

Original Article

Association between RABV G Proteins Transported from the Perinuclear Space to the Cell Surface Membrane and N-Glycosylation of the Sequon Asn²⁰⁴

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SUMMARY: In this study, G proteins of the rabies virus (RABV) Kyoto strain were detected in the cytoplasm but not distributed at the cell membrane of mouse neuroblastoma (MNA) cells. G proteins of CVS-26 were detected in both the cell membrane and perinuclear space of MNA cells. We found that N-glycosylation of street RABV G protein by the insertion of the sequon Asn²⁰⁴ induced the transfer of RABV G proteins to the cell surface membrane. Fixed RABV budding from the plasma membrane has been found to depend not only on G protein but also on other structural proteins such as M protein. However, the differing N-glycosylation of G protein could be associated with the distinct budding and antigenic features of RABV in street and fixed viruses. Our study of the association of N-glycan of G protein at Asn²⁰⁴ with the transport of RABV G protein to the cell surface membrane contributes to the understanding of the evolution of fixed virus from street virus, which in turn would help for determine the mechanism underlying RABV budding and enhanced host immune responses.

INTRODUCTION

Rabies is a fatal viral infectious disease of humans and animals and is caused by the highly neurotropic rabies virus (RABV), which belongs to the genus *Lysnavirus* from the family *Rhabdoviridae*. This bullet-shaped enveloped virus has a nonsegmented negative-strand RNA genome that is approximately 12 kb in length and encodes five viral proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large protein (L) (1). RABVs are generally classified into two categories, street viruses (field isolates) and fixed viruses (laboratory-adapted strains). Street viruses are known to be more pathogenic than fixed viruses after peripheral infections. The characteristic differences between fixed and street viruses are related to regularity and shortening of the incubation period, stabilization of virulence, reduction or loss of infectivity after peripheral inoculations, and increased intracellular propagation and budding of virions from infected cells (2–6).

RABV G protein is the only viral protein that is glycosylated and exposed on the surface of the virion. It is the main factor responsible for the pathogenesis of RABV and is related to its entry into target cells and the production of virus-neutralizing antibodies (1,3,7–11). Recent reports indicated that the number of N-glycosylation sites in G protein is one of the determinants of the

pathogenicity of street viruses (12–14). Most G proteins of street viruses have two N-glycosylation sites at Asn³⁷ and Asn³¹⁹, whereas fixed viruses have additional glycosylation sites on G protein at Asn¹⁵⁸, Asn²⁰⁴, or Asn²⁴⁷ (12,13,15,16) (Fig. 1). Glycosylation is important for the proper folding, expression, transport and function of G protein (17).

In a pseudotyped virus assay, addition of a single N-glycan at amino acid 194 or 247 of the G protein of the street rabies virus 1088 strain was reported to enhance virus production in neural and non-neural cell lines (13). Ultrastructural studies have shown that street viruses bud at intracellular membranes, but fixed viruses preferentially bud from the plasma membrane (18–21). This study investigated the association of G protein that was N-glycosylated at Asn²⁰⁴ with the transport of RABV G protein to the cell surface membrane.

MATERIALS AND METHODS

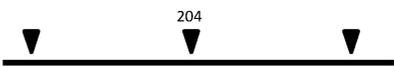
Viruses and Cells: The RABV Kyoto strain (street virus) isolated from an imported human case in 2006 (22,23) and CVS-26 strain (fixed virus) which was kindly provided by Dr. C. E. Rupprecht (former Poxvirus and Rabies Branch, DHCPP, NCEZID, CDC, Atlanta, GA, USA) were used in this study. Viruses were propagated in mouse neuroblastoma (MNA) cells at 37°C in minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum and stored at –80°C until use (24).

Viral titration: Virus titration was performed with a focus assay on confluent monolayers of MNA cells in 96-well plates as described previously (24). Viral nucleoprotein (N) in MNA cells was detected with a UV microscope (Eclipse TE200; Nikon, Tokyo, Japan) after staining with fluorescein isothiocyanate (FITC)-conjugated anti-RABV N monoclonal antibody (Fujirebio

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RABV	Type	Positions of N-glycosylation	RABVs examined in this study	RABV strains ¹⁾
S1		NH ₂ 37  319 COOH	100	Kyoto (22) WH5 (DQ849061) 1088 (AB645847) etc.
Street virus	S2		17	MO1197 (KF484541) BR-NL2 (AB383172) SHBRV (U52946) etc.
	S3		3	A11_30558 (JX871881) A12_1050 (KC7920) GA31940 (JQ595325)
	F1		4	CVS-11 (AF085333) CVS-N2c (AF325714) CVS-26 (LC009634) PM1503 (DQ099525)
Fixed virus	F2		6	Nishigahara (AB044824) RC-HL (AB009663) Ni-CE (AB128149) ERA (GQ406342) SAD Bern (KC178556) Lysvulpen (KC178555)
	F3		2	LEP-Flury (GU565703) HEP-Flury (AB085828)

1): RABV strains (Accession number or reference)

Fig. 1. N-glycosylation of RABV G protein. Putative N-glycosylation sites (▼) are according to the amino acid sequences of RABV G proteins retrieved from GenBank.

Diagnostics, Malvern, PA, USA) diluted 1 : 100 in PBS(-) with Evans blue 0.002% for 30 min at room temperature (r.t.).

Plasmid construction: The expression vectors of Kyoto G and CVS-26 G were constructed as follows. Total RNA of 10% brain homogenates prepared from a patient infected with the Kyoto strain and mouse infected with the CVS-26 strain were extracted using an RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Reverse transcriptase (RTase) reactions with Random Primer (Promega, Madison, WI, USA) were carried out at 42°C for 45 min with AMV Reverse Transcriptase (HC) (Promega). Each G gene was amplified from the corresponding cDNA by PCR with Platinum Taq High Fidelity (Invitrogen-Life Technologies, Gaithersburg, MD, USA) with the following primer sets for the Kyoto G gene: RABVK/Y-G-F 5'-CGCGAATTCACCATGATT CCTCAGGCTCTT-3' (*EcoRI* site and start codon underlined) and RABVK/Y-G-R 5'-TATGCGGCCGCTT ACAGCTTGGTCTCACCT-3' (stop codon and *NotI* site underlined), and for the CVS-26 G gene: RABV-CVS26G-F 5'-CGCGAATTCACCATGGTTCTCAG GTTCTT-3' (*EcoRI* site and start codon underlined) and RABV-CVS26G-R 5'-TATGCGGCCGCTCACAG TCTGATCTCACCT-3' (stop codon and *NotI* site

underlined). PCR amplification was performed for 30 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and extension at 68°C for 60 s/kb of the target. The amplified DNA fragments were inserted into multiple cloning sites of the pcDNA3.1/Zeo vector (Invitrogen). The pcDNA3.1/Zeo vectors with the Kyoto G gene and CVS-26 G gene inserted were named pzKyoto-G and pzC26-G, respectively. Both the G proteins were expressed under the control of the CMV promoter.

Mutation of RABV G protein at the 204 site related to N-glycosylation: Nucleotides were mutated using a PrimeSTAR mutagenesis kit (TAKARA Bio, Shiga, Japan) according to the manufacturer's protocol, with the primer sets Kyoto-G(S204N)-F 5'-GGGAACA AGACTTGTGGATTGTAGATGAG-3' and Kyoto-G(S204N)-R 5'-ACAAGTCTTGTCCCTTGGATG CTCTCT-3' for developing the sequon Asn²⁰⁴ into Kyoto G protein, and primer set CVS26-G(N204S)-F 5'-GGGAGTAAGACCTGCGGCTTTGTGGATGAA-3' and CVS26-G(N204S)-R 5'-GCAGGTCTTACTCCC GTTGGATGCTCTCTT-3' for the mutated sequon Asn²⁰⁴ of the CVS-26 G protein. Mutated nucleotides are underlined. The plasmid pzKyoto-G that was mutated for additional N-glycosylation at 204th amino acid was named pzKyoto-mG, which expressed Kyoto

rG(S204N). The plasmid pzC26-G that was mutated to block N-glycosylation at Asn²⁰⁴ was named plasmid pzC26-mG, which expressed CVS-26 rG(N204S). The nucleotide sequence of G genes reported here has been deposited in the DDBJ/GenBank/EMBL database as Kyoto G (ID: LC009632), Kyoto G (S204N) (ID: LC009633), CVS-26 G (ID: LC009634), and CVS-26 G (N204S) (ID: LC009635).

Indirect immunofluorescence: MNA cells were grown on CELLview (35 mm, 4-compartment cell culture dishes with a glass bottom; Greiner Bio One, Frickenhausen, Germany). MNA cells (50–80% confluent) were infected by the virus at a multiplicity of infection (MOI) of 0.01, and transfected plasmids coding the G gene of RABV were cultured for 120 h and 48 h, respectively. Cell surface antigens were detected by the primary antibody after fixation with 10% formalin neutral buffer solution, pH 7.4 (Wako Pure Chemical, Osaka, Japan) at r.t. for 30 min. Intracellular antigens were detected by the permeabilized cells with 0.2% Triton X-100 in PBS for 5 min at r.t. RABV G proteins were detected by the use of the mouse monoclonal anti-RABV G antibody #7-1-9 (0.4 µg/ml), kindly provided from Dr. A. Kawai (Research Institute for Production Development, Kyoto, Japan) (25,26). Visualization of the primary antibody was performed with FITC-conjugated anti-mouse IgG antibody for anti-RABV G #7-1-9. Nuclei were visualized by DAPI staining. Localization of RABV G proteins in MNA cells was examined and recorded with an FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan).

SDS-PAGE and western blotting: MNA cells grown in 6-well TPP tissue culture plates (Sigma-Aldrich, St. Louis, IL, USA) were infected with RABV or transfected plasmids coding the G gene of RABV. MNA cells infected for 72 h and those transfected for 48 h were lysed with 0.5 ml/well of lysis buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 0.01% [v/v] Tween-20, and 1% [v/v] Triton-X 100) at 4°C for 1 h according to a method described previously (27). Precipitated proteins (5 µl) were suspended in loading buffer and separated by electrophoresis in 8% NuPAGE Novex Bis-Tris Midi Gels (Invitrogen) using MOPS Buffer (Invitrogen). In the upper buffer chamber, NuPAGE Antioxidant (Invitrogen) was added at a concentration of 0.25%. After SDS-PAGE, proteins transferred onto PVDF membranes were blocked for 1 h at r.t. (or overnight at 4°C) with 5% skim milk (w/v) in PBS. G protein was detected using anti-RABV G antibody #7-1-9, with HRP-conjugated anti-mouse IgG, diluted 1 : 5000 with 1% skim milk in PBS-T and visualized by ECL Prime Western Blotting Detection Reagent substrate solution (GE Healthcare, Piscataway, NJ, USA). The signals were detected with VersaDoc (Bio-Rad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as a control, was detected by monoclonal mouse anti-rabbit GAPDH IgG 5G4 (HyTest, Turku, Finland) diluted 1 : 5000 with 1% skim milk in PBS-T.

Inhibition and cleavage of N-linked glycan: After transfection of plasmids coding the RABV G gene into MNA cells by using TransIT-Neural transfection reagent (Mirus Bio LLC., Madison, WI, USA) according to the manufacturer's instructions, N-glycosylation of

G protein was blocked with 1 µg/ml of tunicamycin (Sigma) for 44 h, which was followed by processing for western blotting (12,13). The cleavage of N-linked glycan was performed with N-Glycosidase F (PNGase F) (Promega) according to the manufacturer's instructions.

RESULTS

Localization of G and rG proteins of the Kyoto and CVS-26 strains in MNA cells: Viral G proteins of the Kyoto strain were not observed at the cell surface membrane of MNA cells, but they were detected in the cytoplasm. However, viral G proteins of CVS-26 were detected both in the cell membrane and in the perinuclear space of MNA cells (Fig. 2A). The recombinant G (rG) protein of the Kyoto strain and CVS-26 strain also showed similar localization of G proteins expressed in MNA cells. The expressed rG proteins of CVS-26 were mainly observed around the cell surface membrane but were also detected in the perinuclear space of MNA cells. The expressed Kyoto rG protein was obviously localized around the perinuclear space of MNA cells (Fig. 2B).

Different molecular sizes of Kyoto and CVS-26 rG proteins associated with N-glycosylation: The molecular size of the Kyoto rG protein detected by western blotting after SDS-PAGE was slightly smaller than that of the CVS-26 rG protein. However, the Kyoto and CVS-26 rG proteins showed almost the same mobility after the inhibition of N-glycosylation by tunicamycin and the cleavage of N-linked glycan by PNGase F (Fig. 3A and B).

Localization of CVS-26 rG protein around the cell membrane is associated with N-glycosylation: Putative N-glycosylation at amino acid 204 was not found in the Kyoto rG protein but was present in the CVS rG protein (Fig. 1). Perinuclear localization of Kyoto rG protein in MNA cells did not change with or without treatment with tunicamycin (Fig. 4). However, CVS-26 rG proteins that were localized around the cell surface membrane of MNA cells moved to the perinuclear space after treatment with tunicamycin (Fig. 4).

Mobility of Kyoto and CVS-26 rG proteins with or without N-glycosylation of the sequon Asn²⁰⁴: The Kyoto G gene with the sequence inserted at the sequon Asn²⁰⁴ produced a larger Kyoto rG protein. However, most of the CVS-26 rG proteins translated from the CVS-26 G gene mutated the sequon Asn²⁰⁴ resulting in a decrease in the size of rG proteins on SDS-PAGE (Fig. 5). The mobility of Kyoto rG proteins with the sequon Asn²⁰⁴ was the same as that of CVS-26 rG protein, and CVS-26 rG protein blocked N-glycosylation by the mutation of the sequon Asn²⁰⁴ showed the same mobility as that of Kyoto rG protein (Fig. 5).

Localization of Kyoto rG, Kyoto rG(S204N), CVS-26 rG, and CVS-26 rG(N204S) proteins expressed in MNA cells: Kyoto rG proteins could not be detected at the cell surface membrane, but Kyoto rG(S204N) with the sequon Asn²⁰⁴ was localized at the cell surface membrane. However, both the Kyoto rG and Kyoto rG(S204N) proteins were fully observed around the perinuclear space of MNA cells after membrane permeabilization (Fig. 6A). In contrast, localization of

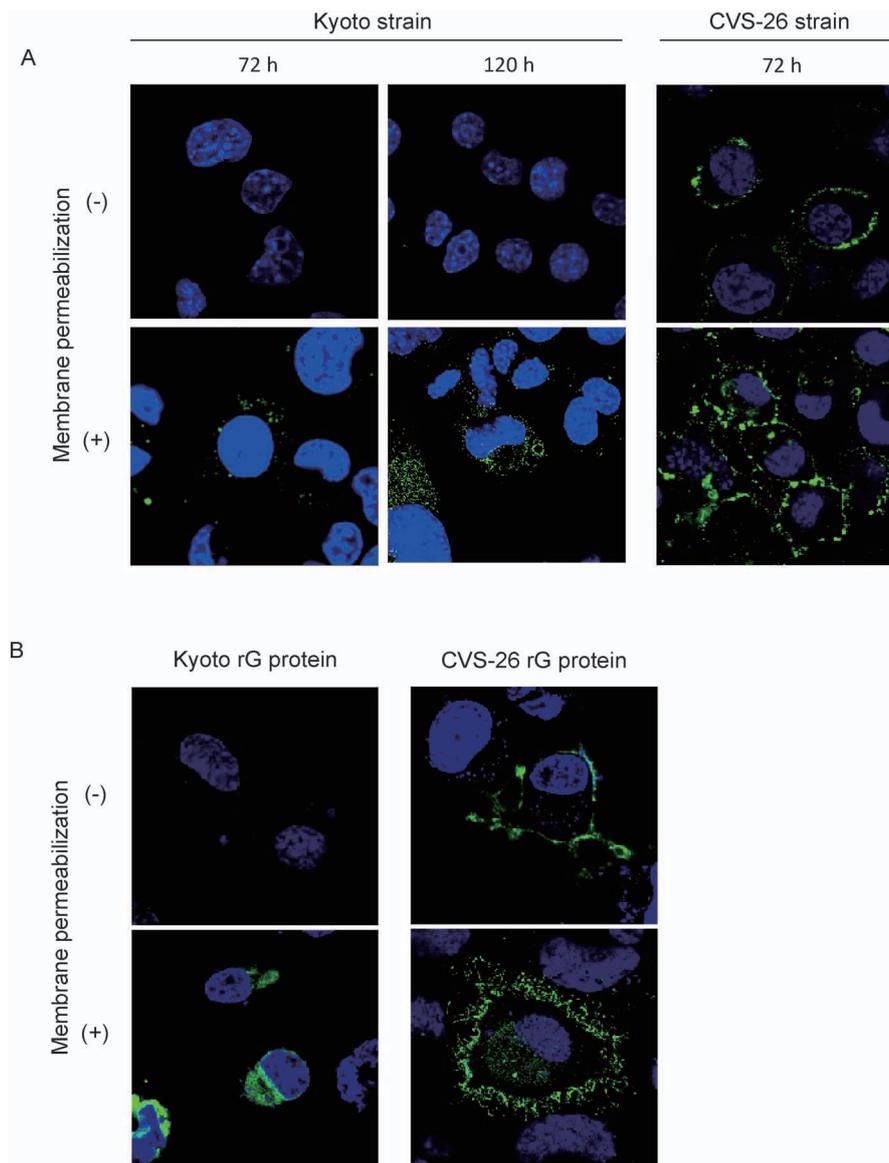


Fig. 2. (Color online) Localization of G and rG proteins in MNA cells. G proteins in MNA cells were detected by indirect immunofluorescence and intracellular G proteins were visualized by membrane permeabilization. Nuclei were visualized by DAPI staining. MNA cells infected with Kyoto strain (after 72 h and 120 h) and CVS-26 strain (after 72 h) were fixed (A). MNA cells expressing rG proteins of the Kyoto strain and CVS-26 strain were fixed after 48 h (B). All experiments were independently performed three times.

CVS-26 rG(N204S) with mutation of the sequon Asn²⁰⁴ was still observed at the cell membrane as seen for the CVS-26 rG proteins, but a greater amount of CVS-26 rG (N204S) proteins than CVS-26 rG proteins was distributed around the perinuclear space (Fig. 6B).

DISCUSSION

RABV G protein plays important roles in viral pathogenesis, for example, in relation to infectivity, intracellular propagation, virus transportation, and immunogenicity. N-glycosylation types of RABV G protein are summarized in Fig. 1. Most G proteins of street viruses have two N-glycosylation sites, that is, at Asn³⁷ and Asn³¹⁹, whereas fixed viruses have three or four potential glycosylation sites on the G protein, that is, at Asn³⁷, Asn¹⁵⁸, Asn²⁰⁴, Asn²⁴⁷, or Asn³¹⁹ (12,13,15,16, 28). The Kyoto strain (street virus) was isolated from a

patient who imported rabies into Japan (21,22), and the CVS-26 strain (fixed virus) was a challenge virus standard strain passaged in mouse brains at the US CDC. N-glycosylation of Kyoto G protein was classified into Type S1, which is the major pattern in street strains. CVS-26 G protein was classified into Type F1 with additional N-glycosylation at the sequon Asn²⁰⁴-X²⁰⁵-Thr²⁰⁶.

G proteins of the Kyoto strain were detected in the cytoplasm but not distributed at the cell surface membrane of MNA cells, whereas those of CVS-26 were detected both at the cell surface membrane and in the perinuclear space of MNA cells (Fig. 2A). These results were similar to those of ultrastructural studies showing differing budding features of street viruses at the intracellular membranes and of fixed viruses at the plasma membrane (18–20). This localization of G proteins in MNA cells was emphasized by the expression of rG proteins. Kyoto rG proteins were obviously localized

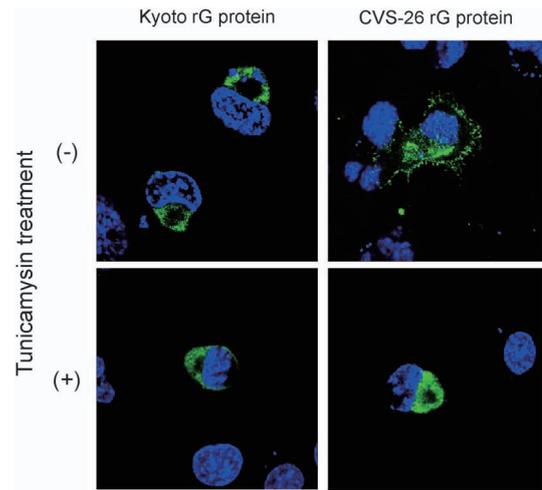
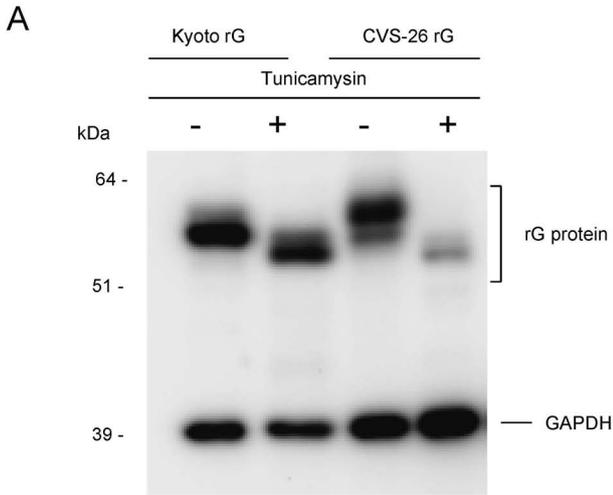


Fig. 4. (Color online) Localization of rG proteins in MNA cells. MNA cells were transfected with 0.5 μ g of pzKyoto-G and pzC26-G into each compartment of CELLview culture dishes. rG proteins of the Kyoto strain and CVS-26 strain were expressed under the presence or absence of 1 μ g/ml tunicamycin for 44 h. Intracellular G proteins were visualized by membrane permeabilization with 0.2% Triton X-100 in PBS. After fixation with 10% formalin neutral buffer solution (pH 7.4), rG protein was detected by indirect immunofluorescence using anti-RABV G antibody #7-1-9 and FITC-labelled anti-mouse IgG antibody. Nuclei were visualized by DAPI staining. Immunofluorescence was analyzed using confocal laser scanning microscopy. All experiments were independently performed three times.

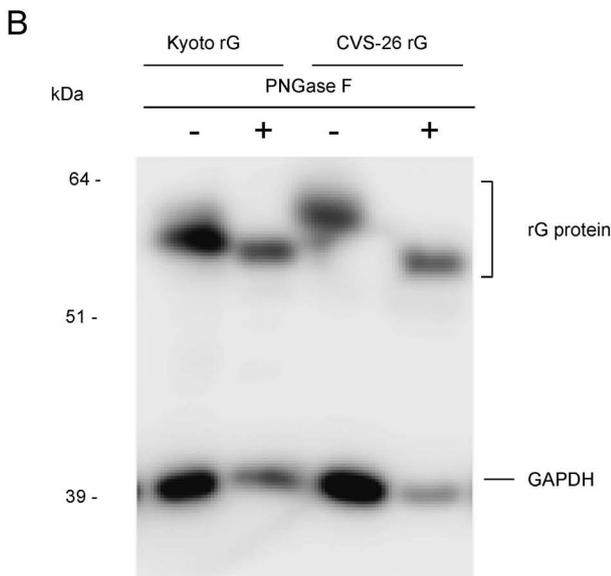


Fig. 3. Molecular sizes of Kyoto and CVS-26 rG proteins expressed in MNA cells. MNA cells were cultured for 48 h after transfection of 2 μ g/well of pzKyoto-G or pzC26-G. Samples after 44 h treatment with or without tunicamycin (A) or with or without cleavage of N-linked glycan of rG proteins by PNGase F at 37°C for 3 h (B) are shown. Molecular sizes of rG proteins extracted from one well of a 6-well TPP tissue culture plate were analyzed by SDS-PAGE and western blotting with anti-RABV G antibody #7-1-9 and with HRP-conjugated anti-mouse IgG. GAPDH was detected by anti-rabbit GAPDH mouse mAb 5G4. All experiments were independently performed three times.

around the perinuclear space of the MNA cells, and CVS-26 rG proteins were mainly observed around the cell surface membrane but also detected in the perinuclear space of the MNA cells (Fig. 2B).

N-glycosylation of the Kyoto and CVS-26 G proteins was detected by the mobility of the G proteins. Additional N-glycosylation of CVS-26 G protein was confirmed by the inhibition of N-glycosylation by tunicamycin and the cleavage of N-linked glycan by PNGase F (Fig. 3A and B). The inhibition of N-glycosylation in Chinese hamster ovary (CHO) cells by tunicamycin has been reported to completely block the surface expression of G proteins produced by the eukaryotic shuttle vector pSG5 in which the G gene of

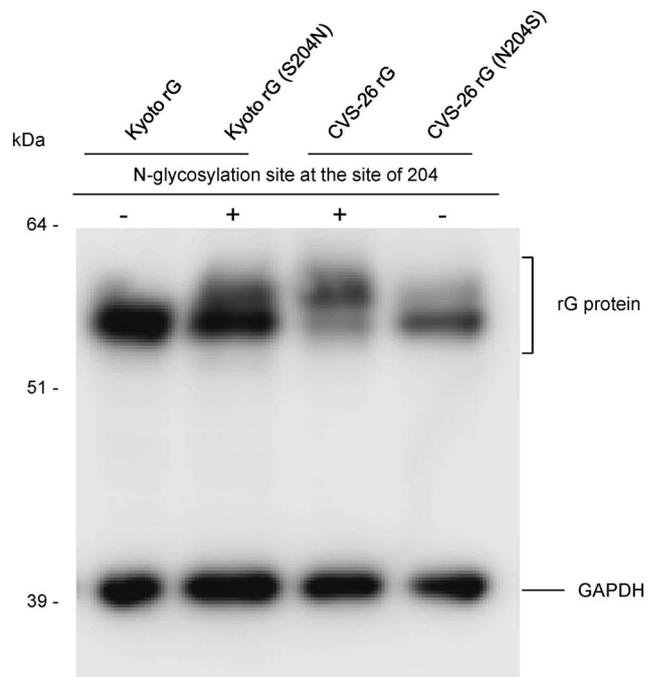


Fig. 5. Mobility of Kyoto rG, Kyoto rG(S204N), CVS-26 rG, and CVS-26 rG(N204S) on SDS-PAGE. Molecular sizes of the rG proteins were analyzed by western blotting with anti-RABV G antibody #7-1-9 and HRP-conjugated anti-mouse IgG. MNA cells were transfected with 2 μ g/well of pzKyoto-G, pzKyoto-mG, pzC26-G, or pzC26-mG into one of 6 wells of a TPP tissue culture plate and were cultured for 48 h. GAPDH was detected using anti-rabbit GAPDH mouse mAb 5G4. All experiments were independently performed three times.

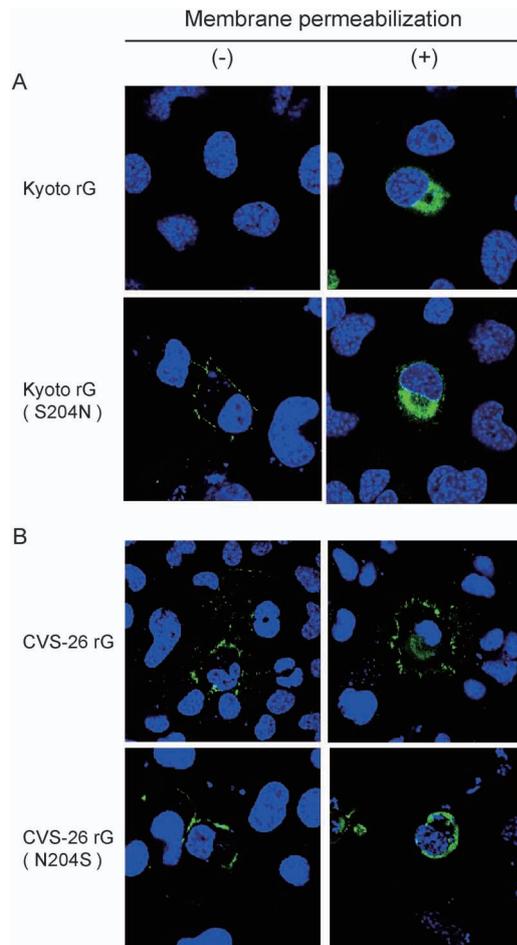


Fig. 6. (Color online) Localization of Kyoto rG, Kyoto rG(S204N), CVS-26 rG, and CVS-26 rG(N204S) proteins expressed in MNA cells. MNA cells transfected with 0.5 μ g of pzKyoto-G, pzKyoto-G(S204N), pzC-26-G, or pzC26-G(N204S) were cultured for 48 h. After fixation with 10% formalin neutral buffer solution (pH 7.4), rG proteins were detected by indirect immunofluorescence with anti-RABV G antibody #7-1-9 and FITC-labelled anti-mouse IgG antibody. Intracellular G proteins were visualized by membrane permeabilization with 0.2% Triton X-100 in PBS. Nuclei were visualized by DAPI staining. Immunofluorescence was analyzed by confocal laser scanning microscopy. All experiments were independently performed three times.

the ERA strain (fixed virus) has been inserted, and the intracellular accumulation of G proteins suggests a role of N-linked oligosaccharides for transport of RABV G proteins to the plasma membrane (29). Deletion of all three sequons at Asn³⁷, Asn²⁴⁷, and Asn³¹⁹ in ERA G proteins also completely blocked cell surface expression in CHO cells (30). In the present study, CVS-26 rG proteins localized around the cell surface membrane disappeared and accumulated in the perinuclear space following treatment with tunicamycin. However, Kyoto rG proteins were localized at the perinuclear space regardless of treatment with tunicamycin (Fig. 4). The G protein of the CVS-26 strain had three potential glycosylation sites at Asn³⁷, Asn²⁰⁴, and Asn³¹⁹, but the Kyoto strain had only two, Asn³⁷ and Asn³¹⁹ (Fig. 1). These results suggested that the transportation of CVS-26 G proteins to the cell surface membrane was associated with N-glycosylation at Asn²⁰⁴.

The role of N-glycosylation at Asn²⁰⁴ in the Kyoto G

and CVS-26 G proteins was also examined using the Kyoto rG(S204N)-constructed sequon Asn²⁰⁴, and CVS-26 rG(N204S) blocked N-glycosylation by the mutation of the sequon Asn²⁰⁴ (Fig. 5). Kyoto rG(S204N) proteins were localized to the cell surface membrane by N-glycosylation of the sequon Asn²⁰⁴ (Fig. 6A), whereas fully N-glycosylated CVS-26 rG proteins were obviously observed around the cell surface membrane, but the amount of CVS-26 rG(N204S) proteins distributed around the perinuclear space was more than that of CVS-26 rG proteins (Fig. 6B). These results demonstrated that N-glycosylation of G protein in the sequon Asn²⁰⁴ induced the transfer of RABV G proteins to the cell surface membrane. The distribution of CVS-26 rG(N204S) in the cell membrane could be acquired by highly sialylated N-glycans and additional changes in the primary structure during the evolution of the fixed virus CVS-26 (13,29,30). N-glycosylation at Asn²⁰⁴ that was found in G protein of the CVS strain (fixed virus) (31) was intensive in CVS-26 G protein, but N-glycosylation of RABV G protein was only partial in Kyoto rG(S204N) proteins (Fig. 5). N-glycosylation of rG protein of the 1088 strain (street virus) was also not efficient with the additional sequon Asn²⁰⁴ (13).

N-glycosylation of street RABV Kyoto G protein with the inserted sequon (Asn²⁰⁴-X²⁰⁵-Thr²⁰⁶) demonstrated expression and transport to the cell surface membrane. Different budding features of RABV in street and fixed viruses could be associated with N-glycosylation of G protein. This study showed that differing intracellular localization of RABV G proteins for the Kyoto (street virus) and CVS-26 (fixed virus) strains was associated with N-glycosylation of RABV G proteins at the sequon Asn²⁰⁴. The number of N-glycosylation sites in G protein may be a determinant of the pathogenicity of street viruses (12–14). A previous report suggested that various factors affect N-glycosylation of individual sequons in RABV G protein (32). The fixed viruses, CVS-24 and CVS-11, with G protein of Type F1, have neuroinvasive characteristics along with peripheral infectivity and highly antigenic features (10,11,23,30,33). Cell surface expression of G protein in nonpathogenic fixed strains is stronger than that in pathogenic fixed strains (34). Over-expression of G protein of fixed virus elevates apoptotic activity and enhances the antibody response (34).

The budding of RABV from the plasma membrane depends not only on G protein but also on other structural proteins such as M protein. However, differing N-glycosylation of G protein could be associated with budding and antigenic features of RABV in street and fixed viruses. Further study of the association between N-glycan of G protein at sequon Asn²⁰⁴ and the transport of RABV G protein to the cell surface membrane will help to clarify the mechanism of RABV budding and enhanced host immune responses after the evolution of fixed viruses from street viruses (4).

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Conflict of interest None to declare.**REFERENCES**

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