of superinfection of ncp NADLcIns--infected MDBK cells with ncp NADLcIns-pac virus and selection with puromycin, no foci was found in cultures of the ncp NADLcIns--infected MDBK cells, demonstrating that homologous superinfecting ncp NADLcIns-pac virus failed to replicate in the acutely infected MDBK cells with ncp NADLcIns- virus. Likewise, no surviving cells were found in cultures of the cells in the absence of overlaid agar (Figure 2C). As a control, the cells primarily infected with only ncp NADLcIns- virus were not survived under puromycin selection. Therefore, our results showed that acute infection with ncp BVDV prevents viral replication of homologous superinfecting ncp BVDV.

Transfected BVD viral RNA in acutely BVDV-infected MDBK cells is competent for translation, but not viral replication. We would like to determine whether the homologous transfected ncp BVDV viral RNA molecules are competent for the translation in the acutely BVDV-infected cells. To determine the translation of the input viral RNA only in the transfected cells, but excluding virus spread into adjacent cells, we generated a self-replicating BVDV viral RNA (ncp NADLcIns-ΔS), whose structural genes (C-E2) are deleted (Figure 3A). We took advantage of the luc gene as a reporter that can be used to measure translation of the viral RNA in a quantitative

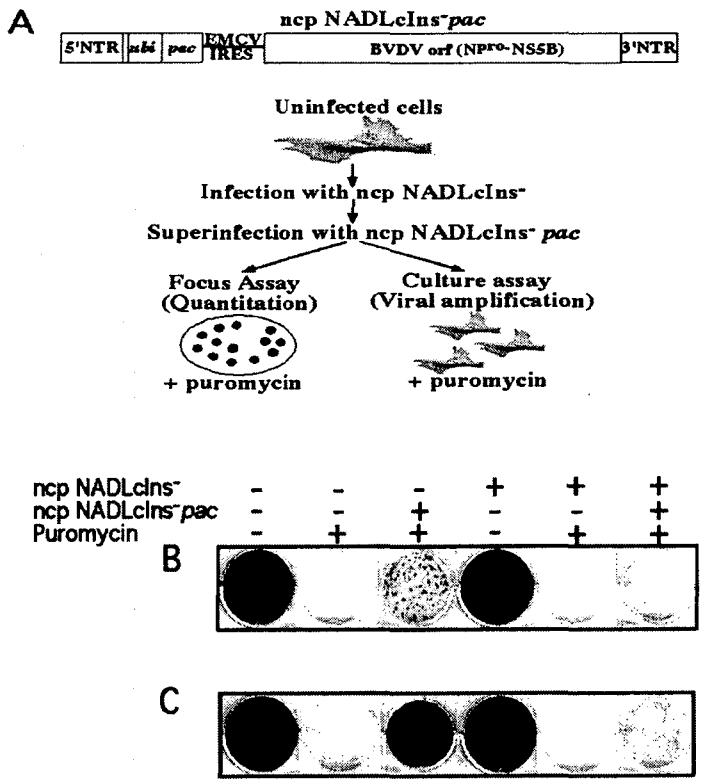

Fig. 2. Superinfection of ncp BVDV-infected MDBK cells with pac gene-encoding ncp BVDV fails to form a puromycin-resistant colony. (A) Schematic diagram outlining BVDV superinfection exclusion using a pac dominant selective marker. (B and C) ncp NADLcIns--infected MDBK cells were either mock-superinfected or superinfected with ncp NADLcIns-pac virus. As a positive control, naive MDBK cells were also either mock-infected or infected with ncp NADLcIns-pac virus. Subsequently, these cells were incubated under 2 ml of overlaid agar and on top of it 2 ml of DMEM containing 10 % horse serum and 10 μg/ml of puromycin (B). Alternatively, these cells were incubated directly in DMEM containing 10% horse serum and 5 μg/ml of puromycin (C). After selection, puromycin-resistant colonies (B) or puromycin-selected cells (C) were visualized by fixing and staining with crystal violet.

manner (Relative Light Unit). First we demonstrated that the ncp NADLcIns-ΔS-luc viral replicon whose luc gene expression was under the control of 5'NTR of BVDV was self-replicating in transfected cells (data not shown). In addition to this replication-competent RNA molecules, we also generated the isogenic replication-incompetent RNA (ncp NADLcIns-ΔS-luc-pol-) by substituting three residues in the catalytic active sites of NS5B RDRP protein (GDD to AAG) as shown in Figure 3A. Luciferase activity from replication-incompetent ncp NADLcIns-ΔS-luc-pol- RNA upon transfection allows us to discriminate between input translation and viral RNA amplification due to replication. Following transfection of these RNA molecules into either naive MDBK cells or the acutely ncp NADLcIns--infected cells, activity of the luciferase was measured at various time points after transfection (Figure 3B).

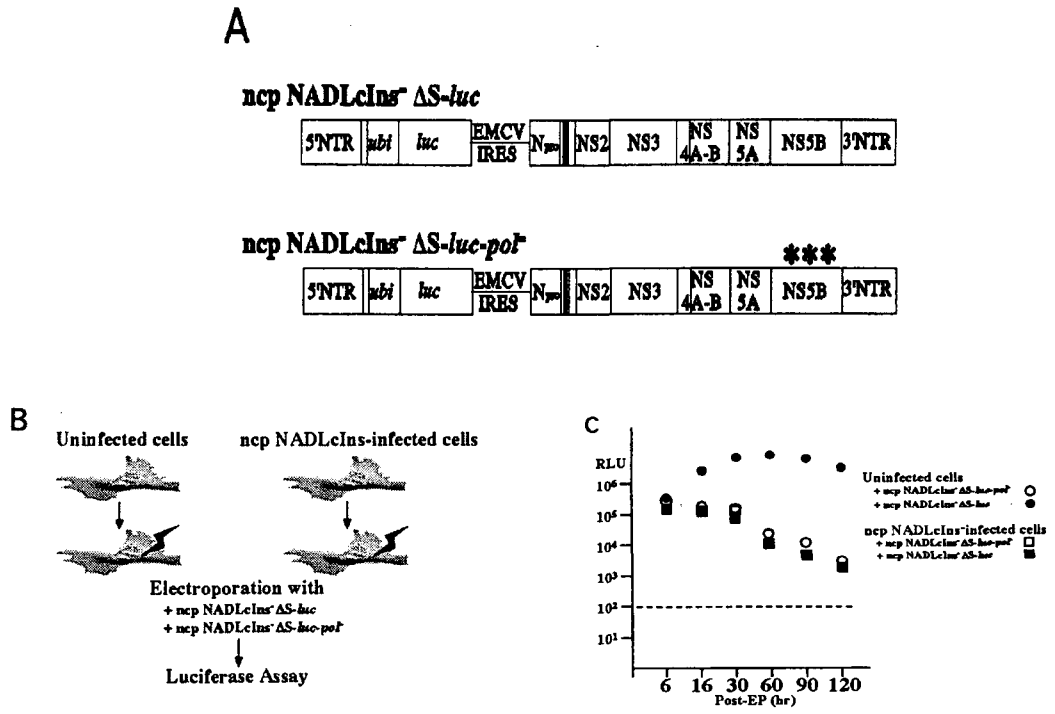

Fig. 3. Transfected BVD viral RNA in BVDV-infected MDBK cells is competent for translation, but not viral replication. (A) Schematic diagram of viral RNAs used in this experiment. ΔS indicates the deletions in the structural genes of BVDV (C-E2) as described in the text. pol- indicates three residues (GDD) in the catalytic active site of NS5B RDRP were substituted into AAG. (B) Schematic diagram outlining transfected BVD viral RNA is translated in BVDV-infected cells. (C) Either 8 x 10^6 cells of naive (circle) or ncp NADLcIn--infected MDBK cells (square) were transfected with 5μg of ncp NADLcIns-ΔS-luc viral RNA by electroporation. In parallel, 5μg of ncp NADLcIns-ΔS-luc-pol- viral RNA was also transfected into these two cells. All of the transfected cells were seeded into a 6-well plate. In the course of incubation, the cells were lysed in 200μl of lysis buffer at a given time point, and the luciferase activity was determined with 5μl of cell lysate. The dash line indicates the luciferase activity from naive MDBK cells without the electroporation, or from the cells transfected with ncp NADLcIns-ΔS-pac.

In the naive MDBK cells transfected with the ncp NADLcIns-ΔS-luc-pol- RNA molecules, luciferase assays at initial 6-hr posttransfection showed approximately 4.65 $\pm$ 1.58 x 10^5 RLU, which is the result of viral translation from the transfected replication-incompetent RNA in the absence of replication (Figure 3C). This initial activity was gradually decreased and eventually estimated to be about 5.72 $\pm$ 1.11 x 10^3 RLU at 120-hr posttransfection (Figure 3C). This allows us to estimate the rate of its decay over time in the absence of new viral RNA template for its translation. Similarly, the luciferase activity from the transfected replication-competent ncp NADLcIns-ΔS-luc RNA molecules at initial 6-hr posttransfection was also estimated to be around 5.04 $\pm$ 1.61 x 10^5 RLU, indicating that the productive viral replication of transfected RNA does not take place until 6-hr posttransfection. As expected, the initial activity from the replication-competent RNA was dramatically increased into about 3.24

± 0.57 x 10^6 RLU at 16-hr posttransfection, and maintained its activity until the time point of 120-hr posttransfection (Figure 3C). This gave us the level of luciferase activity over time in the presence of the viral RNA replication.

In the ncp NADLcIns--infected MDBK cells, the initial luciferase activity at 6-hr posttransfection was about 2.31 ± 0.78 x 10^5 RLU from the transfected replication-competent ncp NADLcIns-ΔS-luc RNA and about 2.56 ± 0.76 x 10^5 RLU from the transfected replication-incompetent ncp NADLcIns-ΔS-luc-pol- RNA (Figure 3C). These findings demonstrated that the transfected viral RNA was an active substrate for viral translation. In addition, the initial activity from the transfected replication-competent RNA molecules was gradually decreased during the course of time period in such a way, almost superimposed with that from the transfected replication-incompetent RNA molecules (Figure 3C). This result further confirmed our finding that transfected ncp BVDV viral RNA fails to replicate in the MDBK cells infected with ncp BVDV as shown in Figure 2.

Homologous superinfecting BVD viral particles fail to deliver their viral RNA into the cytoplasm of the BVDV-infected MDBK cells. In addition to a block occurring at the level of viral replication in the course of superinfection exclusion of BVDV, we further investigated the question whether superinfecting BVDV viral particles efficiently deliver their viral RNA genome into the cytoplasm in the early stages of viral infection. Since our results showed that transfected ncp BVDV viral RNA is fully translated in the ncp BVDV-infected MDBK cells (Figure 3), this question was addressed by determining the luciferase activity translated from the viral RNA of input ncp NADLcIns-luc viral particles upon superinfection, as illustrated in Figure 4A.
In positive control experiments using naive MDBK cells infected with ncp NADLcIns-luc viral particles, the initial luciferase activity (3.05 ± 0.16 x 10^4 RLU) at 6-hr postinfection was readily detected from the cell lysate. Subsequently, the initial activity was increased to 4.63 ± 0.38 x 10^5 RLU and stabilized at the level of about 5-8 x 10^5 RLU during the course of incubation. In negative control experiments using CRIB cells, which have been shown to have a defect in a receptor-mediated viral entry step, the luciferase activity detected from cell lysates of the CRIB cells infected with ncp NADLcIns-luc viral particles at all time points was only at the level of background in our system (Figure 4B). However, CRIB cells have been shown to support BVDV viral replication when viral entry step was bypassed by transfecting infectious viral RNA into the cells. This is also confirmed in our system (data not shown). Therefore, these results demonstrated that detection of the luc gene expression translated from the input viral particles can be used to be an indication of viral RNA delivery from the viral particles into the cytoplasm.

To address an exclusion at the level of viral entry, parental ncp NADLcIns--infected MDBK cells were superinfected with ncp NADLcIns-luc viral particles. Following incubation, the luciferase activity detected from the cell lysate was at the level of background of our system at all time points (Figure 4B). As expected, little luciferase activity was detected from the cell lysate of either naive CRIB cells or the ncp NADLcIns--infected CRIB cells upon superinfection at all time points (data not shown). Therefore, our results showed that superinfecting ncp BVD viral particles have a defect in delivery of their RNA genome into the cytoplasm of the ncp BVDV-infected MDBK cells.

Expression of the viral structural proteins is required for the exclusion at the level of viral entry stage, but dispensable for the block at the level of viral replication.

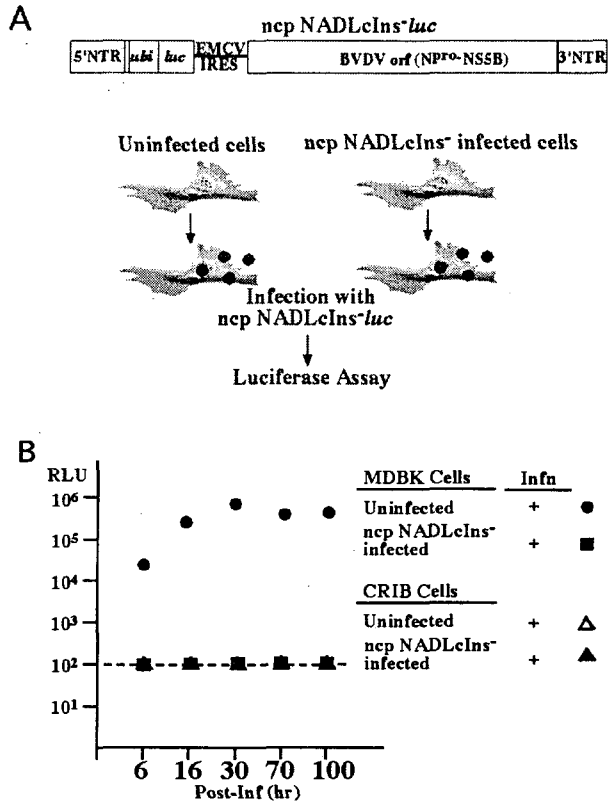

Fig. 4. Superinfecting BVDV viral RNA is not detected in the cytoplasm. (A) Genome organization of ncp NADLcIns-luc viral RNA was schematically illustrated (top panel). Schematic diagram outlining protocol for BVDV superinfection (bottom panel). (B) Either uninfected (circle) or ncpNADLcIns--infected (square) MDBK cells were superinfected with 1 ml of ncp NADLcIns-luc viral particles (9 x 10^6 FFU/ml). After incubation, the cells were lysed in 200μl of lysis buffer at each time point indicated, and luciferase activity was measured with 20μl of cell lysate. In addition, CRIB cells (triangle) were also infected with ncp NADLcIns-luc viral particles, incubated, lysed at a given time point, and used to determine the luciferase activity from the lysate as mentioned above. The dash line presents the background level of our assay as mentioned in Figure 3.

Furthermore, we would like to have a useful system to determine what viral proteins are involved in a block at the level of viral replication or at the level of viral entry step. Based on a recent report describing autonomous BVD viral RNA replicon as being sufficient to support complete RNA replication, we also generated a self-replicating RNA replicon in such a way that the structural genes of BVDV (C-E2) were deleted (ncp NADLcIn-ΔS-pac). Due to the lack of viral structural proteins, we cannot express only the nonstructural proteins without a packaging system through the course of infection. Instead, we took advantage of a pac dominant selective marker to rapidly generate MDBK cell lines expressing only the nonstructural proteins by selecting with puromycin within 60 hours after transfection of in vitro transcribed viral RNA replicon. In parallel, the MDBK cell line expressing both the structural and nonstructural proteins was also generated by transfection of infectious ncp NADLcIn-pac

viral RNA and subsequent selection with the antibiotic.

First of all, we raised the question of whether expression of the structural proteins is required for the exclusion at the level of viral replication. To address this issue, replication-competent ncp NADLcIns-ΔS -luc viral RNA was transfected into either the ncp NADLcIns-ΔS-pac MDBK cell line or ncp NADLcIns-pac MDBK cell line, and luciferase activity was determined at the time points as indicated in the figure legends. As a control, the replication-incompetent ncp NADLcIns-ΔS-luc-pol- viral RNA was also used for parallel transfection and luciferase assays.

In the ncp NADLcIns-pac-selected MDBK cell line, the initial luciferase activity translated from the replication-competent NADLcIns-ΔS -luc RNA at 6-hr posttransfection was determined to be approximately $2.11 \pm 0.45 \times 10^5$ RLU (Figure 5A). Subsequently, this initial luciferase activity was gradually decreased over time to $1.13 \pm 0.23 \times 10^3$ RLU at 120-hr posttransfection (Figure 5A). Likewise, the initial luciferase activity translated from the replication-incompetent NADLcIns-ΔS -luc-pol- viral RNA at 6-ht posttransfection was about $2.32 \pm 0.50 \times 10^5$ RLU, which is gradually decreased into $1.02 \pm 0.24 \times 10^3$ RLU at 120-hr posttransfection. This is consistent with the result observed in the ncp BVDV-infected MDBK cells (Figure 3). This result demonstrated that our system using puromycin selection closely reproduced the acutely infected MDBK cells with BVDV in regards to the ability to exclude superinfecting BVDV.

In the ncp NADLcIns-pac-ΔS-selected cell line, the initial luciferase activity translated from the replication-competent NADLcIns-ΔS -luc RNA was determined to be about $2.05 \pm 0.45 \times 10^5$ RLU at 6-hr posttransfection, which subsequently decreased over time to $1.09 \pm 0.20 \times 10^3$ RLU at 120-hr posttransfection, as seen in the ncp NADLcIns-pac-selected cell line. In the case of luciferase activities translated from the replication-incompetent NADLcIns-ΔS-luc-pol- RNA was $2.22 \pm 0.60 \times 10^5$ RLU at 6-hr posttransfection and $1.06 \pm 0.23 \times 10^3$ RLU at 120-hr posttransfection (Figure 5). Therefore, our results demonstrated that the structural proteins of BVDV are not required for the exclusion at the level of viral replication.

Furthermore, we investigated if expression of the structural proteins is essential for the exclusion at the level of viral entry. This question was addressed by superinfecting the two puromycin-selected MDBK cell lines with ncp NADLcIns-luc viral particles as described in Fig. 4. After superinfection, the initial luciferase activity detected from ncp NADLcIns-pac-ΔS-selected cell line was compared to that from ncp NADLcIns-pac-selected cell line.

In cell lysates of ncp NADLcIns-pac-selected MDBK cells, little luciferase activity was detected at all time points of our assay, which is consistent with the result obtained with ncp BVDV-infected MDBK cells (Figure 5). In cell lysates of ncp NADLcIns-pac-ΔS-selected, however, the initial luciferase activity of about $2.27 \pm 0.18 \times 10^4$ RLU at 6-hr posttransfection was detected. As expected, from cell lysate of naive MDBK cells were infected with ncp NADLcIns-luc viral particles, the initial activity at 6-hr posttransfection was also measured to be around $4.55 \pm 0.78 \times 10^4$ RLU. As a negative control, no luciferase activity was detected in the two selected MDBK cell lines and naive MDBK cells without infection with ncp NADLcIns-luc viral particles. Thus, our results demonstrated that the exclusion at the viral entry step is dependent upon expression of the viral structural proteins. Additionally, while the initial activity from naive MDBK cells ($4.55 \pm 0.78 \times 10^4$ RLU) was increased into $8.17 \pm 0.09 \times 10^5$ RLU, the initial acitivity from ncp NADLcIns-pac-ΔS-selected cells ($2.27 \pm 0.18 \times 10^4$ RLU) was decreased into $1.05 \pm 0.08 \times 10^3$ RLU. These results also showed that translation-competent BVD viral RNA fails to replicate in the MDBK cells expressing only nonstructural proteins of BVDV.

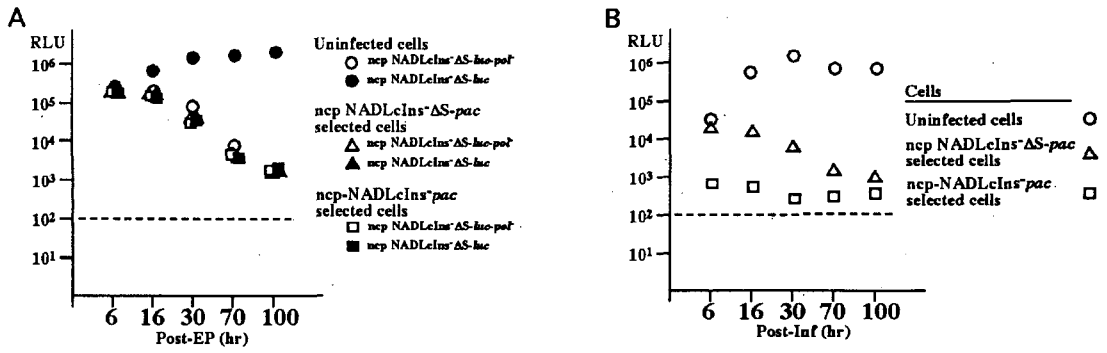

Fig. 5. Expression of BVDV structural proteins is dispensable for exclusion at the level of viral replication (A), but required for a block at the level of viral entry (B). Naive MDBK cells were mock-transfected or transfected with either ncp NADLcIns-ΔS-pac or ncp NADLcIns-pac RNA. These cells were incubated for 12 hr in DMEM with 10% horse serum, and then selected with 10 μg/ml of puromycin for 24 hr. (A) The selected cells (5 x 10^6) were reseeded in a T150 flask and incubated for 24 hr. As a control, naive MDBK cells were prepared at the same time. The selected or naive cells (8 x 10^6) were then transfected with either ncp NADLcIns-ΔS-luc or ncp NADLcIns-luc RNA. All of the transfected cells were seeded in a 6-well plate, and at the indicated time points, the cells in each well were lysed in 200 μl of lysis buffer and luciferase activity was measured with 5 μl of cell lysate. (B) As in A, the selected or naive cells (5 x 10^5/well) were seeded in a 6-well plate for about 24 hr, and subsequently infected with 1 ml of ncp NADLcIns-luc virus (9 x 10^6 FFU/ml). At a given time point, the cells in each well were lysed in 200 μl of lysis buffer and subjected to determine its luciferase activity with 20 μl of cell lysate.

CONCLUSION

For several decades, superinfection exclusion also known as homologous interference has been described in a variety of animal viruses, such as Newcastle disease virus, vesicular stomatitis virus, Sindbis virus, semliki forest virus, and classical swine fever virus. In spite of these extensive reports, the molecular mechanism of superinfection exclusion has not been clearly demonstrated because of technical difficulties to clearly distinguish between primary and secondary superinfecting viruses. In this report, the primary infecting virus of BVDV was clearly distinguished from homologous superinfecting BVDV by several approaches, which allows us to identify and fully characterize dual molecular mechanisms of superinfection exclusion and its requirements. First of all, unlike other viral systems, BVDV can be categorized into two biotypes, noncytopathic (ncp) and cytopathic (cp) viruses based on cytopathogenicity in cell culture. Whereas ncp BVD viral infection is known to establish persistent infections in animals, cp viruses are typically thought to be derived from ncp viruses by rare RNA recombination event. Our recent work has made progress in engineering stable, functional cDNA clones of an isogenic ncp and cp pair, which allows us to easily distinguish primary ncp BVDV infection from homologous superinfection with cp BVDV. Secondly, we also generated infectious ncp BVD viral RNA and viral particles encoding a dominant selective marker, the pac gene, which lets us to determine viral replication of its genome in the isogenic ncp BVDV-infected cells. Third, using the luciferase gene as a

reporter, we were also able to performed our experiments in a sensitive and quantitative manner.

Our findings here demonstrated the fact that acutely BVDV-infected MDBK cells were protected against superinfection with homologous superinfecting BVDV by three independent lines of evidence: 1) ncp NADLcIns-infected MDBK cells were completely protected from CPE when superinfected with homologous cp NADL virus (Figure 1),. 2) the MDBK cells infected with SD-1, another strain of ncp BVDV, were also protected against superinfection with cp NADL virus (data not shown), and 3) ncp BVDV-infected MDBK cells were not able to survive in the presence of puromycin upon superinfection with ncp pac-encoding infectious BVD viral particles (Figure 2).

As the result of efforts to understand molecular mechanisms of the superinfection exclusion of BVDV, we found that the acutely infected MDBK cells successfully exclude homologous superinfecting BVDV by two independent mechanisms. One of the dual mechanisms occurs at the level of viral replication. This was first demonstrated by transfection of the infectious pac-encoding BVD viral RNA into ncp BVDV-infected MDBK cells by electroporation. In these experiments (data not shown), the artificially introduced viral RNA into the cytoplasm did not allows the cells to survive under puromycin selection, indicating the lack of viral replication of the transfected viral RNA. In addition, this was further confirmed by our results (Figure 3), demonstrated that no increment in the luciferase activity translated from the transfected luc-encoding replication-competent BVDV replicon in the acutely BVDV-infected cells, compared to a large increase in the luciferase activity upon transfection into naive MDBK cells. In this experiment, we also showed that the artificially introduced replication-competent BVD viral RNA was fully competent for viral translation in spite of a failure in viral replication (Figure 3). Interestingly, a similar mechanism of superinfection exclusion has been observed in two other viruses. In Sindbis virus with the use of temperature-sensitive mutants, two mutants in RNA-negative complementation groups were failed to exclude superinfecting viruses at the nonpermissive temperature, indicating that the replication of superinfecting virus has been blocked after attachment, penetration, and the translation of the superinfecting RNA. Similarly, the significantly reduced viral RNA synthesis of superinfecting virus has been suggested to account for the superinfection exclusion of arboviruses between Venezuelan equine encephalitis and eastern equine encephalitis viruses.

In addition to a block at the level of viral replication, our results using superinfecting luc-encoding viral particles also showed that superinfecting BVD viral particles have a defect in the early stage of viral life cycle prior to viral protein translation from input particles (Figure 4 and 5). First of all, a high level of the luciferase activity from naive MADK cells infected with luc-encoding viral particles was detected before the productive viral replication occurs. On the other hand, in the ncp BVDV-infected cells, the activity detected was barely above the background of our system (Figure 4). Based on these results, it is suggested that superinfecting BVD viral particles have a defect in viral entry step. This is further supported by two other experiments. First of all, the luciferase activity translated from the superinfecting viral particles was dependent upon expression of the structural proteins of BVDV (Figure 5). Additionally, kinetic analyses also showed that the superinfection exclusion of BVDV could be established within 30 min after primary infection (data not shown), which is consistent with a block at the viral entry step in the course of viral life cycle.

In the evolutionary standpoint, we can ask the question of why viruses develop these mechanisms to achieve superinfection exclusion. Another type of exclusion in T-even phage infection is observed. In the case that E. coli are simultaneously

coinfected with phages T2 and T4, phage T4 is dominant and a number of genetic markers for phage T2 are not easily detected in the progeny. Without superinfection exclusion, phage T2 would be disappeared. As a result, genetic diversity among these viruses would decrease, and a few dominant strains of these viruses would be left. Considering that resistant bacteria are arised by mutating a given receptor, this could be disadvantageous for these T-even phages. Similarly, in animal viruses, if the dominantly selected strains of a virus do not survive against host immune surveillance, it is likely that this viral species would be eliminated from the host. Therefore, it is reasonable to speculate that superinfection exclusion is one of the powerful strategies to maintain genetic diversity among these viruses, and allow to adjust in a highly dynamic environment of host immune surveillance. In a population of cells undergoing viral infection, superinfection exclusion would give advantage to remaining viruses or newly produced viruses, which favor viral entry into uninfected rather than previously infected cells. Additionally, a primary virus successfully infected a cell would have protection from a viral entry of a competing virus. Our system described here will provide a useful strategy to understand molecular mechanisms underlying superinfection exclusion in other viral systems.