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Novel strategy for expression and characterization of rabies virus glycoprotein

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ABSTRACT

Rabies is a fatal zoonosis which could affect all mammals. Glycoprotein (G protein) from the rabies virus plays an important role in the binding of virus to target cells. However, expression of the G protein with native conformation has been a great challenge for many years. In this study, we solved this problem by replacing the original signal peptide of rabies virus G protein with the one from the heavy chain of human IgG. The expression levels of recombinant G protein dramatically increased from a few μg/L to 50 mg/L in the culture supernatants. The identity of the recombinant G protein was confirmed by western blotting using both 6XHis mAb 6E2 and rabies G protein mAb 7G3. The correct conformation of the recombinant G protein was shown by using rabies virus neutralizing antibodies. In addition, the recombinant G protein had immune-reactivities with mice sera raised against rabies vaccines and vice versa. Taken together, our data suggested that by replacing the signal peptide, the expression level of the G protein with native conformation could be significantly improved. This would help the development of a rabies subunit vaccine, structural studies of rabies G protein, elucidation of the signal pathway of RABV infection.

1. Introduction

Rabies is a fatal zoonosis which menaces humans and animals worldwide since antiquity [1]. The disease is caused by rabies virus (RABV), known for neurotropism, belonging to genus Lyssavirus, family Rhabdoviridae and order Mononegavirales [2]. The RABV is composed of a highly stable and organized complex of genomic RNA and nucleoprotein contained in a lipid envelope derived from the host cell membrane [3]. The genome of RABV consists of a single, negative stranded, non-segmented RNA which is approximately 12 kb in size [4]. It encodes for five viral proteins: nucleoprotein, matrix protein, phosphoprotein, glycoprotein and large protein/RNA-directed RNA polymerase [5,6]. The glycoprotein (G protein) plays a pivotal role in viral attachment to neurons [7,8] and determination of tissue tropism [9], retrograde trans-synaptic spread of RABV in the central nervous system [1] and induction of cellular and humoral immune response required for conferring complete protection from lethal challenge [10,11]. The G protein, a type 1 membrane glycoprotein, consists of 524 amino acids including three domains (Fig. 1A) such as cytoplasmic domain, transmembrane domain and ectodomain exposed on the surface of mature virus particle [7,12]. The G protein is anchored in viral envelope by transmembrane domain made up of 22 amino acids from 460 to 480 residues [4]. The cytoplasmic domain consisting of 44 amino acids extends into the cytoplasm of infected cells where it interacts with the matrix protein to complete the viral assembly. The ectodomain exists as homotrimer spikes with each monomer having 439 amino acid residues [13].

As an important viral protein, the G protein has been evaluated for the development of a subunit rabies vaccine and used as reference antigen for Enzyme linked immunosorbent assay (ELISA)/Single radial

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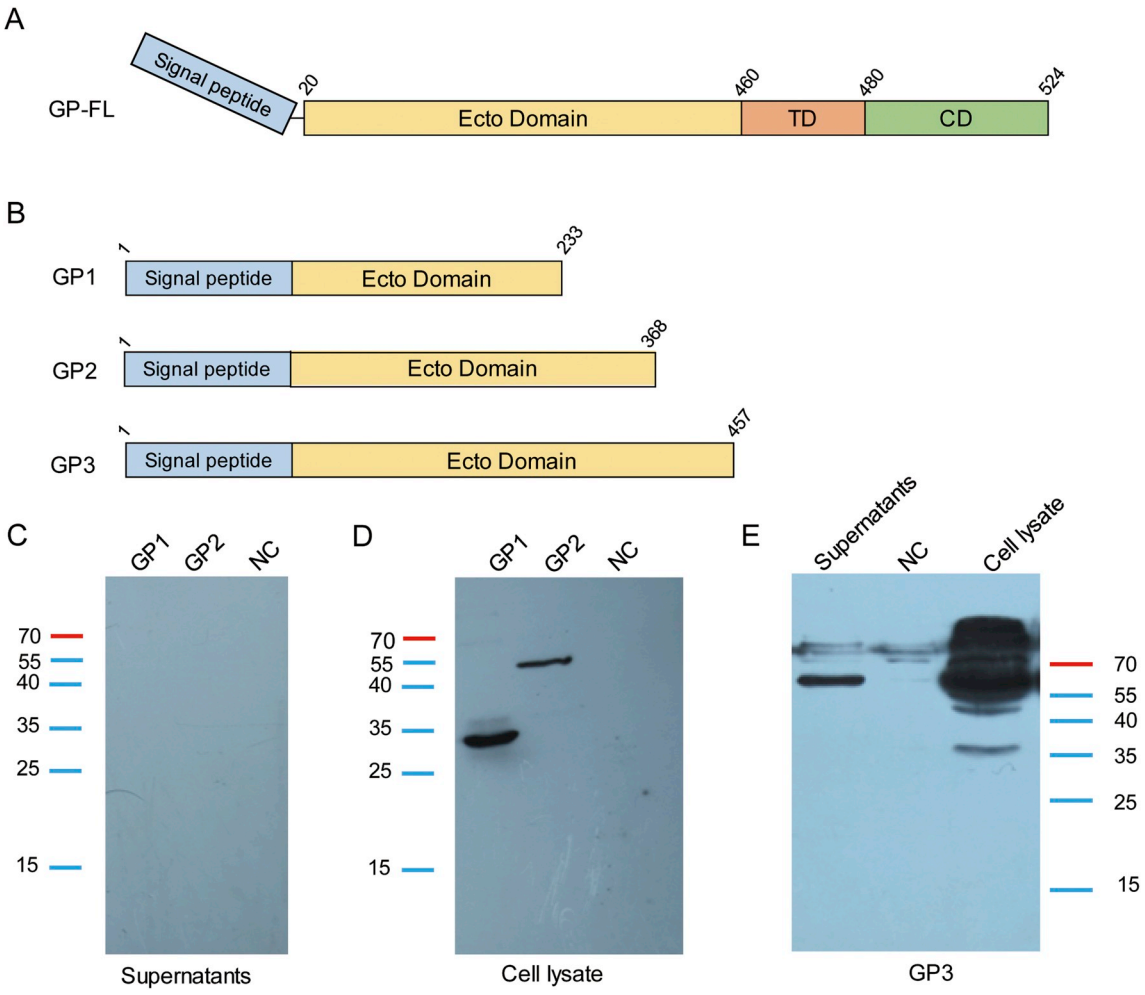

Fig. 1. Construction and expression of the RABV G protein.
A) Domain structure of full length G protein: the signal peptide was colored with blue, ectodomain was colored with yellow, TD colored with orange represented transmembrane domain and CD colored with green represented cytoplasmic domain.B) Different fragments of G protein: GP1 encoding amino acids from 1 to 233, GP2 encoding amino acids from 1 to 368, and GP3 encoding amino acids from 1 to 457.C)~E) Western blot for supernatants and cell lysate of G protein fragments expressed in HEK 293F cells: The G protein band was detected by anti-His 6E2 mAb. The molecular weight marker (kDa) of each band was annotated as appropriate. GP1, GP2 and GP3 were different fragments indicated above, NC referred to negative control (DMEM medium).

immune diffusion (SRID)-based rabies vaccine potency determination. Purified G protein is preferred to whole virus antigen because of its highly defined and homogenous nature devoid of interfering substances. Moreover, it provides better evaluation of specific virus neutralizing antibodies (VNA) in sera of vaccinated subjects [14]. However, the production of native G protein had been shown to be tedium, high cost and long turnaround time. Many attempts have been made to express G protein using a recombinant system. The glycosylated nature of the G protein necessitates a eukaryotic system for expression rather than a prokaryotic system which lacks glycosylation machinery. Thus far, full-length version of G protein has been expressed in eukaryotic systems such as insect cells [15], CHO cells [16], yeast [17] and plant [18] albeit with varying levels of success and expression. In this study, high-level and stable extracellular expression of natively folded recombinant G protein ectodomain in CHO cells was achieved with a yield of 50 mg/L and 95% purity. Most importantly, the recombinant RABV G protein was characterized and found to possess native folding and immunologically relevant antigenic sites including a neutralizing epitope. The immunogenicity of the G protein was demonstrated when the induced antibodies showed broader reactivity with inactivated antigens of at least three fixed RABV strains. The data pertaining to expression and characterization of the recombinant RABV G protein are presented and discussed. Taken together, the purified recombinant RABV G protein has been used to successfully develop an inexpensive rabies serology ELISA kit [19]. It seems to hold promise for the development of a rabies subunit vaccine and elucidation of RABV G protein structure which would help understand the molecular mechanism of RABV pathogenesis.

2. Materials and methods

2.1. Reagents and supplies

Taq DNA polymerase and PCR kits obtained from Transgene, China. Restriction enzymes such as Kpn I and Xho I sourced from New England Biolabs, USA. Kits for DNA purification and plasmid extraction were purchased from TIANGEN, China. Mouse anti-RABV G protein mAb 7G3 raised against a synthetic peptide (YPDYHWLRTVKTTKES; corresponding to aa 135–150 of RABV G protein) was a gift from National Institutes for Food and Drug Control, China. Mouse anti-6XHis mAb 6E2 was provided by AbMax, China. RABV neutralizing human monoclonal antibody (HumAb) NM57 [19,20] was a kind gift from Huabei Pharmaceuticals, Shijiazhuang, China. The human rabies immunoglobulin (HRIG) BRP was purchased from EDQM [21]. DMEM and FBS were from HyClone, USA. Goat anti-mouse IgG Fc specific (GAM Fc) HRP and goat anti-human IgG (GAH H + L) HRP obtained from Jackson

Immunoresearch, USA. Analytical reagent-grade chemicals were used for the preparation of buffers and solutions unless specified otherwise. HEK 293F cells and CHO cells were provided by Zhuhai Kairui Company, China. KD-CHO medium for propagation of CHO cell line came from Sino Biological, China. SDS-PAGE precast gels were purchased from GenScript, China. Rabies vaccines were provided by Liaoning Chengda Co., Ltd (aG strain), Chengdu Kanghua Biological Products Co., Ltd (PM strain) and other Chinese vaccine manufacturers. CTN strain of RABV was sourced from ATCC, USA. Complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) and DMSO were purchased from SIGMA, USA. Female BALB/c mice were obtained from Vital River Co., China.

2.2. Gene synthesis and protein expression of recombinant RABV G protein

The synthetic RABV G protein gene (UniProtKB: O92284) fragments with C terminal 6XHis tag were produced by GENEWIZ, China. Plasmid DNA cloning vector and expression vector pcDNA 3.1 were digested with restriction enzymes at 37 °C overnight. The DNA vector and gene insert were resolved by agarose gel electrophoresis and purified using gel purification kit (QIAGEN, Germany). The gene insert and plasmid vector were ligated overnight and used for the transformation of *DH5α E. coli*. The positive *E. coli* transformants were picked for DNA sequencing to confirm the correct insertion of the sequences. Positive transformants were picked and propagated in 200 mL LB medium. The recombinant plasmid DNA was extracted from *E. coli* culture using plasmid DNA ultrapure extraction kit (QIAGEN, Germany).

The purified plasmid DNA was used to transfect mammalian host cells by lipofection using liposome transfection kit (Invitrogen, USA) following the manufacturer's instructions. The transfected mammalian cells were grown at 37 °C and 5% CO_2 for a few days prior to harvesting.

2.3. Purification of recombinant RABV G protein by immobilized metal affinity chromatography

The harvested cell cultures were pelleted and separated by centrifugation at 4,000 rpm for 10 min at room temperature. The G protein purification was carried out with GE His Trap HP (1 mL) column by fast protein liquid chromatography (GE AKTA Purifier 100 System). The clarified culture supernatant was diluted 1:1 with loading buffer (50 mM MES, 20 mM Imidazole, 150 mM NaCl, pH 6.0). After loading, the Ni column was washed initially with Buffer 1 (50 mM MES 6.0, 300 mM NaCl) and subsequently with Buffer 2 (50 mM MES 6.0, 20 mM Imidazole, 150 mM NaCl). The flow rate was 0.5 mL/min, the pressure was 0.5 MPa. The RABV G protein bound on the Ni column was eluted using Buffer 3 (50 mM MES, 250 mM Imidazole, 150 mM NaCl pH 6.0 and 20% glycerol), and the fractions were collected at 1ml/tube monitored by absorbance at 280 nm. Protein concentration was determined using absorbance at 280 nm.

2.4. SDS-PAGE and western blot analysis

The purification process was ascertained by SDS-PAGE followed by Coomassie brilliant blue R250 staining and Western blotting (WB). Briefly, the samples were mixed with 6X protein loading buffer and loaded onto 12% gels, separated by SDS-PAGE, either stained with Coomassie brilliant blue R250 for purity or transferred onto a PVDF membrane for 2 h at 200 mA for WB blot analysis. The membrane with transferred proteins was immersed in 5% skim milk-PBS at room temperature for 1 h. After two rinses with PBST, the samples were probed with either a mouse anti-6XHis mAb or mouse anti-RABV G protein mAb 7G3 (2 μg/mL) at room temperature for 1 h. The membrane was then incubated with GAM Fc HRP (1:2000) and the visualization of proteins was done based on Enhanced Chemiluminescence (Promega, USA).

2.5. Immunization of animals

All experimental procedures involving mice in this study were in accordance with requirements and guidelines for treatment of experimental animals that were set forth and approved by the National Institutes for Food and Drug Control of China. The Ethics Committee on animal experiments of AbMax Biotechnology has approved all animal experiments conducted in this manuscript.

BALB/c mice (female; 4–6 weeks old) were immunized with recombinant G protein or rabies vaccines emulsified in Freund's adjuvant. Complete Freund's Adjuvant (CFA) and Incomplete Freund's adjuvant (IFA) were used for priming and boosting, respectively. The mice received at least two booster immunizations. Three weeks after the first immunization, tail bleeds were collected and sera were tested for antibody titers by FAVN and indirect ELISA against RABV G protein or rabies vaccine antigens.

2.6. Indirect ELISA

Each well of 96-well high binding ELISA plates (Corning, USA) was coated with purified RABV G protein (1 μg/mL) or rabies vaccine antigens (1:100 dilution in PBS) in PBS overnight at 4 °C. After two washes with PBS and blocking with 5% skim-milk/PBS for 1 h at room temperature, wells were incubated with tail bleeds sera (1:500 to 1: 50,000 dilution) or anti-RABV G protein mAb NM57 (1 μg/mL) or HRIG in 5% skim milk-PBS for another hour at room temperature. After two washes with PBS, wells were then probed with HRP-conjugated GAM Fc-specific or GAH (H + L) secondary antibodies (1: 2,000) in 5% skim milk-PBS for 1 h at room temperature. After five washes with PBST, TMB substrate solution was added and optical density (OD) measured, after 30 min, at 450 nm wavelength using a microplate spectrophotometer.

2.7. Fluorescent antibody virus neutralization test

Viral neutralizing antibody (VNA) titers of immunized mice against RABV were determined by FAVN test using standards set forth by the World Organization for Animal Health (OIE). Briefly, the sample to be tested was subjected to triple gradient dilution and then added to a 96-well plate for detection. WHO reference serum was diluted to 0.5 IU/mL for positive control and non-immunized canine serum was used as negative control. CVS-11 virus was prepared to proper concentration and added 50 μl per well. Plates were incubated at 37 °C for 1 h. The treated BHK-21 cells were diluted to $4X10^5$ cells/ml and added to each well (50 μl/well), and then incubated at 37 °C in incubator with 5% CO_2 for 48 h. Afterwards, the medium was removed and cells were fixed. Then cells were incubated with RABV-specific monoclonal antibody and stained with FITC-conjugated goat anti-mouse IgG. Fluorescence was observed under a fluorescence microscope and the results of each well were recorded.

3. Results

3.1. Novel strategy for expression of recombinant RABV G protein in HEK 293F cells

To obtain the recombinant RABV G protein, we tried to express three different G protein fragments (GP1 from 1 to 233, GP2 from 1 to 368 and GP3 from 1 to 460) by transient transfection of HEK 293F cells with plasmids containing different lengths of G protein genes (Fig. 1B). The culture supernatants and cell lysates were collected on day 6 and the expression levels were examined by Western blot using mouse anti-6XHis mAb 6E2 (Fig. 1C, D and 1E). No signal was detected in the culture supernatants of HEK 293F cells transfected with GP1 and GP2, whereas faint bands at the expected sizes were seen in the cell lysates. Low level of GP3 was detected in the culture supernatant but major

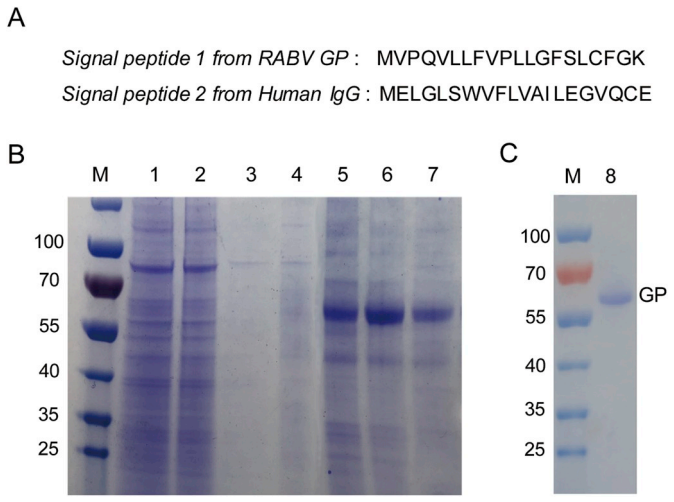

Fig. 2. Expression and purification of the RABV G protein.
A) Signal peptides sequences of the RABV G protein and human IgG heavy chain were mentioned with 1-letter amino acid abbreviation. B) SDS-PAGE of G protein expression with modified peptide in HEK 293F cells: Lane M referred to the MW markers (kDa), lane 1 referred to the culture supernatant, lane 2 referred to the flow-through, lane 3 referred to the 1st wash with buffer 1, lane 4 referred to the 2nd wash with buffer 2, lanes 5, 6 and 7 referred to three different fractions eluted with buffers containing 50 mM MES, 250 mM Imidazole, 150 mM NaCl pH 6.0 and 20% glycerol.C) SDS-PAGE of the RABV G protein after optimization of purification conditions: Lane M refers to the MW markers (kDa), lane 8 refers to the purified G protein as annotated.

portion of the recombinant G protein (about 60 kDa) was retained inside the cells. Our results clearly suggested that the native signal peptide is not suitable for the secretion of recombinant RABV G proteins.

Taking the lessons learnt from large scale productions of recombinant antibodies as therapeutic agents, the native signal peptide of RABV G protein was replaced with the one derived from human IgG heavy chain (Fig. 2A). Human HEK 293F cells were transfected with the newly designed plasmid and culture supernatants were collected on Day 7. Surprisingly, a very strong signal was detected in the culture supernatant by ELISA using the rabies neutralizing mAb NM57 (data not shown), suggesting high level expression of soluble recombinant RABV G protein by transfected HEK 293F cells. Culture supernatant was loaded on to the Ni column and washed sequentially with Washing Buffers 1 and 2 (Lanes 3 and 4), then the bound proteins were eluted with Buffer 3 containing 250 mM Imidazole (Lanes 5–7). A strong band around 60 kDa was observed in all three elution fractions (Fig. 2B) after staining with Coomassie brilliant blue R250. The three elution fractions (5–7) enriched with recombinant rabies G protein were pooled and the protein concentration was determined by UV spectrophotometry, wherein the expression level in the culture supernatant was estimated to be greater than 10 mg/L from HEK 293F cells. To improve the purity, we have tried different washing buffers and elution buffers with different concentrations of imidazole and salt. Finally, a purity of greater than 95% was achieved (Fig. 2C).

3.2. Confirmation of the identity of recombinant RABV G protein

To confirm the identity of the purified recombinant protein, Western blot was done using a mouse anti-RABV G protein mAb 7G3, generated against a synthetic peptide corresponding to amino acids 135–150 of RABV G protein, as a probe. As shown in Fig. 3A, a band around 60 kDa was detected by the mAb 7G3 with the same position as the one stained by Coomassie brilliant blue.

To ensure native conformation of the purified recombinant RABV G protein, an ELISA based testing was done using both a well characterized RABV neutralizing mAb NM57 [19,20] and Human Rabies Immunoglobulin (HRIG). As shown in Fig. 3B, a dose-dependent immunoreactivity was observed between the purified RABV G protein and the mAb NM57 or HRIG. It clearly indicated that the purified recombinant RABV G protein retained at least one neutralizing epitope recognized by the mAb NM57. The HRIG recognition of the RABV G protein also helped confirm its native conformation (Fig. 3C).

3.3. Establishment of stable CHO cell line expressing recombinant RABV G protein

For future industrial applications, a mammalian cell line with stable and high-level expression of the recombinant RABV G protein was desired. Safety is always one of the major concerns for future vaccine development. Thus, the Chinese Hamster Ovarian (CHO) cells were chosen since these cells have been used for production of more than 70 human antibodies approved as therapeutics for human disease treatments. The CHO cells were transfected with recombinant plasmid hIgG-SP-RABV G protein 20-460 and subjected to multiple rounds of antibiotic selection and cloning. Finally, a stable CHO cell line with high-level expression of RABV G protein (20–460) was established.

As rabies is a zoonosis with an extremely wide mammalian host range, we expected the glycosylation of the RABV G protein made by rodent CHO cells to be the same as the one made by human cells. To confirm this, the culture supernatants from G protein 20-460-transfected HEK 293F and CHO cells were tested side by side by indirect ELISA using the neutralizing anti-RABV G protein mAb NM57. As shown in Table 1, the supernatants from stable CHO cells reacted strongly with NM57, suggesting that the CHO cells not only produced the recombinant rabies G protein with correct conformation as did the HEK 293F cells, but also at much higher level than the HEK 293F cells. After the optimization of culture media, culture duration and agitation speed, the expression level of G protein reached to around 50 mg/L. Multiple batches were made to assure the consistency of RABV G protein expressions.

3.4. Assessment of immunogenicity of the recombinant RABV G protein

For G protein immunogenicity validation, tail bleeds sera from mice immunized and boosted with G protein in different days (0,7,14 days) were collected and evaluated by ELISA using G protein coated plates (Fig. 4A). On day 7, the mice began to give an immune response, then reached a strong reaction after 14 days. On day 21, terminal bleeds were collected and evaluated using FAVN test to evaluate virus neutralizing antibody (VNA) titers (Fig. 4B). It can be seen that after the complete immunization program, the titers of VNA in all of the immunized mice were far more than the WHO recommended post-immunization rabies VNA levels (0.5IU/mL), suggesting a very strong protection was achieved. Meanwhile, as shown in Fig. 4C, all the RABV G protein immunized mice generated different levels of IgG antibodies that could bind to all three different fixed strains of RABV (CTN, aG and PM strains) tested, which demonstrated the broad protective activity against different strains of RABV.

Immunogenicity of recombinant RABV G protein was also assessed by ELISA with sera from mice immunized with different strains of rabies vaccines. The wells of 96-well ELISA plates were coated with G protein or rabies vaccine antigens (1:100 dilutions in PBS) belonging to different strains of RABV manufactured by different vaccine companies. As shown in Fig. 4D and E, the sera from mice immunized with two different rabies vaccines using aG or PM strains reacted with recombinant RABV G protein.

Thus, our data suggested that the recombinant RABV G protein could induce antibodies that exhibited broader reactivity with various fixed strains of RABV tested, which made it a good candidate for recombinant rabies subunit vaccine development.

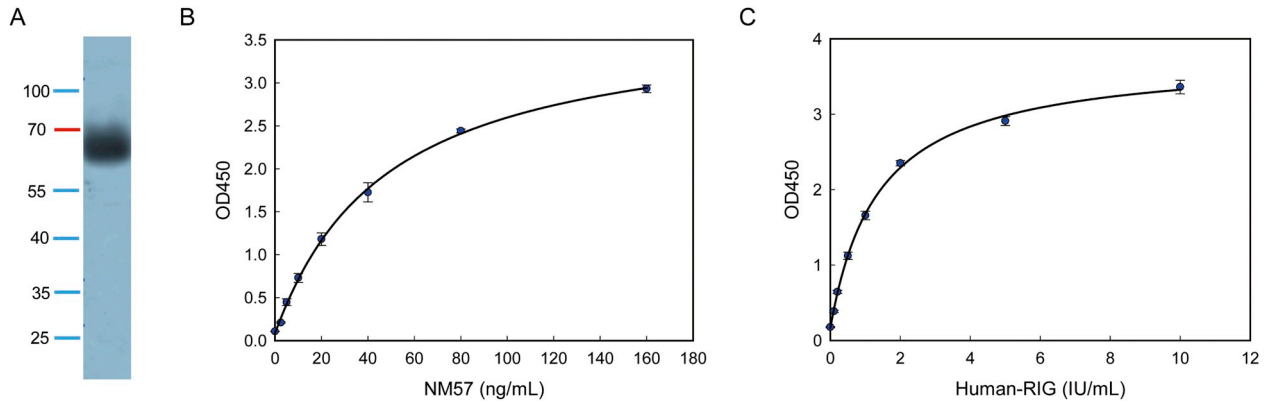

Fig. 3. Identification and characterization of the RABV G protein by Western blot and ELISA.
A) Western blot of purified protein: The RABV G protein strip was probed with mouse anti-RABV G protein mAb 7G3. The molecular weight markers (kDa) were annotated as appropriate. B) ELISA validation for reactivity of the RABV G protein with HumAb NM57: 1 μg/mL RABV G protein was used to coat the wells of the 96 well ELISA plate. The graph was plotted by having various concentrations of NM57 (0–160 ng/mL) on X-axis and OD450 on Y-axis.C) ELISA validation for reactivity of the RABV G protein with HRIG: 1 μg/mL RABV G protein was used to coat the wells of the 96 well ELISA plate. The graph was plotted by having various concentrations of HRIG (0–10 IU/mL) on X-axis and OD450 on Y-axis.

Table 1
Characterization of recombinant RABV G protein from a stable CHO cell line.

Dilution Ratio	Supernatant (HEK 293F)		Supernatant(CHO)	
1:100	***	***	***	***
1:300	1.324	1.254	***	***
1:1000	0.226	0.263	***	3.444
1:3000	0.15	0.185	1.591	1.654
1:10000	0.122	0.116	0.432	0.463
NC	0.048	0.047	0.032	0.045

RABV G protein was expressed and secreted from HEK 293F and CHO cells. Then the wells of 96-well ELISA plates were coated with supernatants in different dilutions as shown in the table. NM57 was used as the primary antibody at a concentration of 2 μg/mL. NC: negative control (5% skim milk –PBS). Secondary antibody was HRP-GAH H + L diluted 1:2000 in PBS. "***" indicates the upper limit of OD_{450} measurement (> 5.0) when read by microplate spectrophotometer.

4. Discussion

The RABV G protein plays key roles in infectivity, neurovirulence, pathogenicity and immunogenicity, with two or more N-glycosylation sites [22]. The glycosylated nature of RABV G protein necessitates a eukaryotic expression system. Prokaryotic expression of RABV G protein using systems e.g. *E. coli,* was proven not ideal since the polyclonal antibodies from RABV vaccine immunized animals failed to recognize it. Expression of viral glycoproteins with the correct conformations in large quantity has been a universal challenge for all researchers.

Our work paved the way for stable expression and purification of natively folded RABV G protein, the final production of RABV G protein achieved a yield of 50 mg/L with 95% purity, thereby setting the stage for further research on structure of RABV G protein and the molecular mechanism of RABV pathogenesis. The RABV G protein expressed and thus has been used to develop inexpensive rabies serology ELISA kits, which could be applied to monitor the efficacy of rabies immunization. Additionally, our work has paved the way to resolve the 3D structure of RABV using X-ray and Cryo-Electron Microscopy while also identifying the recombinant RABV G protein as a potential candidate antigen for the development of a rabies subunit vaccine.

In addition to the rabies G protein, many proteins have the same problem in eukaryotic expression, either with low expression levels and/or with incorrect glycosylation profiles. Employing the same strategy, we demonstrated that the expression levels of glycoproteins from other viruses, such as Ebola and Flu, could be increased significantly as well (data not shown). This solution was also applied on expressions of important immune target proteins, including T-cell immunoreceptor with Ig and ITIM domains (TIGIT), Programmed cell death protein 1 (PD1), Programmed cell death 1 ligand 1 (PDL1) with great success. Thus, our present invention could help solve the universal problem of large scale production of viral glycoproteins and many other eukaryotic proteins.

5. Conclusions

In this study, we found that the native signal peptide for RABV G protein is not strong enough to lead the secretion of RABV G protein in mammalian cells, which caused the failure of production of soluble recombinant protein in large quantities. In fact, a majority of the recombinant G protein remained inside the host cells. Using a novel strategy, by simply replacing the original signal peptide of viral glycoprotein with the one from human IgG heavy chain, the expression level of recombinant RABV G protein in the culture supernatants by HEK 293F cells increased by more than 1,000-folds (from μg/L level to mg/L level). Such drastic increases of RABV G protein expression was also observed in the CHO cells. Our present invention could help solve the universal problem of large scale production of viral glycoproteins and many other eukaryotic proteins.

CRediT authorship contribution statement

Rongqing Zhao: Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing. **Yi Shan:** Conceptualization, Validation, Formal analysis, Writing - review & editing. **Maohua Li:** Conceptualization, Methodology, Formal analysis, Resources. **Zhiyong Lou:** Conceptualization, Methodology, Formal analysis, Resources. **Ye Feng:** Validation, Resources. **Lisong Huang:** Conceptualization. **Wenlin Ren:** Conceptualization, Methodology, Formal analysis, Resources. **Panpan Wang:** Methodology, Investigation. **Yufei Sun:** Methodology, Validation, Investigation. **Ying Sun:** Methodology, Validation, Investigation. **Junchi Su:** Methodology, Validation, Investigation. **Hunter Sun:** Methodology, Validation, Investigation. **Dee Hong:** Methodology, Formal analysis. **Yuhua Li:** Conceptualization, Formal analysis. **Ruifeng Chen:** Funding acquisition, Writing - review & editing. **Le Sun:** Conceptualization, Methodology, Formal analysis, Resources, Funding acquisition, Project administration, Writing - review & editing.

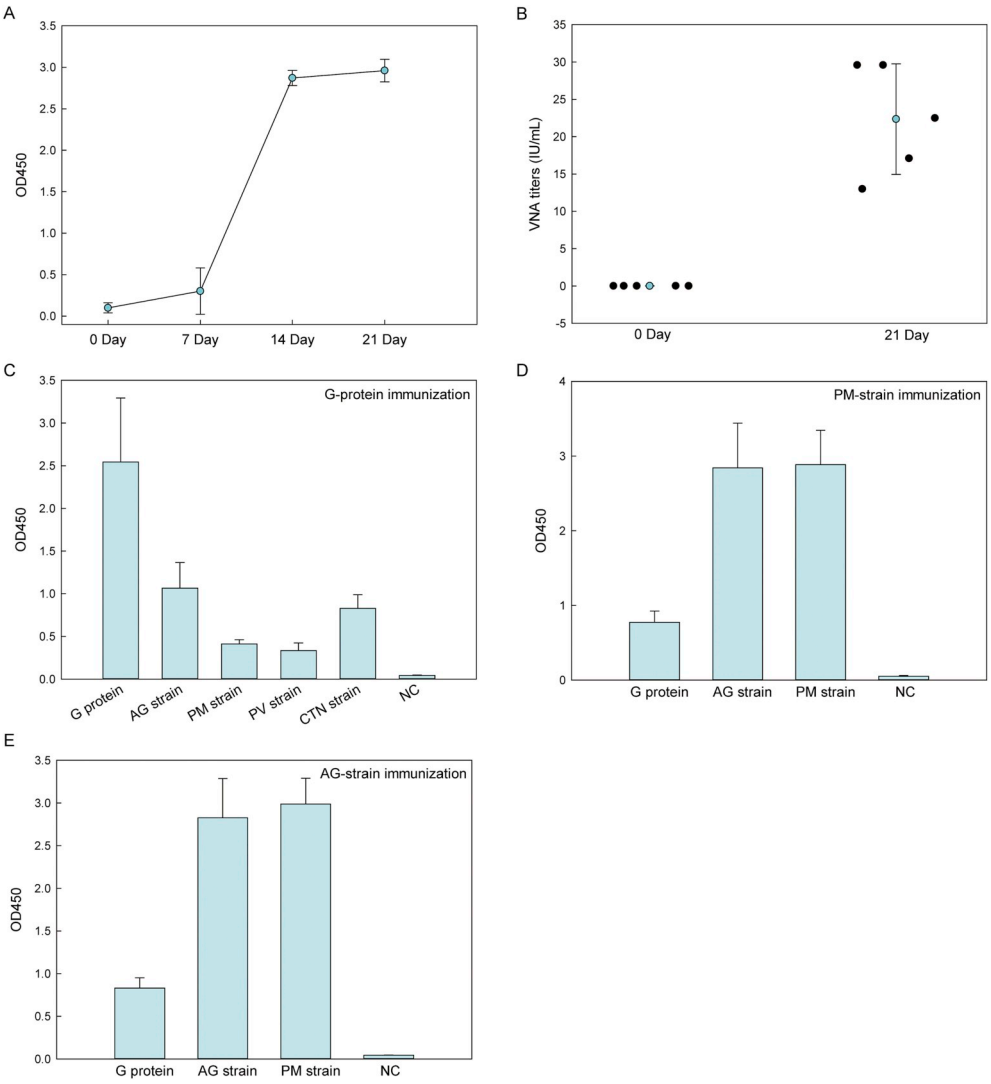

Fig. 4. Evaluation for immunogenicity of the recombinant RABV G protein.
A) ELISA validation for G protein immunogenicity. The 96-well ELISA plates were coated with RABV G protein at 1 μg/mL in PBS. Tail bleeds sera from mice immunized with G protein in different days (0,7,14,21 days) were collected and used for RABV neutralizing antibody evaluation. B) FAVN test for VNA titers: heart blood from 5 mice immunized with G protein after 21 days were collected and the VNA titers were tested using Fluorescent antibody virus neutralization test (FAVN). Points labelled with blue denoted the mean and error of mouse VNA titers.C ~ E) The wells of 96-well ELISA plates were coated with antigens derived from different rabies vaccines (from different Chinese vaccine manufacturers) at 1:100 dilution in PBS. Tail bleeds sera were from mice immunized with aG strain/PM strain or G protein at 1:500 dilutions. Negative control was negative mice sera at 1:500 dilution in PBS. Secondary antibody was HRP-GAM Fc diluted 1:2000 in PBS. Reactivity of different antigens with tail bleeds sera from mice immunized using G protein (C), aG strain (D) and PM strain (E).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pep.2019.105567.

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