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Secretion of Truncated Recombinant Rabies Virus Glycoprotein with Preserved Antigenic Properties Using a Co-Expression System in *Hansenula polymorpha*[§]

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Rabies virus infection remains a serious public health threat in the developing world, where cost-concerns make wide-scale public health interventions impractical. The development of novel and inexpensive ELISA diagnostic antigens is critical in early detection and prevention of complications. The transmembrane glycoprotein (G) of rabies virus (RV) contains an external domain capable of inducing the synthesis of anti-rabies, virus-neutralizing antibodies, in infected or immunized hosts. In our study, the external G domain was synthesized and fused in-frame with a polyhistidine-tag coding sequence present in the expression plasmid. Soluble truncated recombinant G was secreted in *Hansenula polymorpha* (*H. polymorpha*) using *H. polymorpha*-derived calnexin (HpCNE1) overproduction and found to be correctly N-glycosylated. The truncated recombinant G was purified from cell culture supernatant by Ni-agarose affinity chromatography and when compared with the full-length glycoprotein, found to be similarly immunogenic in vaccinated rabbits. These results subsequently led us to explore the potential of truncated recombinant G as a diagnostic antigen in ELISA. Our results show that the truncated recombinant G can detect antibodies directed to both whole virion and native glycoprotein. More sophisticated applications of truncated recombinant G would profit from the correctly N-glycosylated and soluble monomer.

Keywords: Rabies virus, glycoprotein, secretion, *Hansenula polymorpha*

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Introduction

Rabies virus (RV) is a negative-stranded RNA virus that can induce acute encephalitis in mammals (Warrell and Warrell, 2004). Rabies virus glycoprotein (G), a type I transmembrane protein, consists of a cytoplasmic domain, a transmembrane domain, and an external domain exposed as trimers on the surface of the mature virus particle (Rath *et al.*, 2005). Interestingly, the external domain alone has been shown to induce the production of neutralizing antibodies and thereby afford complete protection against RV challenge (Perrin *et al.*, 1985). Although effective pre- and post-exposure treatments exist, rabies remains a serious public health concern in many developing countries due to cost as well as the proximity in which humans and wildlife coexist (Gongal and Wright, 2011). Thus, to reduce the threat of rabies, there is a strong demand for additional inexpensive and potent diagnostic agents for routine detection of potency of rabies vaccines and quantification of anti-glycoprotein antibodies in wild animals, stray dogs, and immunized hosts. The synthesis of a standardized truncated recombinant G used as a coating antigen in ELISAs would provide an attractive alternative as it could be produced on a large scale affordably and safely.

Full-length G has been expressed in insect cells (Prehaud *et al.*, 1989), *Escherichia coli* (Yelverton *et al.*, 1983), plants (Ashraf *et al.*, 2005), and in *Saccharomyces cerevisiae* (Klepfer *et al.*, 1993), albeit with varying degrees of success. For example, an abnormally folded, full-length version of *S. cerevisiae*-derived N-glycosylated G was found to confer intramuscular protection but not intracerebral (i.c.) protection to rabies challenge. In addition, the recombinant protein's preferential association with the yeast cell membrane raised concerns that protein purification might be hindered (Klepfer *et al.*, 1993). Subsequent studies found that a soluble form of truncated G lacking its native transmembrane and cytoplasmic domains was sufficiently antigenic and immunogenic in mammalian-cell expression systems (Gupta *et al.*, 2005). Despite these findings, there have not been any reports to date about the biological efficacy of truncated recombinant G protein in yeast expression systems with preserved N-glycosylation. Given the cost-effectiveness, scalability, and reduced risk of contamination of yeast expression systems (Gellissen *et al.*, 2005), understanding the efficacy of properly N-glycosylated truncated recombinant G is of considerable importance. In our study, we chose the *Hansenula polymorpha* expression system for production and biological evaluation of truncated G since it offers several advantages

over other yeast expression systems.

H. polymorpha is a methylotrophic yeast that has been a preferred organism for the production of eukaryotic proteins with clinical and therapeutic value on an industrial scale due to its fast growth cycle in simple defined media (Gellissen *et al*., 2005). In addition, like methylotrophic *Pichia pastoris*, the glycosylation of *H. polymorha* is less hypermannosylated than the hyperglycosylation of *S. cerevisiae,* with a heterogeneous addition of 50–150 mannose residues to the glycosylation core (Gellissen *et al*., 2005). Characterization of other recombinant glycoproteins expressed in *H. polumorpha* have found preserved glycosylation as well as proper protein folding, presumably due to its endoplasmic reticulum (ER) quality control system (Ellgaard *et al*., 1999). Notable members of this system are the lectin chaperone calnexin and the Hsp70-like chaperone BiP. The modulation of chaperone levels has proven effective in assisting the correct folding and secretion of heterologous proteins in yeast expression systems (Damasceno *et al*., 2007; Klabunde *et al*., 2007).

In this study, we report the secretory expression of truncated recombinant G by over-expression of *H. polymorpha*-derived CNE1 (HpCne1) in *H. polymorpha* and provide another engineering strategy for efficient secretion of heterologous glycoproteins in *H. polymorpha*. In addition, the immunogenic and antigenic property of truncated recombinant G was investigated. The data suggest that the truncated protein has potential for the development of rapid immunoassays for detection and quantification of rabies virus G protein-pecific antibodies in immunized hosts. This information can also contribute to further studies on the effects of glycosylation level on G activity.

Materials and Methods

Strains, plasmid, and chemicals

H. polymorpha DL-1 (ATCC26012) was purchased from ATCC, and served as the standard host for protein expression. *E. coli* DH5α, kept in our laboratory, was used for propagation of recombinant vectors.

pHMOXG-alpha-A, a G418-selectable plasmid, contains a methanol oxidase (MOX) promoter from *H. polymorpha* fused to the *S. cerevisiae*-derived α-mating factor, which guides the recombinant protein through the secretory pathway. pHFMDG-A, a G418-selectable plasmid, contains a formate dehydrogenase (FMD) promoter from *H. polymorpha*. The two plasmids were constructed in our laboratory (Song *et al*., 2003) and used for cloning and expression in *H. polymorpha*. pMD18-RG containing the extracellular domain of RVG was kindly provided by Dr. Wenjun Liu (Institute of Microbiology, Chinese Academy of ScieInstitute of Microbiology, Chinese Academy of Sciences, Beijing, China).

Ni-NTA agarose resin was purchased from Bio-Rad (USA). Taq DNA polymerase and endonucleases were purchased from TaKaRa Biotechnology Co. Ltd. (China). Protein concentrations were quantitated by the Bradford method using reagents supplied by Bio-Rad. All other chemicals were of analytical grade and obtained from local commercial resources (China).

Native viral glycoprotein from purified virions was detergent-extracted and purified using a protocol previously described by Gaudin *et al.* (1992). The anti-rabies-virus glycoprotein polyclonal antibody from guinea pigs was kindly provided by Dr. Guoqiang Zhang (Institute of Biological Products, China).

Media and culturing

H. polymorpha was cultivated in YPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose. The transformants were grown at 37°C in YPD selective medium supplemented with 300 μg/ml G418. For protein expression, transformants were cultured in YPD medium to generate biomass and YPG medium containing 2% (w/v) glycerol for induction.

Construction of plasmid

The truncated G gene, lacking its native signal peptide sequences, was synthesized by PCR using primers RG-F (5′-CGGAATTCATGAAGTTCCCCATTTACACGAT-3′, the *Eco*RI site is underlined) and RG-R (5′-ATAAGAATGCGGCCGCTGTTTGTACACATCGGGGAG-3′, the *Not*I site is underlined) from pMD18-RG. The resulting truncated G fragment was cut by *Eco*RI/*Not*I, cloned into pHMOXG-α-A, and digested by the same restriction enzymes to construct pHMOXG-alpha-RG-A. Similarly, the HpCNE1 fragment was amplified from *H. polymorpha* genomic DNA using primers HpCne1-F (5′-CATGTCATGAAAGTCAGCCGTCCAATC-3′, the *Xba*I site is underlined) and HpCne1-R (5′-ATAAGAATGCGGCCGCCTATCCTCTCTTGACAGCTTCT-3′, the *Not*I site is underlined) and cut by *Not*I/*Xba*I, and then ligated with the same enzyme digested pHFMDG-A to generate pHFMDG-HpCne1. Finally, the truncated G and HpCne1 co-expression plasmid pHFMDG-HpCne1-MRG was constructed by inserting the G expression cassette from pHMOXG-alpha-RG-A cut by *Bgl*II/*Bam*HI into the *Bam*HI site of plasmid pHFMDG-HpCne1.

Transformation of yeast

H. polymorpha was transformed by electroporation as described by Faber *et al.* (1994) with minor modifications. For gene expression, DraI-linearized pHMOXG-alpha-RG-A and KpnI-linearized pHFMDG-HpCne1-MRG were each used to transform *H. polymorpha* competent cells. FMD-F (5′-TCTCTCAGAGGGGGGAATG-3′) and HpCne1-R and MOX-F (5′-ATCACAGATGGGGTCAGCG-3′) and RG-R were used to confirm whether or not the respective target genes were integrated into the genome of *H. polymorpha*.

Secretory expression of truncated recombinant G in shake flasks

After transformants were cultured in 2 ml YPD liquid selective medium, supplemented with 300 μg/ml G418, for 12 h at 37°C, the cells were collected by low-speed centrifugation (2,000×g, 15 min) and resuspended with 30 ml of YPD medium in a 250 ml flask at 37°C for a 24 h incubation. The cells were then harvested and resuspended in 500 ml of YPD medium in a 2-L flask, and adjusted to an initial OD_{600} of approximately 1.0. For induction, glycerol (100%) was added

every 12 h to a final concentration of 2% (v/v) throughout the induction phase. After induction for 72 h, the supernatants of the culture containing truncated G were harvested by centrifugation (4,000×g, 15 min). To detect the presence of RVG, 50 µl of culture supernatant were used directly for ELISA analysis. The purified native rabies virus G was used as a standard.

Purification of soluble, truncated, recombinant G from yeast culture supernatant

The soluble, truncated, recombinant G in the yeast culture supernatant was purified under native conditions using Ni-NTA agarose resin (BD Biosciences Clontech). Briefly, the supernatant was concentrated and dialyzed against the binding buffer (50 mM $Na_2HPO_4{\cdot}12H_2O$, 300 mM NaCl, pH 7.5) by tangential flow filtration using a 5,000 MWCO PES membrane (Vivaflow 200, Sartorius Stedim Biotech, Germany) and then loaded onto Ni-NTA resin equilibrated with the same buffer. The bound proteins were eluted using a gravity-flow column starting with extraction/wash buffer (20 mM $Na_2HPO_4{\cdot}12H_2O$, 20 mM $NaH_2PO_4{\cdot}H_2O$, 300 mM NaCl, and 20 mM imidazole, pH 7.5) to remove any loosely associated proteins and finishing with the same buffer containing 150 mM imidazole. Fractions were pooled and then desalted with a desalting column (Sephadex G-25, Amersham Pharmacia) in Tris/HCl buffer (20 mM Tris, pH 7.5) at 4 ml/min. Protein content was quantified via Bradford assay using BSA as a standard (Bradford, 1976).

SDS-PAGE and Western blotting analysis

For SDS-PAGE, the sample of eluted protein was mixed with 5×reducing or non-reducing sample buffer and separated by 12% SDS-PAGE gel. The gel was stained with Coomassie blue R-250. The purity of protein preparations was evaluated by coomassie- and silver-stained 12% SDS-PAGE by standard methodology (Laemmli, 1970; Morrissey, 1981). For Western blotting, after the gel was equilibrated in transfer buffer [25 mM Tris, 192 mM glycine and 20% (v/v) methanol] for 5 min, the proteins present in the gel were transferred onto Hybond-P PVDF membranes (Amersham Pharmacia) using the Mini Trans-Blot cell (Bio-Rad Laboratories, USA). The membrane was blocked with 2.5% casein in PBST (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0.1% Tween-20) at room temperature for 2 h followed by three washes with PBST. The membrane was then incubated with mouse-derived polyclonal antibody raised against the truncated recombinant G (diluted 1:10000 in PBST with 1% BSA) at room temperature for 1 h. After three consecutive washes with PBS containing 0.1% Tween 20, the membrane was incubated with HRP-labeled goat anti-mouse IgG (diluted 1:4000 in PBST with 1% BSA) at room temperature for 1 h followed by three washes. Finally, the membrane was developed with ECL Western blotting reagent (Amersham Biosciences). The results were further analyzed with Bandscan 5.0 software.

Deglycosylation analysis

The purified, truncated, recombinant G were digested with PNGaseF (New England Biolabs) following the manufacturer's instructions. Following digestion with PNGaseF, the protein was precipitated using acetone and subjected to SDS-PAGE analysis. As a control, the same procedure was performed in the absence of PNGaseF.

Immunogenic property of truncated recombinant G

The immunogenic properties of truncated recombinant G were determined according to the protocol reported by Gupta *et al.* (2005). Briefly, two rabbits each were immunized (100 µg) with truncated recombinant G on days 0 and 21 by subcutaneous injection. Then the sera were collected and pooled 7 days after final vaccination, and applied to examine the reactivity with inactivated purified RV using Western blotting. The purified native G was solubilized under reducing conditions with 2-merceptoethanol-treatment or non-reducing conditions without 2-merceptoethanol-treatment and both preparations were then separated by 12% SDS-PAGE, respectively, and transferred onto nitrocellulose. The separated viral proteins were investigated with rabbit-derived immune sera induced by the truncated recombinant G as described above.

Antigenic property of soluble truncated recombinant G as diagnostic antigen in ELISA

To explore the potential use of recombinant truncated G as an ELISA diagnostic antigen, we first developed an ELISA method where the wells of the plate were coated with truncated recombinant G to detect anti-rabies antibodies in serum samples. Inactivated purified whole virus as coated antigen was used as the positive control. Additionally, sera from healthy rabbits were included as a negative control. Briefly, the 96-well microtiter plates were coated with 50 µl of 0.05 M carbonate-bicarbonate buffer (pH 9.6) containing either soluble truncated recombinant G (2 µg/well) or inactivated purified rabies virus (1 µg/well). After overnight incubation at 4°C, the coated plates were blocked with 5% BSA in PBST (PBS-0.05% Tween 20) at 37°C for 1 h. Following three washes with PBST, 50 µl of serum samples (two-fold serial dilution in PBST) were added in duplicate wells and incubated at 37°C for 1 h. Then plates were washed as above and 100 µl of goat anti-rabbit (1: 4000) antibodies conjugated with horseradish peroxidase (Sigma) were added to each well at 37°C for a further 1 h. Subsequently, the plates were washed before loading 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) solution. Finally, absorbance was quantitated by measuring absorbance at 492 nm after stopping the reaction with 5 N H_2SO_4. Moreover, the antibody titre was the reciprocal of the serum dilution at which the mean absorbance ratio of the sample versus the control was ≥2.

To further investigate the sensitivity and specificity of the truncated recombinant G as coating antigen, 56 serum samples from dogs were analyzed (Sauerbrei and Wutzler, 2006). To this end, the sera were graded as positive or negative according to the methods described by Ishikawa *et al.* (1989). Sensitivity was determined by calculating the ratio of positive samples to actual positive samples. Specificity was calculated as the proportion of negative samples among all negative samples. Finally, the diagnostic sensitivity and specificity of the truncated recombinant G was calculated

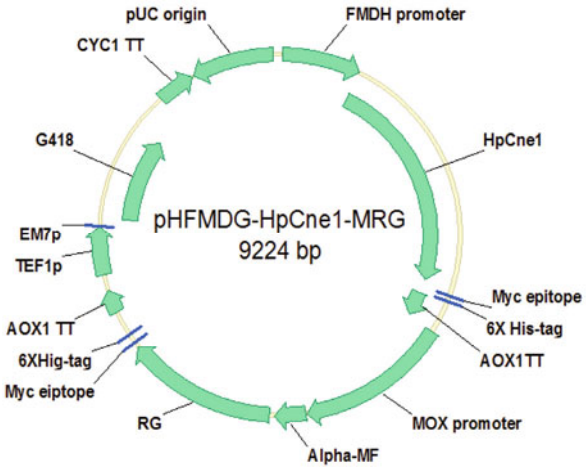

Fig. 1. Physical map of the plasmid pHFMDG-HpCne1-MRG. This plasmid consists of a trucated rabies-glycoprotien expression cassette and an *H. polymorpha*-derived CNE1 gene expression cassette.

by comparing the ELISA titre with virus neutralizing titre following previously described methods (Singh *et al*., 2004).

Results

Construction of expression vector

As shown in Supplementary data Figs. S1 and S2, the sequence encoding the truncated recombinant G fragment corresponding to amino acid residues 20–445 (extracellular domain of full-length G) was amplified by PCR using pMD18-RG as the template. The resulting 1,269 bp DNA fragment was digested with *Eco*RI and *Not*I restriction enzymes, and then inserted into the expression plasmid pHMOXG-α-A, yielding the recombinant plasmid pHMOXG-alpha-RG-A. The fragment was cloned upstream of, and in-frame with, a 306 bp coding sequence composed of a C-terminal polyhistidine tag and myc epitope, generating a 1,575 bp chimeric DNA fragment. Similarly, the 1674 bp *H. polymorpha*-derived calnexin fragment was amplified using *H. polymorpha* genomic DNA as the template, then cut by *Not*I/*Xba*I, and ligated with pHFMDG digested by the same enzyme. The construct was designated as pHFMDG-HpCne1. Finally, as shown in Fig. 1 and Supplementary data Fig. S3, to generate the co-expression plasmid pHFMDG-HpCne1-MRG of truncated G

Table 1. Purification protocol of truncated recombinant G in shake flask culture

Fraction[a]	Yield[b]	Recovery (%)
I	14.6	100
II	8.3	56.8
III	3.7	44.5

[a] I, total protein content before purification; II, the truncated recombinant G bound to the resin after wash steps; III, the truncated, recombinant G eluted with 150 imidazole.
[b] Total truncated recombinant G in the 1 L yeast cell culture supernatant was estimated with a standard curve based on known amounts of native rabies-virus proteins by quantitative ELISA. The truncated recombinant G content, purified from a Ni-NTA resin column, was determined quantitatively by the Bradford Method.

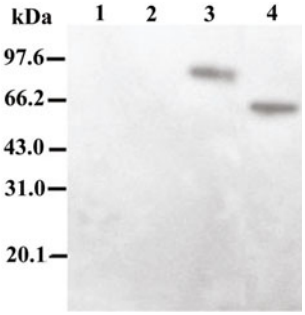

Fig. 2. Expression of soluble, truncated, recombinant G by Western blot analysis. Lanes: 1, the supernatants of yeast transformed with the plasmid pHMOXG-α-A without any insert; 2, the culture supernatant of *H. polymorpha*/RVG transformed with the plasmid pHMOXG-alpha-RG-A; 3, the supernants of *H. polymorpha* RVG/HpCne1 transformed with the plasmid pHFMDG-HpCne1-MRG; 4, the native rabies virus G protein.

and chaperone CNE1, the truncated G expression cassette from pHMOXG-alpha-RG-A cut by *Bgl*II/*Bam*HI was inserted into the *Bam*HI site of plasmid pHFMDG-HpCne1.

Secretion and purification of truncated G by overproduction of HpCNE1

To achieve secretory expression of truncated recombinant G, the *H. polymorpha* competent cells were transformed with the linearized pHMOXG-alpha-RG-A. The resultant positive clones designated *H. polymorpha*/RVG/HpCne1, along with the clones of *H. polymorpha*/RVG only transformed with linearized pHMOXG-alpha-RG-A, were grown in a shake-flask to compare their expression levels. As is shown in Fig. 2, overproduction of HpCNE1 enabled the secretion of truncated G, whereas soluble truncated G was not detected in the supernatants of *H. polymorpha*/RVG. These results showed that overproduction of HpCNE1 might help proteins efficiently fold into the correct three-dimensional structure, thereby reducing intracellular protein degradation due to incorrect folding of proteins and enabling the secretion of a protein of interest. Moreover, the expression of the truncated recombinant G at relatively high levels in

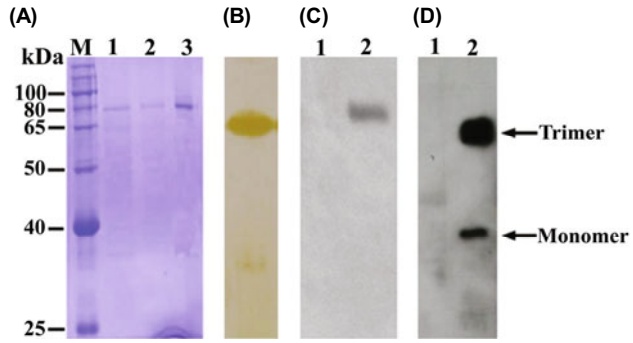

Fig. 3. Purification and analysis of the soluble, truncated, recombinant G. (a) Coomassie-(A) or silver-stained (B) SDS-PAGE of truncated G purified by Ni^{2+}-affinity chromatography. A, lanes: 1, molecular mass markers; 2–4, proteins eluted with 20, 50, 150 mM imidazole, respectively. B, silver-stained SDS-PAGE gel of proteins eluted with 150 mM imidazole. (b) Western blot analysis of truncated G under reducing (C) and non-reducing conditions (D). Lane 1 in both C and D, the culture supernatant of *H. polymorpha*/RVG transformed with the plasmid pHMOXG-alpha-RG-A; lane 2 in both C and D, the pooled fractions eluted with 150mM imidazole of the bound material., After the purified, truncated, recombinant G (25 ng/lane) proteins were subjected to separation under reducing or non-reducing conditions, they were subjected to Western blot analysis. The arrow shows the two bands corresponding to trimer and monomer of purified, truncated recombinant G.

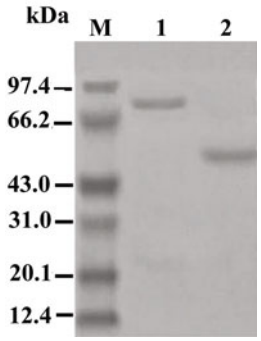

Fig. 4. Deglycosylation analysis of truncated recombinant G. 10 μg of RNase B were denatured with 1×glycoprotein denaturing buffer at 100°C for 15 min. After the sample was cooled, the purified, truncated, recombinant G, the manufacturer supplied G7 reaction buffer and the NP-40 [final concentration 1% (v/v)] were added. Subsequently, the sample was digested with or without PNGaseF at 37°C for 24 h. Finally, the sample was harvested and subjected to SDS-PAGE analysis. Lane 1–2, the truncated, recombinant G digested without or with PNGaseF, respectively.

yeast is evident from Fig. 2, where the expected positive band can be seen even in a crude protein preparation from culture supernatants.

To facilitate the purification of soluble, truncated, recombinant G, an effective restriction-ligation cloning approach was employed. Because the truncated G DNA fragment was in frame with the C-termini 6×His tag coding sequence present on the pHMOXG-α-A, an efficient one-step purification was made possible with high stringency washing via Ni-NTA affinity chromatography. As is shown in Fig. 3A, the resin-bound truncated recombinant G was eluted with 150 mM imidazole. The purity of the preparation was estimated by Coomassie- and silver-stained SDS-PAGE to be over 93% (Figs. 3A and 3B). The yield of secreted truncated G reached approximately 14.6 mg/L culture in the *H. polymorpha*/RVG/HpCne1 clone as determined by quantitative ELISA (data not shown). Table 1 summarizes the purification scheme of the recombinant proteins.

Characterization of truncated recombinant G

To analyze the molecular weights of soluble truncated recombinant G, purified samples from the culture supernatant of *H. polymorpha*/RVG/HpCne1 were subjected to SDS-PAGE and Western blotting. Fig. 3 shows that *H. polymorpha* expressed the recombinant protein at 74.6 kDa, which was larger than predicted theoretically (http://www.cbio.psu.edu) for the non-glycosylated truncated G protein encoded by the synthetic gene constructed in this study. Here, the synthetic gene consisted of the extramembrane domain of glycoprotein (predicted to be a 49 kDa protein) and polyhistidine fusion at the C-terminal end of this protein including 101 amino acids (predicted to be a 10 kDa protein). To investigate the glycosylation status of the truncated recombinant G, we employed the PNGaseF assay described above. The results indicate that glycosylation is responsible for the observed size difference between the digested and undigested truncated G (Fig. 4). In other words, the results indicate that the truncated recombinant G is indeed N-glycosylated. The size of the glycosylated protein, together with the fact that it appears as a distinct band, suggests that it contains a typical yeast-modified core oligosaccharide without fully extended outer chains.

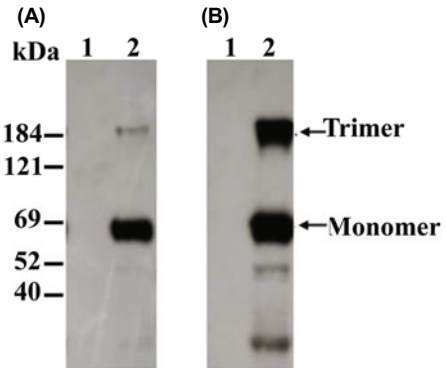

Fig. 5. The reactivity of polyclonal antibodies induced by the truncated, recombinant G under reducing (A) and non-reducing conditions (B). After the purified native rabies virus proteins (25 ng/lane) were subjected to separation under reducing or non-reducing conditions, the proteins were subjected to Western blot analysis. Lane 1 in both (A) and (B), the sera from healthy rabbits unimmunized with truncated, recombinant G; lane 2 in both (A) and (B), anti-recombinant-soluble-glycoprotein-specific, polyclonal antibodies raised in rabbits immunized with truncated recombinant G. The arrow shows the two bands corresponding to trimer and monomer of purified rabies virus proteins.

Immunogenic property of truncated recombinant G

To investigate the immunogenic property of recombinant soluble glycoprotein, the sera pooled from rabbits immunized with purified, truncated, recombinant G was subjected to Western blotting analysis. The results show that the sera had significant reactivity to the monomer form of native rabies viral glycoprotein under reducing conditions (Fig. 5A, lane 2) as well as the monomer and trimer forms under non-reducing condition (Fig. 5B, lane 2). This indicates the antigenic similarity of soluble truncated recombinant G to native viral glycoprotein, as monomer and trimer forms of rabies viral glycoprotein have been reported (Doms *et al.*, 1993).

Antigenic property of soluble truncated recombinant G as diagnostic antigen in ELISA

The immunoreactivities of *H. polymorpha*-expressed truncated recombinant G were examined in an ELISA format using rabies-positive dog serum samples, in which equal molar amounts of the truncated recombinant G were coated onto ELISA plate wells. Fig. 6 shows that the soluble, truncated,

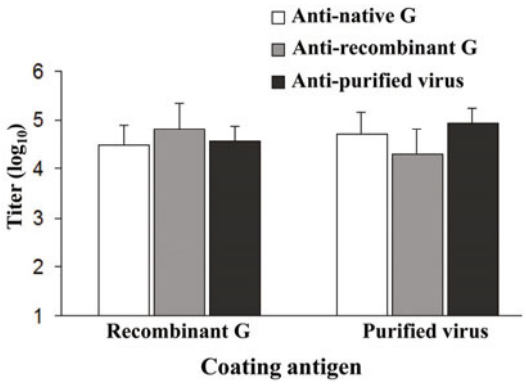

Fig. 6. Characterization of *H. polymorpha*-expressed truncated G protein reactivity with anti-recombinant G, anti-native glycoprotein, and anti-virus sera in ELISA. The purified virion was used in parallel as the control. The results are expressed as the means±standard deviation from three representative independent experiments.

recombinant G successfully detected antibodies against rabies virus in serum samples. The ELISA titre of anti-recombinant G sera was higher than that of anti-native glycoprotein and anti-purified virus sera, indicating that the truncated recombinant G could detect anti-glycoprotein antibodies in sera induced by either purified virus or native glycoprotein. However, anti-recombinant G gave a lower antibody titre than sera against either purified virus or native glycoprotein. Moreover, the diagnostic sensitivity and specificity of the truncated recombinant G as an ELISA coating antigen was found to be 82.1% (32/39) and 88.2% (15/17), respectively. The Sen/Spe index was found to be 0.93.

Discussion

The methylotrophic yeast *H. polymorpha* expression system preserves a glycoprotein's native conformation, including posttranslational modifications such as N-glycosylation. Thus, this system represents an attractive method for the production of glycoproteins. Although several laboratories have reported expression of full-length G in *S. cerevisiae* (Klepfer *et al*., 1993; Sakamoto *et al*., 1999), most of these full-length G protein molecules were incorrectly processed, resulting in abnormally folded versions that largely associated with yeast membranes. This can be attributed mainly to the fact that full-length glycoprotein contains a signal sequence and hydrophobic anchor domain, which results in the glycoprotein being expressed as a glycosylated, membrane-bound protein on the surface of infected cells and on the mature virion (Gaudin *et al*., 1992). Consequently, the purification of these target proteins would generally prove to be time-consuming and tedious. Therefore, to overcome these problems, we investigated the possibility of secretory expression of a soluble, truncated, recombinant G, using the *H. polymorpha* co-expression system, and the potential use of soluble, truncated, recombinant G as a diagnostic agent. Here, to achieve the secretion of recombinant protein, the extracellular domain of the G gene alone was cloned and synthesized. In addition, the MF-alpha signal sequence from *S. cerevisiae* present on the recombinant plasmid was added at the N-terminal end of the expressed soluble, truncated, recombinant G, which directed the expressed protein to be secreted by the expression cells.

The extracellular domain of G has three potential N-linked carbohydrate moieties at Asn37, Asn247, and Asn319, of which the latter two are efficiently core glycosylated (Shakin-Eshleman *et al*., 1992). More importantly, appropriate G N-glycosylation plays a vital role in ensuring its proper expression and function (Wojczyk *et al*., 2005). In order to assist the N-glycosylation process and achieve proper conformation, we explored the feasibility of using a chaperone overexpression system. In the present study, the combination of Western blot analysis and deglycosylation analysis of the truncated recombinant G revealed that the soluble 74.6 kDa protein produced in engineered *H. polymorpha* cells with HpCNE1 overproduction migrated slower than native G protein (Fig. 2) and was indeed N-glycosylated (Fig. 4). This indicated that the secretion of truncated recombinant G was obtained with the help of overproduction of calnexin, a chaperone protein encoded by HpCNE1. Calnexin may modulate the secretion of truncated recombinant G through multiple mechanisms. It may serve as an N-glycosylation-binding lectin or an ER chaperone (Parlati *et al*., 1995). When acting as a lectin, calnexin is known to interact with N-linked glycans present on glycoproteins until the correct conformation is reached (Rutkevich and Williams, 2011). Regardless of how HpCNE1 achieves this enhanced secretion expression of truncated recombinant G, the results obtained here are consistent with the previous reports that N-glycosylation of Asn247 and/or Asn319 allowed high levels of surface expression (Shakin-Eshleman *et al*., 1992).

Although the truncated recombinant G obtained here was N-glycosylated, structural differences in glycan structure do exist between glycoprotein obtained from mammalian cell lines (for example, CHO or BHK cell lines) and yeasts (Wildt and Gerngross, 2005). To determine whether or not differences in N-glycan structure affected antigencity, the truncated recombinant G was injected into rabbits to elicit antibody response. The antibodies induced by the truncated recombinant G showed significant reactivity with monomer and trimer forms of rabies virus glycoprotein, as demonstrated by immunoblotting analysis (Fig. 5). No band was observed with the negative serum (Fig. 5). Taken together, this demonstrated that the antigencity of the soluble, truncated, recombinant G was similar to that of native viral glycoprotein, suggesting that the glycosylation status of truncated recombinant G had no effect on the formation of the correct three-dimensional structure of the protein. Furthermore, the anti-recombinant G serum reacted efficiently with both the soluble, truncated, recombinant G protein and the purified Pasteur virus strain. This is important because the solubility of the recombinant protein is an important criterion for antigen selection (Zhang *et al*., 1997). Therefore, it is reasonable to presume that when applied as a coating antigen, the soluble truncated recombinant G will detect antibodies directed to both the whole virion and native glycoprotein. The use of a soluble, truncated, recombinant G for the development of rapid immunoassays that are suitable for detection and quantification of rabies virus, G protein-specific, antibodies in immunized hosts would offer the advantage of obtaining scalable protein production without the necessity of handling the live RV.

The ease with which the yeast expression system can be scaled up in comparison with higher eukaryotic cell systems, makes the resulting standardization of production of recombinant glycoprotein antigen in bulk an attractive alternative to the National Institutes of Health (NIH) rabies vaccine potency test, with the added advantage that live RV is not handled (Barth *et al*., 1988). Taken together, these advantages make *H. polymorpha*-produced, truncated, recombinant G an attractive candidate for use in therapeutic applications.

In summary, the soluble, truncated, recombinant G was obtained using the *H. polymorpha* expression system, with genetic engineering to modulate the ER folding environment. Moreover, the present study is the first report assessing the potential of yeast-derived, soluble, truncated, recombinant G as a diagnostic antigen in ELISA capable of detecting antibodies directed at both the whole virion and native gly-

coprotein. This expression system would be very useful for the production and investigation of the glycoprotein, permitting standardization of its production in bulk without the necessity of handling live RV.

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