FULL PAPER *Virology*

Akabane virus nonstructural protein NSm regulates viral growth and pathogenicity in a mouse model

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ABSTRACT. The biological function of a nonstructural protein, NSm, of Akabane virus (AKAV) is unknown. In this study, we generated a series of NSm deletion mutant viruses by reverse genetics and compared their phenotypes. The mutant in which the NSm coding region was almost completely deleted could not be rescued, suggesting that NSm plays a role in virus replication. We next generated mutant viruses possessing various partial deletions in NSm and identified several regions critical for virus infectivity. All rescued mutant viruses produced smaller plaques and grew inefficiently in cell culture, compared to the wild-type virus. Interestingly, although the pathogenicity of NSm deletion mutant viruses varied in mice depending on their deletion regions and sizes, more than half the mice died following infection with any mutant virus and the dead mice exhibited encephalitis as in wild-type virus-inoculated mice, indicating their neuroinvasiveness. Abundant viral antigens were detected in the brain tissues of dead mice, whereas appreciable antigen was not observed in those of surviving mice, suggesting a correlation between virus growth rate in the brain and neuropathogenicity in mice. We conclude that NSm affects AKAV replication *in vitro* as well as *in vivo* and that it may function as a virulence factor.

KEY WORDS: Akabane virus, nonstructural protein, pathogenicity, reverse genetics

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Akabane virus (AKAV), an orthobunyavirus, was first isolated in central Japan in 1959 [10]. It is transmitted by hematophagous arthropod vectors, such as Culicoide biting midges, and epizootic and sporadic Akabane disease has been reported in Australia, Southeast Asia, East Asia, Middle East and Africa. AKAV infects cattle, sheep and goats, and the infected animals show abnormal deliveries, such as abortion, stillbirth, premature birth and congenital deformities known as arthrogryposis-hydranencephaly syndrome. Outbreaks of Akabane disease occurred during 1972–74 in Japan and caused severe economic damage to the livestock industry; the virus was isolated from a naturally infected bovine fetus and named as OBE-1 strain [6]. Despite widespread use of the live or inactivated vaccine, sporadic emergences continue to occur. There are considerable variations in antigenic and pathogenic properties among field isolates of AKAV [1, 2, 8]. In 1989, a variant of AKAV that not only causes abnormal deliveries, but also induces encephalitis in calves, was isolated from the southern part of Japan, and this strain was named as the Iriki strain [8]. Young mice inoculated with the Iriki strain via the intraperitoneal route show encephalitis unlike other strains resulting in animal death [9], indicating that the Iriki strain is neuropathogenic. However, the molecular basis of its neuropathogenicity is unknown.

Orthobunyaviruses have four structural proteins and two nonstructural proteins. The largest RNA segment (L RNA) encodes a viral RNA-dependent RNA polymerase (L), the medium segment (M RNA) encodes two virion surface glycoproteins (Gc and Gn) and a nonstructural protein (NSm), and the smallest RNA segment (S RNA) encodes a viral nucleoprotein (N) and a nonstructural protein (NSs). The ribonucleoprotein consists of three viral RNAs and two structural proteins (N and L). Gn may function in virus attachment to insect cells, and Gc is responsible for neutralization and virus attachment to mammalian cells [7]. NSs of the Rift Valley fever virus (RVFV) (phlebovirus) and Bunyamwera virus (BUNV) (orthobunyavirus) counteracts the induction

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of α/β interferon [3, 15] and is involved in regulation of host protein synthesis [15]. NSm of RVFV suppresses virus-induced apoptosis [16], and NSm of BUNV is a key factor for the tubular structure, which harbors the viral replication complexes and is involved in virus assembly in the Golgi [4]. These findings suggest that bunyavirus nonstructural proteins may function as virulence factors.

In this study, in order to gain insight into the biological function of AKAV NSm, we generated a series of mutant viruses with various deletions in NSm and investigated their biological phenotypes *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cells and virus: Hamster lung (HmLu-1) cells, African green monkey kidney (Vero) cells and baby hamster kidney cells expressing T7 RNA polymerase (BHK/T7-9) [5] were used. HmLu-1 and Vero cells were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS). BHK/T7-9 cells were kindly provided by Dr. N. Ito (Gifu University, Gifu, Japan) and were maintained in MEM supplemented with 5% FBS. The plaque-cloned Iriki strain of AKAV [8] was propagated in HmLu-1 cells under serum-free conditions.

Plasmids: The plasmids pT7riboSM2/IL, -/IM and -/IS carrying the L, M and S cDNAs of the Iriki strain, respectively, were constructed in our previous study [13]. pT7riboSM2/IM was modified to delete several parts of the NSm gene. First, *Kpn*I restriction sites were introduced in the NSm coding region of pT7riboSM2/IM using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.), resulting in the generation of three plasmids, pT7riboSM2/IM-PM367/368/446, -PM322/460 and -PM322/446, each of which has two *Kpn*I sites in the NSm coding region. Using these plasmids, we constructed deletion plasmids pT7riboSM2/IM-Δ369-447, -Δ323-460, -Δ323-446, -Δ323-367/447–460, -Δ323-367 and -Δ447-460 (Fig. 1B). In addition, by using PCR amplification with primers having a *Kpn*I site followed by enzyme digestion and ligation, five deletion plasmids, pT7riboSM2/IM-Δ369-393, -Δ369-412, -Δ369-420, -Δ369-430 and -Δ369-440, were constructed (Fig. 1B). All the generated constructs were authenticated by DNA sequencing.

Generation of recombinant viruses: We generated recombinant viruses containing NSm deletion mutants by T7 polymerase-based reverse genetics as described in our previous study [14]. Briefly, a sub-confluent monolayer of BHK/T7-9 cells in a 12-well plate was transfected with 0.6 μg of pT7riboSM2/IL, 0.3 μg of either of the original pT7riboSM2/IM or its modified plasmids described above, and 0.6 μg of pT7riboSM2/IS, using TransIT-LT1 (Mirus, Madison, WI, U.S.A.) at 3 μl per μg of DNA in 50 μl of serum-free medium (OPTI-MEM; Invitrogen, Carlsbad, CA, U.S.A.). After 5 days incubation, the culture supernatant was inoculated into fresh HmLu-1 cells to facilitate virus replication. When the cytopathic effect became apparent, supernatants were harvested and plaque-purified. The resulting viruses were grown in HmLu-1 cells. The titers of recombinant viruses were determined using a plaque assay as described previously [13].

Growth kinetics of recombinant viruses: Sub-confluent monolayers of HmLu-1 or Vero cells were infected with the recombinant wild-type Iriki strain (rIriki-wt) or recombinant viruses at a multiplicity of infection (MOI) of 0.01 for 1 hr at 37°C. The infected cells were washed twice with PBS and overlaid with DMEM supplemented with 2% FBS. The supernatants were collected every 12 hr post-infection (hpi) and titrated. Viral titers were determined by a plaque assay.

Experimental infection: The experimental protocol was approved by the Animal Care Committee at the University of Tokyo, and the experiments were conducted according to the guidelines. Groups of six to eleven 3-day-old BALB/c mice were inoculated intraperitoneally with 0.1 m*l* of rIriki-wt, the recombinant viruses (5×10^4 PFU) or the vehicle DMEM. Lethality in animals was observed for 21 days following inoculation. Mice that died immediately after inoculation (on day 0) were not included in the experiments.

Enzyme immunoassay: Brains tissues were collected from infected animals at 5 days post-infection (dpi) or dead animals, fixed in 10% buffered formalin, embedded in paraffin and sectioned into 6 μm sections. The sections were stained with hematoxylin and eosin and used for immunohistochemical detection of AKAV antigens [10]. In brief, the primary antibody was a rabbit antiserum against the OBE-1 strain of AKAV (1:100) (kindly provided by Dr. T. Tsuda, National Institute of Animal Health, Tsukuba, Japan). Goat anti-rabbit IgG antibody labeled with horseradish peroxidase conjugated polymers (Dako Envision system, Agilent Technologies, Tokyo, Japan) was used as the secondary antibody. Finally, the sections were developed with 3.3′-diaminobenzidine and counterstained with Mayer's hematoxylin. The sections were qualitatively evaluated by microscopy.

Virus neutralization test: The serum samples from surviving mice after the observation period were subjected to the virus neutralization test as described earlier [9].

RESULTS

Generation of NSm deletion mutant viruses: AKAV NSm is encoded on the M segment and is flanked by Gn and Gc (Fig. 1A). It comprises of residues at positions 310 to 465 of the M segment precursor protein with 1,401 amino acid residues and is processed by proteolytic cleavage of the precursor protein. Three hydrophobic (amino acids at positions 317 to 339: domain I, 371 to 393: domain III and 448 to 465: domain V) and two hydrophilic (amino acids 340 to 370: domain II and 394 to 447: domain IV) regions are predicted in NSm by the computer program SOSUI WWW server (http://bp.nuap.nagoya-u.ac.jp/sosui/), which demonstrates similar molecular features as the BUNV NSm, which includes five domain structures [12].

We first tested whether the point mutations (NSmA322G, M367G, I368I, Y446G and V460G) that were introduced to construct the further deletion plasmids, could affect virus infectivity. Three mutant viruses with altered NSm (rAKAV-

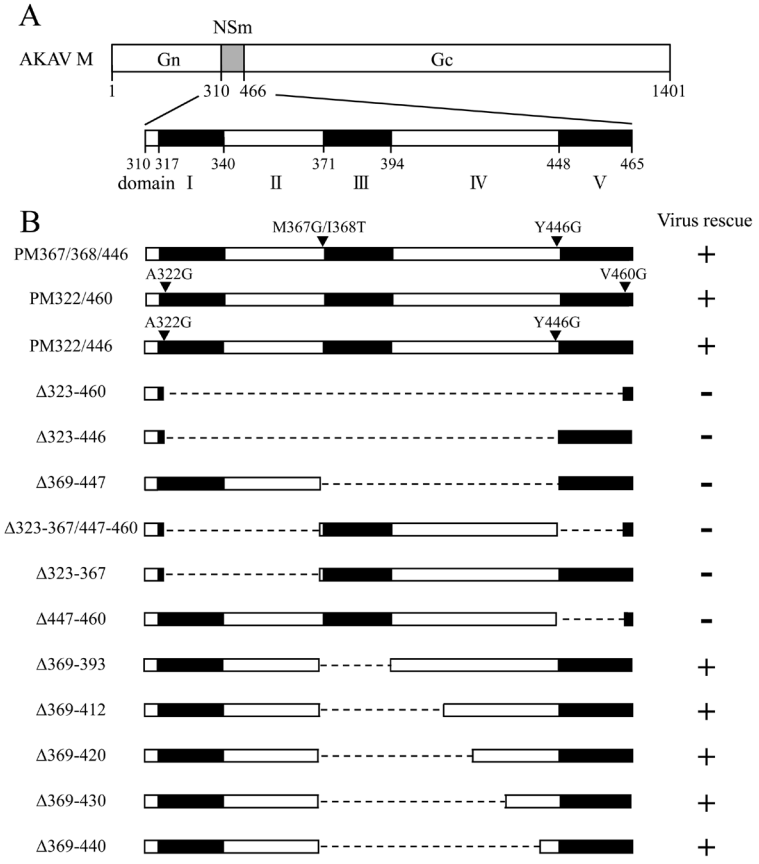

Fig. 1. Schematic diagrams of NSm mutants. (A) The AKAV M segment and the enlarged NSm gene are illustrated. Hydrophobic domains (I, III and V) are indicated by the black box, whereas hydrophilic domains (II and IV) are shown by the white box. (B) Construction of NSm mutants with point mutations or deletions. Each of the three point mutants (PM) contains 2 or 3 changes in amino acids shown by arrowheads. In each of the deletion mutants, the deleted amino acid positions are shown on the left, and the deletion region(s) are shown by the dashed line. The results of virus rescue experiments are shown on the right.

NSm M367G/I368I/Y446G, -A322G/V460G and -A322G/Y446G) could be generated by reverse genetics, indicating that these point mutations in NSm are acceptable for virus infectivity.

To investigate which domains of NSm are important for virus infectivity, we tried to generate mutant viruses with near complete NSm deletion (rAKAV-NSmΔ323-460) and with several NSm deletions lacking the domains I/II/III/IV (rNSmΔ323-446), III/IV (-Δ369-447), I/II/V (-Δ323-367/447–460), I/II (-Δ323-367), V (-Δ447-460) and III (-Δ369-393), respectively (Fig. 1B). Among these, only the mutant virus lacking NSm domain III (rAKAV-NSmΔ369-393) was rescued, indicating that this domain was dispensable for virus infectivity. Additionally, we tried to generate NSm deletion mutant viruses lacking domain III and a part of domain IV. Four such mutant viruses (rAKAV-rNSmΔ369-412, -Δ369-420, -Δ369-430 and -Δ369-440) could be successfully rescued, indicating that almost the entire region of domain IV was dispensable for virus infectivity.

Growth properties of NSm deletion mutants in cell culture: We tested the effects of NSm deletion on plaque size and growth kinetics using the rIriki-wt and the NSm deletion mutant viruses (rAKAV-NSmΔ369-393, -Δ369-412, -Δ369-420, -Δ369-430 and -Δ369-440). The plaque sizes of NSm deletion mutant viruses in HmLu-1 cells were significantly smaller than those of the rIriki-wt (Fig. 2A). To compare growth kinetics, HmLu-1 or Vero cells were infected with these viruses and then monitored. In Vero cells, viral titers decreased at every time-point with increasing deletion of domain IV of NSm (Fig. 2B), indicating a clear connection between NSm deletion size and virus replication. However, in HmLu-1 cells, this connection was vague, especially at later time-points; all mutant viruses but one (rAKAV-NSmΔ369-440) reached maximal yields almost equivalent to rIriki-wt at 48 hpi. This discrepancy in growth kinetics may be related with interferon-dependent anti-viral activity; Vero cells lack interferon signaling unlike HmLu-1 cells.

Experimental infections of NSm deletion mutant viruses in mice: To evaluate viral pathogenicity, suckling mice were

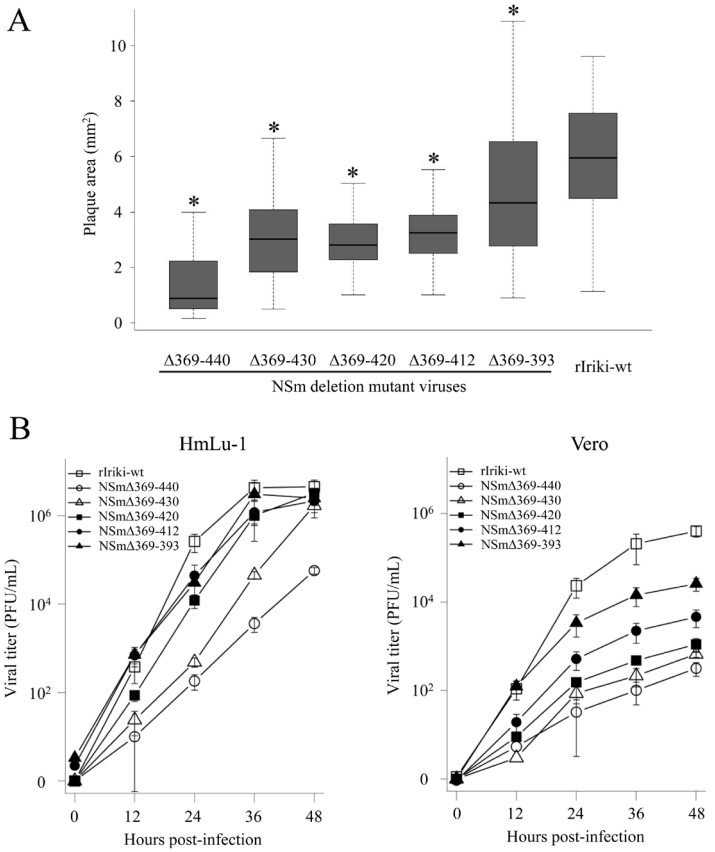

Fig. 2. Growth properties of NSm deletion mutant viruses. (A) Plaque sizes of the mutant viruses on HmLu-1 cells were measured by the computer program ImageJ [11] at 4 dpi and are shown by a box plot. Significant differences (*) between each of the mutant viruses and the rIriki-wt were observed (P<0.05; Student t-test). (B) Growth kinetics of mutant viruses on HmLu-1 and Vero cells. After cells were infected with the viruses at an MOI of 0.01, the supernatants were harvested at different time-points, and virus titers were determined by plaque assay. The averages of three independent experiments are shown.

inoculated intraperitoneally with the rIriki-wt and the NSm mutant viruses under similar conditions. Survival curves (Fig.3) indicated that the deletion mutant virus (rAKAV-NSmΔ369-393) has pathogenic phenotype as do the rIriki-wt and the point mutant virus (rAKAV-NSm M367G/I368I/Y446G), indicating that deletion of domain III alone in NSm did not affect virus pathogenicity in this animal model. In contrast, 4 mutant viruses having more deletion sizes were less pathogenic than the wild-type (P<0.01 for rAKAV-NSmΔ369-430 and P<0.05 for -Δ369-412, -Δ369-420 or -Δ369-440; Log-Rank Trend test). Among these 4 mutant viruses, there is no significant difference in pathogenicity, except for one case (rAKAV-NSmΔ369-430 vs. -Δ369-440; P<0.05). Therefore, the mutant viruses possessing the NSm deletion with a part of domain IV in addition to domain III were less pathogenic compared with the wild-type virus and the domain III-deletion mutant virus. More than half the mice inoculated with the NSm deletion mutant viruses died over an observation period of 21 days (Fig. 3). However, all the surviving mice showed neurological symptoms and pathological changes, in addition to possessing specific antibodies

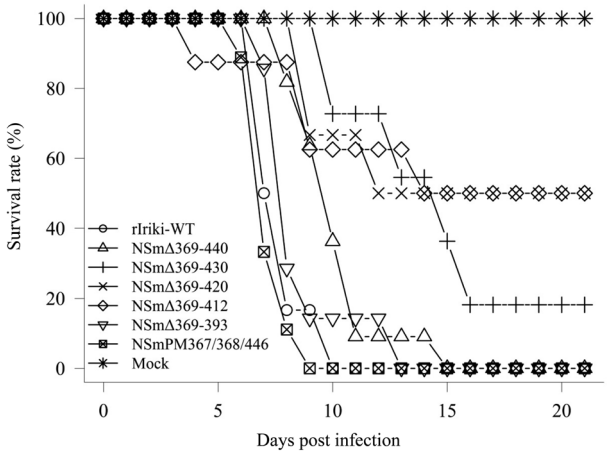

Fig. 3. Survival curve of mice inoculated with NSm mutant viruses. Groups of 6 to 11 3-day-old mice were inoculated intraperitoneally with 0.1 ml of virus suspension (5×10^4 PFU) or DMEM (mock), and mortality was observed.

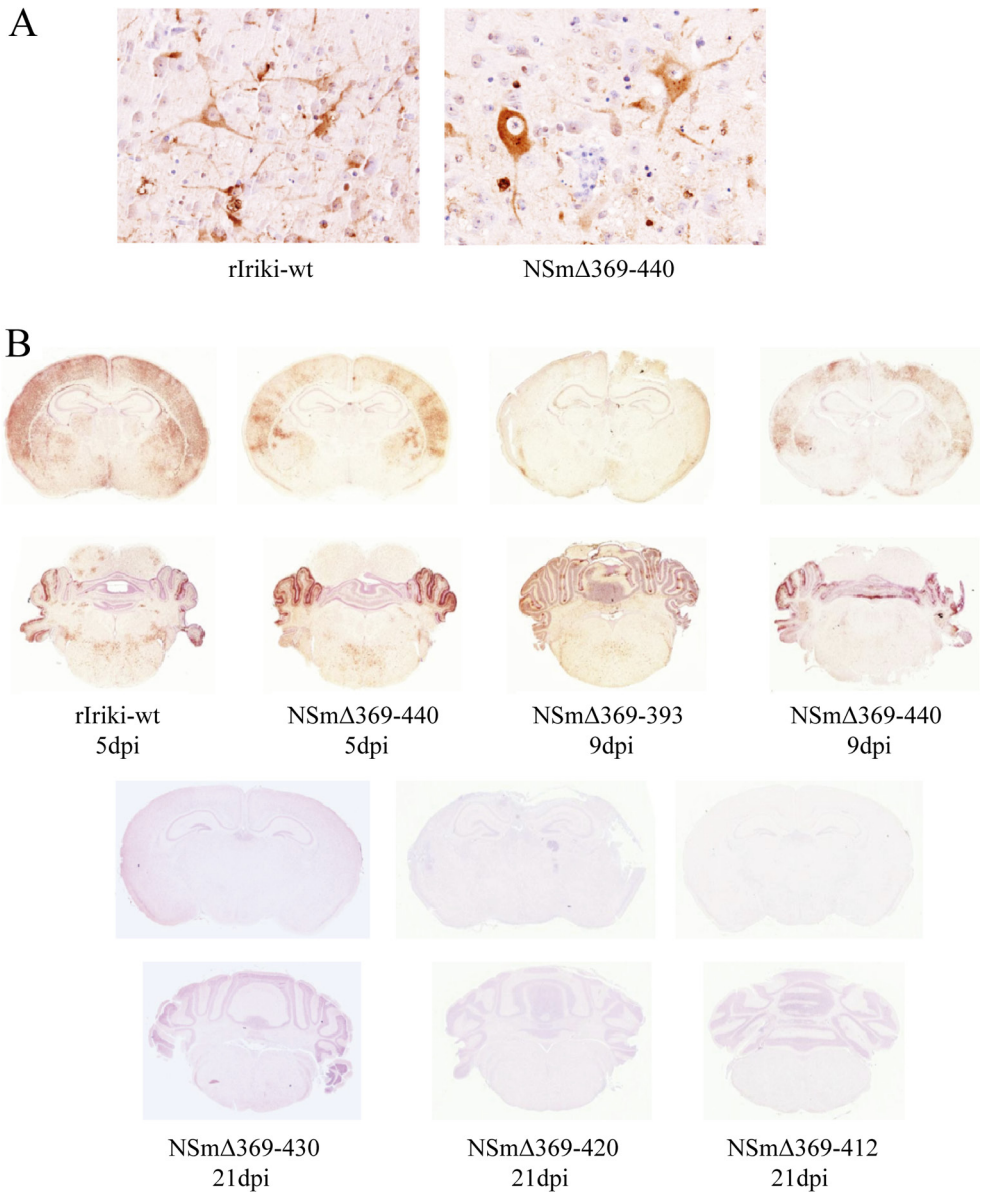

Fig. 4. Pathology of the mouse brain inoculated with NSm mutant viruses. (A) Brain tissues were fixed with 10% buffered formalin and embedded in paraffin. Brain sections were stained with hematoxylin and eosin. Viral antigens were detected by immunohistochemical staining with a specific antibody. Typical staining patterns in the cerebrums of infected mice at 5 dpi are shown. (B) Pathological examination of the infected mouse brain. The upper pictures show the cerebrums, and the lower pictures show the cerebellums and brainstems. The virus names and sampling points are shown at the bottom.

against the viruses with more than 1:80 of virus neutralizing titers (data not shown). At 5 dpi, the viral antigens were observed in neurons (Fig. 4A) and were widely distributed in the cerebrum, the cerebellum and the brainstem, demonstrating the neuroinvasiveness of the mutant viruses (Fig. 4B). At 9 dpi, the viral antigen was distributed in the cerebrum and cerebellum, but was rarely seen in the brainstem. The viral antigen was not seen in the brain at 21 dpi, but signs of infection, such as degeneration, necrosis, glial nodules and vascular response, were observed.

DISCUSSION

The biological function of the nonstructural protein NSm of AKAV is unknown. To address those in the present study, we generated a series of NSm deletion mutant viruses by reverse genetics and compared their phenotypes. Although the mutant virus lacking almost the entire sequence of NSm could not be rescued, several mutant viruses possessing various partial deletions in NSm could be rescued. *In vitro* and *in vivo* analyses with these mutant viruses demonstrated that

NSm plays a role in AKAV replication and pathogenicity.

Computational analysis predicted that AKAV NSm contains five structural domains: three hydrophobic (domains I, III and V) and two hydrophilic (domains II and IV) domains similar to the NSm of another orthobunyavirus BUNV [12]. Previous reports have shown that the N-terminal region of BUNV NSm that contains domain I and part of domain II is required for virus assembly and that the C-terminal domain V may function as an internal signal sequence for Gc [12]. In addition, NSm domains I and V of BUNV might function as anchors to fix Gn and Gc at the Golgi membrane, as supported by an observation in which NSm localizes to the Golgi complex along with Gn and Gc [12]. Our result that all the mutant AKAVs lacking either NSm domains I, II or V (i.e., rNSmΔ323-460, Δ323-446, -Δ369-447, -Δ323-367/447–460, -Δ323-367 and -Δ447-460) were not rescued supports these findings. Therefore, these NSm regions might be essential for orthobunyavirus infectivity.

On the other hand, mutant BUNVs with deletions in the NSm domains III and IV could be generated, even though they replicated less efficiently in cell culture [12]. In the present study, mutant AKAVs lacking either domain III (rAKAV-NSmΔ369-393) or domain III plus the most region of domain IV (rAKAV-NSmΔ369-440) were successfully rescued, but their replication in cell culture was limited compared with that of rIriki-wt. This result was consistent with the previously reported findings [12]. These data suggest that domains I, II and V should form the essential structure of NSm required to exhibit its biological functions. The hydrophobic domains I and V may play an important role in membrane association of functional NSm, but hydrophobic domain III may not play any such role. Interestingly, the virus with complete deletion of both domains III and IV (rAKAV-NSmΔ369-447) could not be rescued in our study, indicating that a short peptide consisting of the amino acids 441 to 447 of NSm domain IV, was required for viral infectivity, which appears to be an additional factor required for NSm function. In addition, different pathogenicity observed between rAKAV-NSmΔ369-430 and -Δ369-440 may provide an undefined function with another short peptide consisting of the amino acids 431 to 440 of NSm.

In experimental infection, the mice inoculated with one NSm deletion mutant virus lacking only the domain III (rAKAV-NSmΔ369-393) exhibited similar pathogenicity to rIriki-wt, indicating that the reduced growth observed in cell cultures did not affect its pathogenic phenotype in mice. In contrast, mice inoculated with other NSm deletion mutant viruses exhibited lower pathogenicity than rIriki-wt. However, neurological signs were observed even in mice surviving infection with these mutant viruses, suggesting that they retain both their neuroinvasiveness and neurovirulence. Pathological examination of the surviving mice revealed that injured brain tissues were repaired at 21 dpi possibly by host defense, indicating that mouse lethality could be determined by surpassable immune response over viral replication in the brain. This notion is supported by our observed correlation between virus growth rate and neuropathogenicity in mice infected with mutant viruses.

In conclusion, our study revealed that the AKAV NSm is responsible for virus infectivity *in vitro* as well as *in vivo* and that AKAV pathogenicity could be reduced by partial deletion of NSm, demonstrating that NSm is a determinant of virus pathogenicity. These findings may contribute not only to understanding the molecular features of AKAV replication, but also to developing an alternative live vaccine strategy using an NSm deletion mutant virus in the future.

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