FULL PAPER *Virology*

The effect of glycosylation on cytotoxicity of Ibaraki virus nonstructural protein NS3

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ABSTRACT. The cytotoxicity of Ibaraki virus nonstructural protein NS3 was confirmed, and the contribution of glycosylation to this activity was examined by using glycosylation mutants of NS3 generated by site-directed mutagenesis. The expression of NS3 resulted in leakage of lactate dehydrogenase to the culture supernatant, suggesting the cytotoxicity of this protein. The lack of glycosylation impaired the transport of NS3 to the plasma membrane and resulted in reduced cytotoxicity. Combined with the previous observation that NS3 glycosylation was specifically observed in mammalian cells (Urata *et al.,* Virus Research 2014), it was suggested that the alteration of NS3 cytotoxicity through modulating glycosylation is one of the strategies to achieve host specific pathogenisity of Ibaraki virus between mammals and vector arthropods.

KEY WORDS: cytotoxicity, intracellular distribution, NS3 glycosylation, Orbivirus

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Orbivirus is one of 15 genera in the Reovirus family and is characterized as an arthropod borne virus (arbovirus) whose infection is mediated by arthropod vectors [23]. Bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV) and African horse sickness virus (AHSV) are well known orbiviruses, because of the severe diseases they cause in livestock [7]. The infection of orbiviruses is mediated by biting midges with *Culicoides* spp. characterized as the most prevalent vector [16, 20].

Similar to many other arboviruses, orbiviruses do not kill or cause serious disease in vector arthropods, although they establish pathogenic infections in mammalian hosts. It is believed that arboviruses have developed mechanisms to achieve host specific pathogenicity in order to protect vector arthropods from their own virulence, thereby maintaining the virus's ability to spread [26]. In fact, it was reported that BTV showed mild or no cytopathic effect (CPE) when it was infected to insect cells, whereas severe CPE was observed in cells of mammalian origin [8].

In a previous report, we compared the growth efficiency and virulence of Ibaraki virus (IBAV) in cell lines from mammals and insects [22]. IBAV is one of the strains of EHDV serotype 2 and causes Ibaraki disease characterized by the paralysis of swallowing muscles resulting in debilitation in cows [20, 21]. In 2013, the reemergence of Ibaraki disease, in combination with the co-circulation with other EHDV, was detected in Japan. It is considered as a potential threat for the emergence of mutated IBAV due to the genomic reassortment [12]. The growth pattern of IBAV in two different cell cultures clearly reflected its pathogenicity in living hosts. It grew rapidly and showed strong CPE in mammalian cells, whereas the growth was slower and displayed no obvious CPE in insect cells. Glycosylation of IBAV's nonstructural protein NS3 occurred only in mammalian cells. This glycosylation pattern correlated with the release efficiency of progeny virus where greater release efficiency was observed when NS3 was not glycosylated. In BTV, NS3 was reported to play a role for virus release via interaction with host endosomal sorting complexes required for transport (ESCRT) machinery [3–5, 27]. When the amino acid sequence of NS3 was compared between IBAV and BTV, we found that many domain structures including the viral late domain required for ESCRT interaction were conserved, although the homology was relatively low (Fig. 1). Therefore, it is possible that the difference in NS3 glycosylation controls the function of NS3. To better understand the function of NS3 and the contribution of glycosylation, we focused on the cytotoxicity of NS3 and evaluated the effect of glycosylation by using NS3 glycosylation mutants produced in this study.

MATERIALS AND METHODS

Cells and molecular cloning of IBAV NS3: 293T (human embryonic kidney) cells were maintained in Dulbecco's modified Eagle medium (DMEM, Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/m*l*), streptomycin (100 μg/m*l*) and L-glutamine (0.29 mg/m*l*).

Molecular cloning of the open reading flame for IBAV NS3 was performed as follows. The open reading frame of NS3 was amplified by polymerase chain reaction (PCR) with primers NS3Fw and NS3Rv using the IBAV S10 genome cloned into the pGEM vector as a template. PCR products were cloned into the pGEM®-T easy vector (Promega, Madison, WI, U.S.A.), and the nucleotide sequence of cloned

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IBAV        MLSRLVSGTET---RINMKQSDEMSLVPYQENVR-PPSYVPTAPTPTSMPRVALDILDKA 56
BTV10       MLSGLIQRFEEEKMKHNQERVEELSLVRVDDTIYQPPRYAPSAPMPSSMPTVALEILDKA 60
            *** *:.  *    : * :: :*:***  ::.:  ** *.*:** *:*** ***:*****

IBAV        MSNQTGATMAQKVEKVAYASYAEAFRDDLRLRQIKKRVNEQVLPKMRVELTMMKRKRAMA 116
BTV10       MSNTTGATQTQKAEKAAFASYAEAFRDDVRLRQIKRHVNEQILPKLKSDLGGLKKKRAII 120
            *** **** :**.**.*:**********:******::****:***:: :*  :*:***:

IBAV        HMILIIAAVVALITSASTLTSDLGIILKNTTATEIIQKQIKPFCAAFGIINLAATMIMMF 176
BTV10       HMTLLIAAVVALLTSVCTLSSDMSVAFKLNGTSAEIPQWFKSLNPMLGVVNLGATFLMMV 180
            ** *:*******:**..**:**:.: :* . ::  * : :*.: . :*::**.**::**.

IBAV        MAKNEKVISQRIDHTRKEIMKKDAYNEAVRMSITELSEVPLDGFDIPPELIR 228
BTV10       CAKSERSLNQQIDMIKKEVMKKQSYNDAVRMSFTEFSSVPLDGFELPLT--- 229
             **.*: :.*:**  :**:***::**:*****:**:*.******::*
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Fig. 1. The alignment of NS3 amino acid sequence between IBAV and BTV10. The deduced amino acid sequence of IBAV NS3 was aligned with BTV NS3 by the ClastalW2 program. Putative N-linked glycosylation sites are underlined, predicted transmembrane domains are indicated by bold, and viral late domains are surrounded by square.

DNA was confirmed by the BigDye® v3.1 Terminator Cycle Sequence Kit and ABI 3100 gene analyzer (Life Technologies, Carlsbad, CA, U.S.A.). The nucleotide sequences of all oligonucleotide primers used in this study are summarized in Table 1. These oligonucleotide primers were designed based on the published sequence (GenBank Accession No. AB262177). The amino acid sequence of IBAV NS3 was deduced from the obtained nucleotide sequence and aligned with NS3 of BTV-10 ([15] GenBank Accession No. M28981.1) using the ClastalW program. The resulting plasmid, pGEM/NS3, was used for further mutagenesis and subcloning to the mammalian expression plasmid pCAG [17].

Production of NS3 glycosylation mutants by site-directed mutagenesis: Glycosylation mutants of NS3 were produced by site-directed mutagenesis using the KOD-Plus-Mutagenesis Kit (TOYOBO, Osaka, Japan). The schematic of glycosylation mutants generated in this study is shown in Fig. 2a. Briefly, primers, NS3N59Q-1/2 and NS3N145Q-1/2, were designed to include nucleotides which achieve amino acid substitution of asparagine to glutamine at 59 and 145. PCR was performed using pGEM/NS3 as a template. After digestion of the template plasmid by DpnI, PCR products were self-ligated. The clone with correct mutation was selected, and its nucleotide sequence was confirmed as described above.

All plasmids were digested by EcoRI and KpnI, and cDNA of NS3 was cloned into the EcoRI/KpnI site of the mammalian expression vector pCAG. The final plasmids, pCAG/NS3, pCAG/NS3N59Q, pCAG/N145Q and pCAG/N59,145Q, were transfected to 293T cells with polyethylenimine max (MW=40,000, PolyScience, Warrington, PA, U.S.A.). The expression of each NS3 was confirmed by western blot analysis using mouse anti-NS3 antisera.

Western blot analyis and density analysis: The expression of NS3 in plasmid transfected cells was analyzed by western blot analysis using mouse anti-NS3 sera raised against recombinant IBAV-NS3 expressed in *E. coli*. Collected cells were mixed with 2 × SDS sample buffer (100 mM Tris-Cl

Table 1. Oligonucleotide primers used for wild type and mutated NS3 cloning

Primer name	Sequence (5′–3′)
NS3Fw	ggggtaccatgctatccagattagtatc
NS3Rv	tagtctatctcaccacggcttaagg
NS3N59Q-1	cagcaaacgggtgcaactatggcg
NS3N59Q-2	tgacattgccttgtcgagtatatc
NS3N145Q-1	cagactaccgcaacggaaataatac
NS3N145Q-2	ctttagaatgattcctaaatcgctc

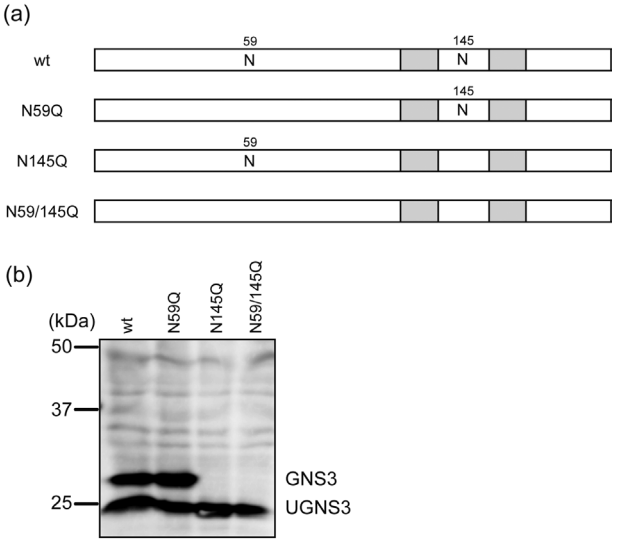

Fig. 2. Production of NS3 glycosylation mutants. (a) Schematic illustration of NS3 glycosylation mutants generated in this study. Gray area represents the transmembrane domain. (b) Western blot analysis of 293T cells expressing wt-NS3, NS3/N59Q, NS3/N145Q and NS3/N59,145Q. NS3 was detected by western blot analysis using mouse anti-NS3 sera with both glycosylated (GNS3) and unglycosylated (UGNS3) proteins indicated.

(pH 6.8), 4% SDS, 200 mM dithiothreitol (DTT), 20% glycerol and 0.4% bromophenol blue) and subjected to sonication to disrupt cellular genomic DNA. After separating with 15% sodium dodecyl sulfate-polyacrylamide gel electrophoreses (SDS-PAGE), proteins were transferred to a PVDF membrane (Immobilon-P, Merck Millipore, Billerica, MA, U.S.A.) and incubated with mouse anti-NS3 sera followed by anti-mouse IgG (H+L) conjugated with HRP (Jackson ImmunoResearch, West Grove, PA, U.S.A.). After incubation with substrate solution (ImmunostarLD, Wako), the chemiluminescent signal was detected by LAS-3000 mini (Fujifilm, Tokyo, Japan). After visualization, the intensity of protein band was quantified using ImageJ software [19].

Quantification of NS3 cytotoxicity: Cytotoxicity of NS3 was evaluated by the amount of lactate dehydrogenase (LDH) released from the cytoplasm as measured with the Pierce™ LDH Cytotoxicity Assay Kit (Thermo Scientific, Waltham, MA, U.S.A.). In brief, 293T cells were prepared in a 12-well plate, transfected with each plasmid and incubated at 37°C for 36 hr. Fifty μl of culture supernatant was collected at several time points post transfection and transferred to a 96-well flat-bottom plate. Reaction mix containing substrate was added to each well and incubated for 30 min at room temperature. After the reaction was stopped, the absorbance was read at 490 nm and 655 nm with an iMark™ Microplate reader (Bio-Rad, Hercules, CA, U.S.A.). Transfected cells were also harvested and analyzed by western blot analysis as described earlier. To quantify cell number for standardization, β-actin was detected by anti-β-actin monoclonal antibody (A5441, Sigma, St. Louis, MO, U.S.A.).

Detection of NS3 by indirect immunofluorescence assay (IFA): 293T cells were seeded on collagen coated glass coverslips and transfected with either pCA7/NS3 or pCA7/NS3N59,145Q. Cells were fixed at 36 hr post transfection in 4% paraformaldehyde at room temperature. After permeabilization with 0.05% IgePAL® CA-630 (Sigma), cells were incubated with mouse anti-NS3 sera, followed by DyLight™ 488 goat anti-mouse IgG (Jackson ImmunoResearch). After washing with PBS (−), cells were reacted with 2.5 μg/m*l* of WGA conjugated with Alexa Fluor® 594 (Molecular Probes, Eugene, OR, U.S.A.). Coverslips with stained cells were sealed with ProLong® Gold antifade reagent with DAPI (Molecular Probes). The fluorescent signal was observed using confocal laser scanning microscopy (LSM710, Carl Zeiss, Oberkochen, Germany).

Statistical analysis: Statistical analysis was performed using R [18]. To test the significance in the difference between cytotoxicities provided by pCA7/NS3 and pCA7/MCS, student's *t*-test was used. Tukey's test was used to perform multiple comparison in the cytotoxicity provided by NS3 and its mutants.

RESULTS

Several characteristic domains and motifs in NS3 are shared between IBAV and BTV10: PCR with NS3Fw and NS3Rv amplified a 644 bp product containing the open reading frame of IBAV NS3. The amino acid sequence of IBAV NS3 was deduced from the nucleotide sequence. This sequence was consistent with the published sequence (GenBank Accession No. AB262177) showing 1 amino acid substitution (N29H). More than 83.8% (mean=89.7%) sequence identities were shared among all EHDV serotypes [1]. Two transmembrane domains, two putative N-linked glycosylation sites and two viral late domains were identified (Fig. 1). When the IBAV NS3 amino acid sequence was compared to that of BTV10, it was revealed that all of the above domains and motifs were conserved even though the sequence homology was relatively low (51.75%).

The first glycosylation site (N59) was not used for NS3 glycosylation: To confirm the glycan attachment to two putative N-linked glycosylation consensus sequences in NS3, we introduced amino acid substitutions to asparagine residues at 59 and/or 145. The resulting glycosylation mutants were expressed in 293T cells, and the expression was confirmed by western blot analysis (Fig. 2b). In wild type (wt) NS3 and NS3/N59Q, two clear protein bands with molecular weights of 24 and 28 kDa were detected by anti-NS3 antisera. Conversely, only a single protein band with 24 kDa was observed in the cells expressing NS3/N145Q and NS3/N59,145Q. NS3 with 28 kDa was eliminated by PNGaseF treatment with only the 24 kDa protein band remaining (Data not shown). These results suggest that the 28 kDa NS3 was glycosylated NS3 (GNS3) and the 24 kDa NS3 was unglycosylated NS3 (UGNS3). Since the NS3/N59Q mutant still produced 28 kDa GNS3, it was suggested that no glycan was attached to N59. On the other hand, 28 kDa GNS3 was not observed in NS3/N145Q mutant, suggesting that N145 was used for glycan attachment.

IBAV NS3 showed membrane permeabilization ability in mammalian cells: From earlier studies, it was suggested that NS3 from other orbiviruses possesses the ability to permeabilize the plasma membrane of the host cell [9, 13, 25]. Thus, it might be possible that IBAV NS3 also permeabilizes the host plasma membrane. To confirm this hypothesis, various quantities of pCA7/NS3 were transfected to 293T cells, and LDH activity in the culture supernatant was evaluated. As shown in Fig. 3a, a reduction of LDH activity was observed in the supernatant when lesser amounts of plasmids were used for transfection. The amount of NS3 protein was also reduced in a dose dependent manner, whereas the cell number in the culture was not affected as confirmed by β-actin detection and quantification (Fig. 3b). The result of student's *t*-test revealed that the supernatant from NS3 expressing cells showed significantly higher LDH activity compared to control cells (P<0.001 when 0.5 μg/well of plasmid DNA were used).

NS3 showed higher cytotoxicity when GNS3 was synthesized: Given the cytotoxic effect of wt NS3, we attempted to determine, if glycosylation affects cytotoxicity. Culture supernatants from cells expressing wt NS3 and NS3 mutants were subjected to the LDH cytotoxicity assay. As shown in Fig. 4a, both wild type and mutants showed higher LDH activity compared to mock transfection. However, when results were analyzed by Tukey's test, it was revealed that the expression of GNS3 (NS3 and NS3/N59Q) resulted in

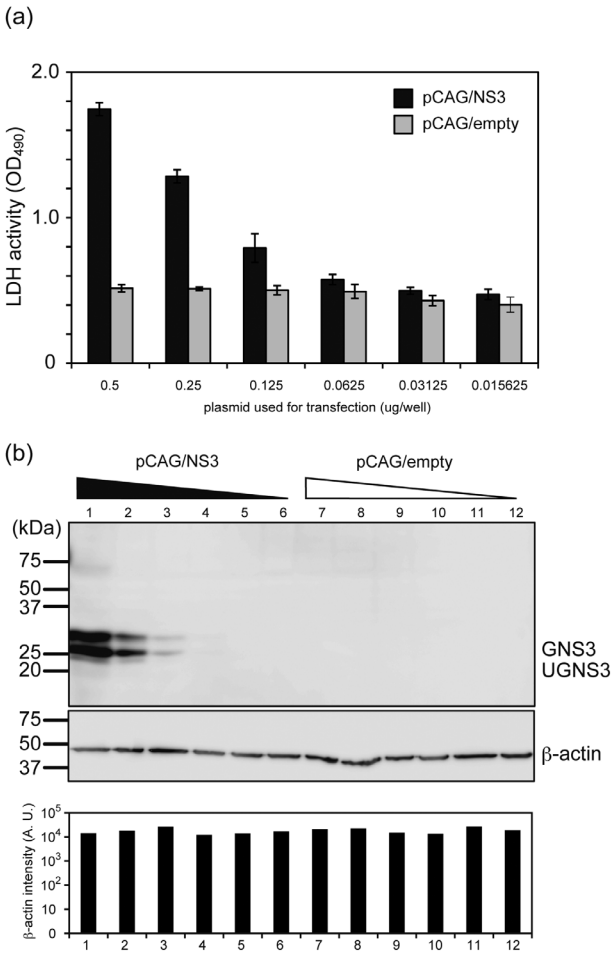

Fig. 3. IBAV NS3 possesses cytotoxicity on mammalian cells. (a) LDH activity in the culture supernatant of 293T cells transfected with various amounts of wt-NS3 expression plasmid (black bar) or control plasmid pCAG/empty (gray bar). The result is the representative of three independent experiments. (b) Western blot analysis of cells used for LDH assay with anti-NS3 and anti-β-actin antibodies. The bottom graph shows the intensity of β-actin protein band quantified by ImageJ software.

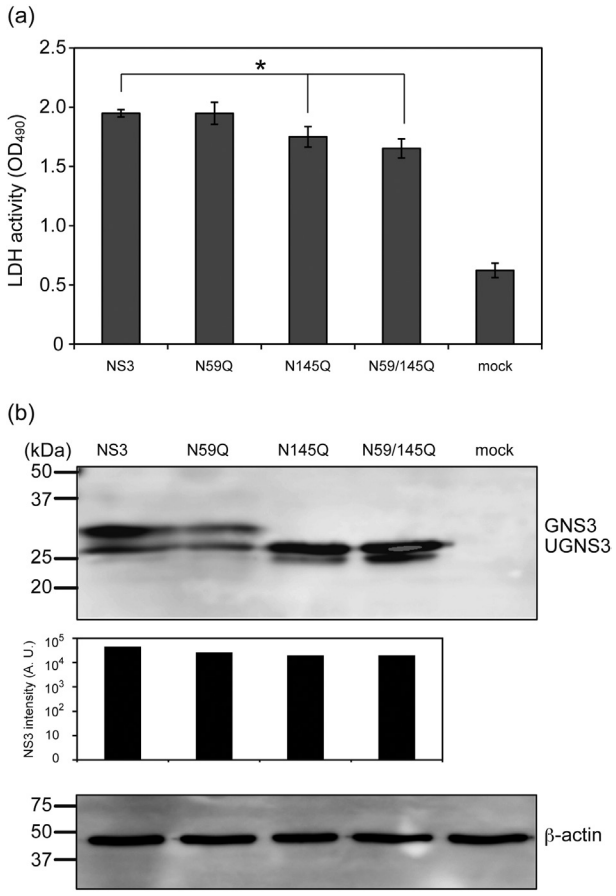

Fig. 4. NS3 glycosylation mutants showed lower cytotoxicity in mammalian cells compared to wt NS3. (a) LDH activity in the culture supernatant of 293T cells transfected with expression plasmids for wt-NS3 and various glycosylation mutants. The result is the representative of three independent experiments. n=6 for each experiment. (*P<0.001) (b) Western blot analysis of cells used for LDH assay with anti-NS3 and anti-β-actin antibodies. The middle graph shows the intensity of total NS3 (GNS3 plus UGNS3) quantified by ImageJ software.

a higher LDH activity than the expression of UGNS3 (NS3/N145Q and NS3/N59,145Q). On the other hand, no significant difference was observed when glycosylation pattern was the same (P=0.99 for NS3 and NS3/N59Q, P=0.18 for NS3/N145Q and NS3/N59,145Q). The expression of each NS3 mutant was confirmed by western blot analysis. The amount of total NS3 including GNS3 and UGNS3 was quantified (Fig. 4b). As previously shown in Fig. 2b, only UGNS3 was observed in NS3/N145Q and NS3/N59,145Q. All mutants expressed almost equal amount of total NS3 (Fig. 4b graph). In addition to UGNS3, the protein band corresponding to the molecular weight of NS3A was detected under UGNS3. Since we collected cells at early time point after transfection (36 hr), no CPE was observed neither in cells expressing glycosylated NS3 nor NS3 lacking glycosylation. This lack of CPE was also confirmed by the result of β-actin detection in the western blot analysis (Fig. 4b). This result suggested that modification by N-glycan enhanced the cytotoxic effect of NS3 in mammalian cells.

Intracellular distribution of wt-NS3 and glycosylation deficient NS3 in 293T cells: The immunofluorescence assay was performed to confirm, if glycosylation of NS3 affects the intracellular distribution of NS3. When both NS3 and the plasma membrane were stained, most NS3 co-localized with the plasma membrane in the cells transfected with pCA7/NS3 (Fig. 5a). In contrast, NS3 tended to remain clustered near the nucleus and did not distribute to the plasma membrane in the cells transfected with pCA7/NS3N59,145Q. Moreover, when the longitudinal and cross-section images were created, wt-NS3 had merged with the plasma membrane, whereas a merged image was not observed in cells expressing UGNS3 (Fig. 5b). These results indicated that glycosylation could aid in the transport of NS3 to the cell

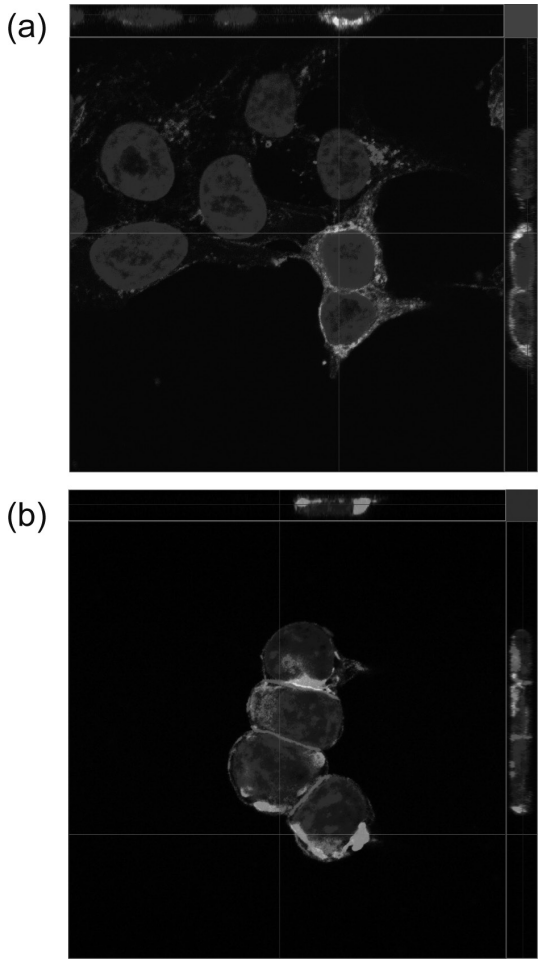

Fig. 5. Visualization of NS3 in 293T cells expressing wt-NS3 and NS3 glycosylation mutants by immunofluorescence assay. (a) pCA7/NS3 and (b) pCA7/NS3N59,145Q were transfected to 293T cells. NS3 was visualized by IFA with protein specific antisera. The longitudinal and cross-section images were also created. NS3, the plasma membrane and the nucleus are shown in green, red and blue, respectively. Co-localization of NS3 and the plasma membrane is shown in yellow.

surface and enhance distribution on the plasma membrane.

DISCUSSION

In our previous study [22], we observed a correlation between NS3 glycosylation and host specific pathogenicity of IBAV in two different cell lines. NS3 glycosylation and a severe CPE occurred only in mammalian cells infected with IBAV. In this study, we constructed NS3 expression plasmids with or without mutation to its glycosylation site (s) to gain insight into the significance of NS3 glycosylation observed only in mammalian cells in IBAV replication. Here, we tried to identify the correlation between cytotoxic activity of IBAV NS3 with glycosylation when it was expressed in mammalian cells without a live virus infection. We did not test NS3 cytotoxicity in insect cells, since no NS3 glycosylation was observed in the previous study. Significantly greater quantities of LDH were released into the culture supernatant as the quantity of cells expressing IBAV NS3 was increased (Fig. 3a). Moreover, wt-NS3 exhibited significantly higher cytotoxicity than its glycosylation mutants (Fig. 4a). Those results confirmed the cytotoxicity of IBAV NS3 and suggested the influence of glycosylation on its cytotoxicity.

Some studies suggest that orbivirus NS3 has a cytotoxic effect on host cells and that it might be one of the causative agents for severe symptoms observed in orbivirus infection [13, 25]. Also, it has been proposed that orbiviruses utilize this cytotoxicity to achieve the exit of progeny virus particle from host cells [9]. In fact, BTV with altered expression of NS3 exhibited less cytotoxicity and slower growth kinetics than wild type BTV [6, 24]. We confirmed this cytotoxicity was conserved in IBAV and showed the link between cytotoxicity and NS3 glycosylation. Furthermore, we tried to reveal how glycosylation controls NS3 cytotoxicity.

As a possible explanation regarding the importance of NS3 glycosylation toward cytotoxicity, we propose the contribution of glycans on the transition of NS3 to the plasma membrane. It is well known that glycans can act as a tag to control protein transport and quality [10]. Many glycoproteins receive glycosylation in the process of passing the ER and Golgi apparatus, and such modification is required for protein transport to the plasma membrane and secretion [11]. BTV NS3 is believed to enter the ER and then transit to the plasma membrane via the Golgi apparatus while receiving modification to its glycan [14, 28]. A past study revealed that when glycosylation was impaired in BTV NS3, it could not exit the Golgi apparatus and NS3 distribution on the plasma membrane was suppressed [2]. This result shows that glycosylation on NS3 facilitates NS3 transition to the plasma membrane. In fact, wt-NS3 was distributed to the plasma membrane in the cells when detected with IFA in the case of IBAV (Fig. 5a). On the other hand, the glycosylation mutant NS3/N59,145Q tended to remain around the nucleus, and less NS3 signal was detected at the plasma membrane (Fig. 5b). Combined with the reduced cytotoxicity of unglycosylated NS3 mutants observed in Fig. 3a, we suggest that glycosylation on NS3 controls its cytotoxicity by changing cell surface distribution of NS3. This is the first report showing the effect of glycosylation on orbivirus NS3 to both cell surface expression and cytotoxic effect.

The exit from host cells is an important step for viruses to initiate the next round infection in new hosts. To achieve an efficient exit, many viruses have developed different mechanisms including the hijacking of host intracellular transport mechanism and disrupting the plasma membrane of host cells. In BTV, both virus release pathways have been suggested including hijacking of the host cellular ESCRT system [5] and disruption of the host membrane by a viroporin-like structure [9]. Although the latter system is useful for the release of large quantities of progeny viruses simultaneously, the damage incurred by the host cells is extensive, potentially impairing further virus replication. When this occurs in vector arthropods, the virus might kill

its vector, decreasing its ability to spread. In the previous report, we reported that unglycosylated form of NS3 as well as NS3A was the main form of NS3 in IBAV infected insect cells. Also, other group showed that the replication of BTV in insect cells is supported by both NS3 and NS3A [24]. In combination, it is possible that even they were not transported to the cell surface, unglycosylated form of NS3 contributes for the formation of budding vesicles at the inside of the infected insect cells. The correlation between NS3 glycosylation and its ability to disturb the plasma membrane coincides with our earlier observation about the relationship between NS3 glycosylation and severe CPE in mammalian cells [22]. Although, the detailed regulatory mechanism to control NS3 glycosylation remains unclear, it is likely that IBAV has developed a mechanism to achieve host specific pathogenicity by controlling glycosylation on NS3.

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