Association between RABV G Proteins Transported from the Perinuclear Space to Cell Surface Membrane and N-glycosylation of the Sequon at Asn$^{204}$

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Association between RABV G Proteins Transported from the Perinuclear Space to Cell Surface Membrane and N-glycosylation of the Sequon at Asn^{204}

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RABV G N-glycosylation at Asn204
SUMMARY: Viral 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. Viral 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 at Asn^{204} induced the transfer of RABV G proteins to the cell surface membrane. Fixed RABV bud from the plasma membrane depend not only on G protein but also on other structural proteins such as M protein. However, different N-glycosylation of G protein could be associated with budding and antigenic features of RABV in street and fixed viruses. Our study of the association of N-glycan of G protein at Asn^{204} with the transport of RABV G protein to the cell surface membrane contributes to understanding of the evolution of fixed virus from street virus for determining the mechanism of RABV budding and enhanced host immune responses.
INTRODUCTION

Rabies is a fatal viral infectious disease of humans and animals caused by the highly neurotropic virus named rabies virus (RABV), which belongs to the genus Lyssavirus, family Rhabdoviridae. This bullet-shaped enveloped virus has a nonsegmented negative-strand RNA genome of about 12 kb in length that encodes five viral proteins: the 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 involve 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. This is the major factor responsible for the pathogenesis of RABV as related to its entry into target cells and production of virus-neutralizing antibodies (1,
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 Asn\textsuperscript{37} and Asn\textsuperscript{319}, whereas fixed viruses have additional glycosylation sites on G protein of Asn\textsuperscript{158}, Asn\textsuperscript{204} or Asn\textsuperscript{247} (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 194\textsuperscript{th} or 247\textsuperscript{th} amino acid of G protein of the street rabies virus 1088 strain resulted in the enhancement of virus production in neural and non-neural cell lines (13). Ultrastructural studies have shown that street viruses bud at intracellular membranes, but fixed viruses prefer to bud from the plasma membrane (18-21). This study investigated the association of N-glycosylated G protein at Asn\textsuperscript{204} 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) kindly provided by Dr. C.
E. Rupprecht (former Poxvirus and Rabies Branch, DHCPP, NCEZID, CDCP, Atlanta, GA, USA) were used in this study. Viruses were propagated in mouse neuroblastoma (MNA) cells at 37°C in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and stored at -80°C until use (24).

**Viral titration:** Virus titration was performed by 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 the fluorescein isothiocyanate (FITC)-conjugated anti-RABV N monoclonal antibody (Fujirebio Diagnostics, Inc., Malvern, PA, USA) diluted 1:100 in PBS(-) with 0.002% Evans blue 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 by using an RNeasy Mini kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer’s protocol. Reverse transcriptase (RTase) reactions with Random
Primer (Promega Corporation, Madison, WI, USA) were carried out at 42°C for 45 min by AMV Reverse Transcriptase (HC) (Promega). Each G gene was amplified from the corresponding cDNA by PCR with Platinum Taq High Fidelity (Invitrogen, Life Technologies Inc., MD, USA) with the following primer sets for the Kyoto G gene:

RABVK/Y-G-F (+) 5’-CGCGAATTCACCATGATTCCTCAGGCTCTT-3’ (EcoR I site and start codon underlined) and RABVK/Y-G-R (+)

5’-TATGCGGCCGCTTACAGCTTGGTCTCACCT-3’ (-) (stop codon and Not I site underlined), and for the CVS-26 G gene: RABV-CVS26G-F (+)

5’-CGCGAATTCACCATGTTTCCTCAGGCTCT-3’ (-) (EcoR I site and start codon underlined) and RABV-CVS26G-R (+)

5’-TATGCGGCCGCTCACAGTCTGATCTCACCT-3’ (-) (stop codon and Not I site underlined). PCR amplification was carried out 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 target. The amplified DNA fragments were inserted into the multiple cloning site of the pcDNA3.1/Zeo vector (Invirogen). The pcDNA3.1/Zeo vectors inserted in the Kyoto G gene and CVS-26 G gene were named pzKyoto-G and pzC26-G, respectively. Both of the G proteins were expressed under the control of CMV promotor and T7 promotor.
Mutation of RABV G protein at the site of 204 related to the N-glycosylation:

Mutation of the nucleotide was developed by a PrimeSTAR mutagenesis kit (TAKARA Bio Inc., Japan) according to the manufacturer’s protocol with the following primer sets for developing the sequon at Asn\(^{204}\) into Kyoto G protein by Kyoto-G(S204N)-F (+)

5’-GGGAACAAGACTTGTGGATTTGTAGATGAG-3’ (-) and Kyoto-G(S204N)-R (+)

5’-ACAAGTCTTTGGCCTTTTGGATGCTCTCTT-3’ (-), and the mutated sequon at Asn\(^{204}\) of CVS-26 G protein by CVS26-G(N204S)-F (+)

5’-GGGAGTAAGACCTGCGGCTTTGTGGATGAA-3’ (-) and CVS26-G(N204S)-R (+) 5’-GCAGGTCTTACTCCCGTTGGATGCTCTCTT-3’ (-). Mutated nucleotides are underlined. A plasmid pzKyoto-G that was mutated for additional N-glycosylation at Ser\(^{204}\) was named plasmid pzKyoto-mG expressing Kyoto rG (S204N). In contrast, a plasmid pzC26-G that was mutated to block N-glycosylation at Asn\(^{204}\) was named plasmid pzC26-mG expressing 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 GmbH.

Germany). MNA cells (50-80% confluent) were infected by the virus at a multiplicity of infection (MOI) = 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 Industries, Ltd. Japan) at r.t. for 30 min. In contrast, 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 use of mouse monoclonal anti-RABV G antibody #7-1-9 (0.4 μg/ml) (25, 26), respectively. Visualization of the primary antibody was performed by 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 Corporation, Tokyo, Japan).

**SDS-PAGE and Western blotting:** MNA cells grown in 6-well TPP tissue culture plates (Sigma-Aldrich Co. LLC., St. Louis, 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% [vol/vol] Tween-20, and 1% [vol/vol] Triton-X 100) at 4°C for 1 h according to the method described previously (27). A volume of 5 µl precipitated proteins was 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 (wt/vol) in PBS. The detection of G protein was performed by 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, WI, USA). The signals were detected with VersaDoc (Bio-Rad Laboratories, Inc., CA, USA). As a control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected by monoclonal mouse anti-rabbit GAPDH IgG (HyTest Ltd. 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., WI, USA) according to the manufacturer’s instructions, N-glycosylation of G
protein was blocked by 1 μg/ml of tunicamycin (Sigma) for 44 h and processed for Western blotting (12, 13). The cleavage of N-linked glycan was also performed by N-Glycosidase F (PNGase F) (Promega) according to the manufacturer’s instructions.

**RESULTS**

**Localization of G and rG protein 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 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 the 204th amino acid residue was not found in the Kyoto rG protein but was present in 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 sequon at Asn204:** Kyoto G gene with the sequon at Asn204 produced an additional larger size of Kyoto rG protein. However, most of the CVS-26 rG proteins translated from the CVS-26 G gene mutated the sequon at Asn204 resulting in a decrease in the size of rG proteins on SDS-PAGE (Fig. 5).
proteins with the sequon at Asn$^{204}$ was the same as that of CVS-26 rG protein, and
CVS-26 rG protein blocked N-glycosylation by the mutation of the sequon at Asn$^{204}$ 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 at Asn$^{204}$ 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). Contrastingly, localization of CVS-26 rG(N204S) with mutation of the sequon at Asn$^{204}$ was still observed at the cell membrane as with the CVS-26 rG proteins, but CVS-26 rG(N204S) proteins were more distributed around the perinuclear space than were CVS-26 rG proteins (Fig. 6B).

**DISCUSSION**

RABV G protein has important roles in viral pathogenesis such as infectivity, intracellular propagation, transportation of virus 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 at Asn$^{37}$ and Asn$^{319}$, whereas fixed viruses have three or four potential glycosylation sites on the G protein at Asn$^{37}$, Asn$^{158}$, Asn$^{204}$, Asn$^{247}$ and Asn$^{319}$ (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 by mouse brains in the US CDC. N-glycosylation of Kyoto G protein was classified into Type S1, which is a major pattern in street strains. CVS-26 G protein was classified into Type F1 with additional N-glycosylation at the sequon Asn$^{204}$-Thr$^{206}$.

Viral 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 ultrastructural studies showing different budding features of street viruses at intracellular membranes and of fixed viruses from 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 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 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 was reported to completely block the surface expression of G proteins produced by the eukaryotic shuttle vector pSG5-inserted G gene of the ERA strain (fixed virus), 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 Asn\textsuperscript{37}, Asn\textsuperscript{247} and Asn\textsuperscript{319} in ERA G proteins also completely blocked cell surface expression in CHO cells (30). In the present study, CVS-26 rG proteins localized around 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 Asn\textsuperscript{37}, Asn\textsuperscript{204} and Asn\textsuperscript{319}, but the Kyoto strain had only two, Asn\textsuperscript{37} and Asn\textsuperscript{319} (Fig. 1). These results suggested that the transportation of CVS-26 G proteins to the cell surface membrane was associated with N-glycosylation at Asn\textsuperscript{204}. 
The role of N-glycosylation at Asn\textsuperscript{204} in the Kyoto G and CVS-26 G proteins was also examined by using the Kyoto rG(S204N)-constructed sequon at Asn\textsuperscript{204}, and CVS-26 rG(N204S) blocked N-glycosylation by the mutation of the sequon at Asn\textsuperscript{204} (Fig. 5). Kyoto rG(S204N) proteins were localized to the cell surface membrane by N-glycosylation of the sequon at Asn\textsuperscript{204} (Fig. 6A), whereas fully N-glycosylated CVS-26 rG proteins were obviously observed around the cell surface membrane, but rCVS-26 (N204S) proteins were more distributed around the perinuclear space than were rCVS-26 G proteins (Fig. 6B). These results demonstrated that N-glycosylation of G protein in the sequon at Asn\textsuperscript{204} induced the transfer of RABV G proteins to the cell surface membrane. The distribution of CVS-26rG(N204S) in the cell membrane could be acquired by highly sialylated N-glycans and additional changes in the primary structure during the evolution of fixed virus CVS-26 (13, 29, 30). N-glycosylation at Asn\textsuperscript{204} 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. 6A). N-glycosylation of rG protein of the 1088 strain (street virus) was also not efficient with the additional sequon at Asn\textsuperscript{204} (13).

N-glycosylation of street RABV Kyoto G protein with the inserted sequon (Asn\textsuperscript{204}-X\textsuperscript{205}-Thr\textsuperscript{206}) 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 different intracellular localization of RABV G proteins between the Kyoto (street virus) and CVS26 (fixed virus) strains was associated with N-glycosylation of RABV G proteins at the sequon Asn^{204}. The number of N-glycosylation sites in G protein may be one 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). Fixed viruses, CVS-24 and CVS-11, with G protein of Type F1, have neuroinvasive characteristics 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). Overexpression of G protein of fixed virus elevates apoptogenic activity and enhances antibody response (34).

Whether RABV buds from the plasma membrane depends not only on G protein but also other structural proteins such as M protein. However, different 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 Asn^{204} 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.
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Figure legends:

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

Fig. 2. 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 and CVS-26 strain were fixed after 72 h and 120 h (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.

Fig. 3. Molecular size of Kyoto and CVS-26 rG proteins expressed in MNA cells. MNA cells were cultured for 48 h after transfection of 2 μg/well pzKyoto-G or pzC26-G. After 44 h of treatment with tunicamycin (A) or cleavage of N-linked glycan of rG proteins by PNGase F at 37°C for 3 h (B). Molecular sizes of rG proteins extracted from one well of a 6-well TPP tissue culture plate were analyzed by SDS-PAGE and Western
Fig. 4. Localization of rG proteins in MNA cells. MNA cells were transfected with 0.5 μl of pzKyoto-G and pzC26-G into each compartment of CELLview cell culture dishes. rG proteins of the Kyoto strain and CVS-26 strain were expressed under the presence of 1 μg/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 with anti-RABV G antibody #7-1-9, and FITC-labelled anti-mouse IgG antibody. Nuclei were visualized by DAPI staining. Immunofluorescence was analyzed by confocal laser scanning microscopy. All experiments were independently performed three times.

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 μg/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 by anti-rabbit GAPDH mouse mAb 5G4. All experiments were independently performed three times.

Fig. 6. 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 μl pzKyoto-G, pzKyoto-G(S204N), pzC26-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.
| RABV Type | Positions of N-glycosylation | RABVs examined in this study | RABV strains¹ |
|-----------|-----------------------------|-----------------------------|---------------|
| S1        | NH₂ 37 ▼ 319 ▼ COOH         | 100                         | Kyoto (22) WH5 (DQ849061) 1088 (AB645847) etc. |
| Street virus | S2                          | 17                          | MO1197 (KF484541) BR-NL2 (AB383172) SHBRV (U52946) etc. |
|           | 247 ▼                        | 3                           | A11_30558 (JX871881) A12_1050 (KC7920) GA31940 (JQ595325) |
| S3        |                             |                             |               |
| F1        | 204 ▼                        | 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) |
|           | 158 ▼                        | 2                           | LEP-Flury (GU565703) HEP-Flury (AB085828) |

1: RABV strains (Accession number or reference)
▼: Indicates the putative N-glycosylation site
Fig. 2

A

|       | Kyoto strain | CVS-26 strain |
|-------|--------------|---------------|
| 72h   |              |               |
| 120h  |              |               |

Membrane permeabilization

(-)

(+)

B

|       | Kyoto rG protein | CVS-26 rG protein |
|-------|------------------|------------------|
| (-)   |                  |                  |
| (+)   |                  |                  |
Fig. 3

A

|                | Kyoto rG | CVS-26 rG |
|----------------|----------|-----------|
| Tunicamycin    |          |           |
| -              | +        | -         |
| +              |          | +         |

| kDa | 64 - | 51 - | 39 - |
|-----|------|------|------|
| rG protein |    |      |      |
| GAPDH      |    |      |      |

B

|                | Kyoto rG | CVS-26 rG |
|----------------|----------|-----------|
| PNGase F       |          |           |
| -              | +        | -         |
| +              |          | +         |

| kDa | 64 - | 51 - | 39 - |
|-----|------|------|------|
| rG protein |    |      |      |
| GAPDH      |    |      |      |
Fig. 4

Kyoto rG protein
CVS-26 rG protein

Tunicamycin treatment

(-)

(+)

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Fig. 5

- N-glycosylation site at the site of 204

| kDa | Kyoto G | Kyoto G (S204N) | CVS-28 G | CVS-28 G (N204S) |
|-----|---------|----------------|----------|------------------|
| 64  | -       | +              | +        |                  |
| 51  |         |                |          |                  |
| 39  |         |                |          |                  |

- rG protein
- GAPDH
| Membrane permeabilization | (-) | (+) |
|---------------------------|-----|-----|
| **A**                     |     |     |
| Kyoto rG                  | ![Image](image1.png) | ![Image](image2.png) |
| *Kyoto rG (S204N)*       | ![Image](image3.png) | ![Image](image4.png) |
| **B**                     |     |     |
| CVS-26 rG                 | ![Image](image5.png) | ![Image](image6.png) |
| *CVS-26 rG (N204S)*      | ![Image](image7.png) | ![Image](image8.png) |