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Engineering, expression, and immuno-characterization of recombinant protein comprising multi-neutralization sites of rabies virus glycoprotein

Xiangdong Li [a], Jinyan Luo [a], Shuiming Wang [b], Yang Shen [a], Yafeng Qiu [a], Xiaodu Wang [a], Xufang Deng [a], Xuehui Liu [b], Weidong Bao [b], Peihong Liu [c], Jinping Zhou [c], Chan Ding [a], Zhiyong Ma [a,*]

[a] Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Science, No. 518, Ziyue Road, Shanghai 200241, PR China
[b] Nanjing Entry–Exit Inspection and Quarantine Bureau, No. 99, Zhonghua Road, Nanjing 200001, PR China
[c] Shanghai Municipal Animal Husbandry and Veterinary Station, Shanghai 201103, PR China

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ABSTRACT

The rabies virus (RV) glycoprotein (G protein) induces neutralizing antibodies, which are important in protection against rabies. In the present study, three gene fragments that encode polypeptides (corresponding to amino acid residues 19–60, 181–219, and 300–458) comprising the linear neutralization sites of the G protein were spliced together in tandem by PCR-based site-directed mutagenesis and heterologously expressed in *Escherichia coli* (DE3). The recombinant protein (designated rRVg) was purified under denaturing conditions and solubilized by stepwise dialysis against an alkaline buffer (0.05 M Na_2CO_3 pH 9.6). Western blot analysis of the antigenicity of rRVg showed that it was recognized by rabies-immune serum. Competitive neutralization assay revealed that rRVg significantly reduced the RV-neutralizing activity of the rabies-immune serum. These results show potential of rRVg as a diagnostic antigen for detecting RV-neutralizing antibodies in immunized hosts.

Rabies is a fatal viral zoonosis caused by rabies virus (RV)[1], a member of the Lyssavirus genus of the Rhabdoviridae family. The transmission of RV from wildlife or domestic animals to humans remains a serious public health problem in many countries [1]. The viral genome is a non-segmented, negative-strand RNA, which is used to produce five monocistronic mRNAs encoding nucleoprotein, phosphoprotein, matrix protein, glycoprotein (G protein), and viral RNA-dependent RNA polymerase, respectively [2]. The G protein is the only protein on the surface of mature virus and plays an important role in virus infection [3]. The G protein is also a major antigenic stimulus of the host immune system during infection and vaccination, specifically inducing neutralizing antibodies [4]. It is a 65 kDa type I transmembrane protein consisting of an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The extracellular domain is involved in the induction of RV-neutralizing antibody production [5]. Its main antigenic sites are localized into two clusters; the first is formed from two sites between amino acid residues 34–42 and amino acid residues 198–200 [6], the second is between amino acid residues 330 and 338 [7].

Virus-neutralizing antibodies serve as an important factor responsible for protection against rabies. A World Health Organization working group recommended that RV-neutralizing antibodies should be determined by mouse neutralization test, plaque reduction assay, or rapid fluorescent focus inhibition test (RFFIT) [8]. However, these assays are expensive, somewhat time-consuming, and laborious. Enzyme linked immunosorbent assay (ELISA) has been broadly used for the qualitative and quantitative assessment of virus-specific antibodies in many diseases, but the current rabies-ELISA kit uses the whole virus as the diagnostic antigen and detect antibodies against antigens other than the G protein, which may lead to erroneous interpretation of immune state of vaccinated hosts [8]. The G protein, which truly reflects the titer of the RV-neutralizing antibodies [7], is a useful and safe antigen for monitoring RV-neutralizing antibodies in vaccinated hosts. Because purification of the G protein from RV is unsafe and laborious [7], several truncated fragments of the G protein have been heterologously expressed and investigated as potential diagnostic antigens for monitoring RV-neutralizing antibodies [9,10].

To develop safer and cheaper sources of diagnostic antigens, we spliced together three gene fragments in tandem, encoding polypeptides (corresponding to amino acid residues 19–60, 181–219, and 300–458) that comprise the linear neutralization sites of the G protein, then expressed it in *Escherichia coli* (*E. coli*). The recombinant protein, designated rRVg, was able to react with rabies-immune serum and reduce its neutralizing activity, thus demonstrating its

* Corresponding author. Fax: +86 21 54081818.
E-mail address: zhiyongma@shvri.ac.cn (Z. Ma).

[1] *Abbreviations used:* RV, rabies virus; RFFIT, rapid fluorescent focus inhibition test; ELISA, enzyme linked immunosorbent assay; SPF, specific-pathogen-free; DMEM, Dulbecco's modified Eagle's medium; IPTG, isopropyl-β-D-thiogalactopyranoside; His, hexahistidine.

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potential as a diagnostic antigen for detecting RV-neutralizing antibodies.

Materials and methods

Virus, plasmids, and cells

HEP-Flurry RV strain was purchased from the China Institute of Veterinary Drug Control (Beijing, PR China) and propagated in 6-day-old embryonated specific-pathogen-free (SPF) chicken eggs. *E coli* BL21 (DE3) and plasmid pET28a (Novagen, Madison, WI, USA) were cultured according to standard techniques. Rabies-immune serum was a gift from Dr. Dongliang Hu (Laboratory of Epidemiology, Veterinary Institute, Academy of Military Medical Sciences, PR China). BHK-21 hamster kidney cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in an atmosphere containing 5% CO_2.

Cloning of the extracellular domain gene of the G protein

The total RNA was extracted from the allantochorion of 6-day-old embryonated SPF chicken eggs infected with HEP-Flurry RV strain, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using AMV reverse transcriptase (TaKaRa, Otsu, Japan). The fragment encoding the extracellular domain (amino acid residues 19–458) of the G protein was amplified by PCR using a primer pair RVg-F/RVg-R (Table 1) that was designed using the nucleotide sequence of the G protein (GenBank Accession No. M32751). The amplification parameters were an initial 5 min at 95 °C followed by 28 cycles consisting of 1 min at 95 °C, 1 min at 55 °C, 2 min at 72 °C, and a final 10 min extension step at 72 °C. PCR amplification was confirmed by electrophoresis of 10 μL of the PCR products on an agarose gel. The PCR product was inserted into the EcoRI–SalI cloning site of the expression vector pET28a to construct a recombinant plasmid, designated pET-RVg. The pET-RVg was characterized by PCR, double enzyme digestion, and DNA sequencing analysis.

Gene splicing

To construct an expression plasmid expressing recombinant protein (rRVg) consisting of the multi-neutralization sites of the G protein, modified PCR-based site-directed mutagenesis [11] was performed using the recombinant plasmid pET-RVg as the template. The primer pairs used were RV1F/RV1R and RV2F/RV2R (Table 1), which excised two sections of the undesired nucleotides sequences between the antigenic sites and spliced together three gene fragments encoding the main antigenic sites (amino acid residues 19–60, 181–219, and 300–458) in tandem (Fig. 1). Briefly, in a total volume of 50 μL, 10 ng plasmid pET-RVg, 100 ng each primer (RV1F, RV1R, RV2F and RV2R), 200 μM each dNTP, 2.5 U cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA), and the buffer supplied by the manufacture were combined. Amplification parameters were an initial 5 min at 95 °C, followed by 18 cycles consisting of 1 min at 95 °C, 1 min at 55 °C, 15 min at 68 °C, and a final 7 min extension step at 68 °C. After PCR amplification, 10 U of restriction enzyme DpnI (New England Biolabs, Beverly, MA, USA) was added directly to the PCR reaction and incubated at 37 °C for 2 h. Following the DpnI digestion, 2 μL of the DpnI-treated DNA was used to transform competent *E. coli* BL21 (DE3) cells. Clones carrying the resulting recombinant plasmid, designated pET-rRVg, was characterized by PCR, double enzyme digestion, and DNA sequencing analysis.

Table 1
Primer sequences.

Primers	Sequence
RVg-F	5′-CGCACAGAATTCAAGTTCCCC ATTTAC-3′
RVg-R	5′-ATTAGTCGAC TCACTTCCCCCATTTCGG-3′
RV1F	5′-CCAACCTGTCCGGGTTCTCTACGGTGTCCTCGACCTACTG-3′
RV1R	5′-CAGTAGGTCGAGGACACCGTAGAGAACCCGGACAGGTTGG-3′
RV2F	5′-GCAGAGGGAAGAGAGCATCCGAGTGTCTGGATGCACTAGA-3′
RV2R	5′-TCTAGTGCATCCAGACACTCGGATGCTCTCTTCCCTCTGC-3′

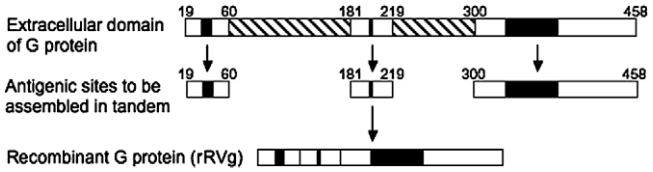

Fig. 1. Schematic representation of the generation of the recombinant G protein (rRVg). The black blocks represent the neutralization sites. The shadow blocks (360 and 240 bp) represent the undesired sequences to be excised from G protein. The numbers indicate amino acid positions.

Expression and purification of rRVg

The *E. coli* cells containing the plasmid pET-rRVg were pre-cultured in 5 mL of LB medium supplemented with 50 μg/mL of kanamycin overnight at 37 °C with vigorous shaking. The overnight cultures were transferred into 500 mL of the same medium and were aerobically grown at 37 °C until mid-log phase (OD_{600} = 0.7–0.8). Expression was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 4 h. After induction, the bacterial cells were harvested by centrifugation at 10,000*g* for 10 min and the total cell pellet was frozen at −20 °C before preparation of the cell extracts. The cell pellet was re-suspended in 30 mL of lysis buffer (10 mM Tris–Cl, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5 mM PMSF, pH 8.0) for sonication. The suspended cells were sonically disrupted in an ice-water bath. The recombinant protein rRVg, which was expressed as insoluble inclusion bodies in *E. coli*, was separated by centrifugation at 5000*g* for 15 min. The insoluble pellet was washed with 2% sodium deoxycholate 3 times and then solubilized in 6 M urea buffer (6 M urea, 500 mM NaCl, 20 mM Tris–Cl, 5 mM imidazole, pH 7.9). The urea-solubilized rRVg was purified under denaturing conditions using a His Band kit (Novagen), according to the manufacturer's instructions. Briefly, the supernatant of urea-solubilized rRVg was loaded on a pre-equilibrated His Bind Resin column. The column was first washed with 10 volumes of the 6 M urea buffer, followed by 6 volumes of a wash buffer (6 M urea, 500 mM NaCl, 20 mM Tris–Cl, 20 mM imidazole, pH 7.9). The rRVg was finally recovered by elution with an elution buffer (6 M urea, 500 mM NaCl, 20 mM Tris–Cl, 1 mM imidazole, pH 7.9). The purity of the preparation were assessed by 15% SDS–PAGE followed by Coomassie blue staining. To remove the urea from the preparation, three types of dialysis buffer (buffer A, 0.01 M PBS pH 7.2; buffer B, 0.05 M Tris–HCl pH 8.0; buffer C, 0.05 M Na_2CO_3 pH 9.6) were tested. The stepwise dialysis was performed at 4 °C against 20 volumes of these dialysis buffers containing decreasing concentrations of urea (4, 2, 1, and 0 M). The dialysis buffers were changed every 4 h to gradually remove the urea. After being filtered through a 0.22 μm cellulose acetate membrane, the rRVg was stored at −20 °C. The protein content was determined by a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

Western blot

An aliquot of protein (20 μg) was subjected to SDS–PAGE and transferred to an Immobilon-P membrane (Millopore Corp., Bed-

ford, MA, USA), which was incubated first in blocking buffer (5% non-fat milk power in Tris-buffered saline containing 0.2% Tween 20 [TBS-T]) for 2 h at room temperature and subsequently in a solution of primary antibodies for 16 h at 4 °C. Finally, the membrane was washed in TBS-T to remove the unbound primary antibodies and then incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. After a further wash in TBS-T, the antibody–antigen complex was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Competitive neutralization assay

The neutralizing titer of rabies-immune serum was determined by RFFIT according to the standard methodology [12]. To determine whether rRVg reacted with neutralizing antibodies present in the rabies-immune serum, a twofold serial dilution of the refolded rRVg (from 0.01 to 3.2 μM) was mixed with 100 μL rabies-immune serum and incubated at 37 °C for 1 h. The serum/rRVg mixtures were then incubated at 37 °C for 1 h with a constant dose of challenge RV that causes infection in 80% of the cells. After incubation, BHK-21 cells were added to the serum/rRVg/RV mixtures and cultured for 24 h. The BHK-21 cell monolayer was then fixed with acetone and stained with a fluorescent antibody in order to detect the presence of non-neutralized virus (fluorescent foci). The neutralizing tilter (IU/mL) was then compared with and without incubation with the rRVg. Statistical analysis was performed using Duncan's multiple range test.

Results

Construction of the expression vector

The amplified DNA fragment encoding the extracellular domain of the G protein was 1317 nucleotides in length and showed 99.6% similarity with the G protein sequence deposited in GenBank (GenBank Accession No. M32751). The DNA fragment was cloned into the expression vector pET28a (designated pET-RVg) and the pET-RVg was characterized by double digestion with EcoRI and SalI (Fig. 2A, lane 1) and PCR analysis (Fig. 2B, lane 1). To assemble the three DNA segments encoding the antigenic domains in tandem, 240- and 360-bp sections of undesired nucleotide sequences between the antigenic domains were excised from pET-RVg using modified PCR-based site-directed mutagenesis (Fig. 1), which resulted in the generation of a recombinant expression plasmid, pET-rRVg. pET-rRVg was characterized by double enzyme digestion (Fig. 2A, lane 2), PCR analysis (Fig. 2B, lane 2), and DNA sequencing analysis (data not shown).

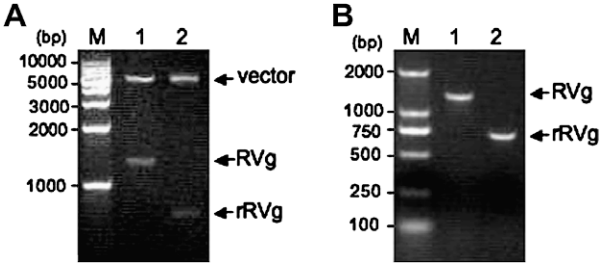

Fig. 2. (A) Characterization of the recombinant plasmids by double enzyme digestion. The recombinant plasmids were double digested with EcoRI and SalI and separated by agarose gel electrophoresis. Lane M, DNA ladder; lane 1, pET-RVg; lane 2, pET-rRVg. (B) Characterization of the recombinant plasmids by PCR analysis. The inserted fragments were amplified by PCR using the recombinant plasmids as templates and checked on agarose gel electrophoresis. Lane M, DNA ladder; lane 1, pET-RVg; lane 2, pET-rRVg.

Expression and purification of rRVg

Induction of *E. coli* carrying the plasmid pET-rRVg with IPTG resulted in expression of a 20.1 kDa protein with a molecular mass corresponding to that of rRVg (Fig. 3A, lanes 1 and 2). The rRVg with a hexahistidine (His) tag at its N-terminal end was expressed as insoluble protein aggregates in the form of inclusion bodies in *E. coli* (Fig. 3A, lanes 3 and 4). Although several induction protocols based on variations in incubation temperature, induction period, and IPTG concentration were tested, no significant improvements on the solubilization of rRVg were achieved (data not shown). We therefore expressed the rRVg as inclusion bodies and purified it under denaturing conditions. The inclusion bodies of rRVg were solubilized in 6 M urea and purified by Ni^{2+} affinity chromatography using His Bind Resin. Peak A_{280} fractions eluted from the column were pooled and showed a predominant single rRVg band (Fig. 3A, lane 8). The yield of purified rRVg was 42 mg/L of cell culture (Table 2).

The purification of rRVg from *E. coli* was carried out under denaturing conditions (6 M urea). To remove the urea from the preparation, the purified rRVg was dialyzed against different types of dialysis buffers. Dialysis buffers A and B led to protein precipitation, whereas no precipitate was observed using dialysis buffer C (Fig. 3B) – nearly all of the purified rRVg was recovered in a soluble state (Table 2). The rRVg remained soluble even when stored at 4 °C or −20 °C.

Immunodetection of rRVg

To investigate the reactivity of rRVg, rabies-immune serum, which was determined to be positive for RV by RFFIT, was used

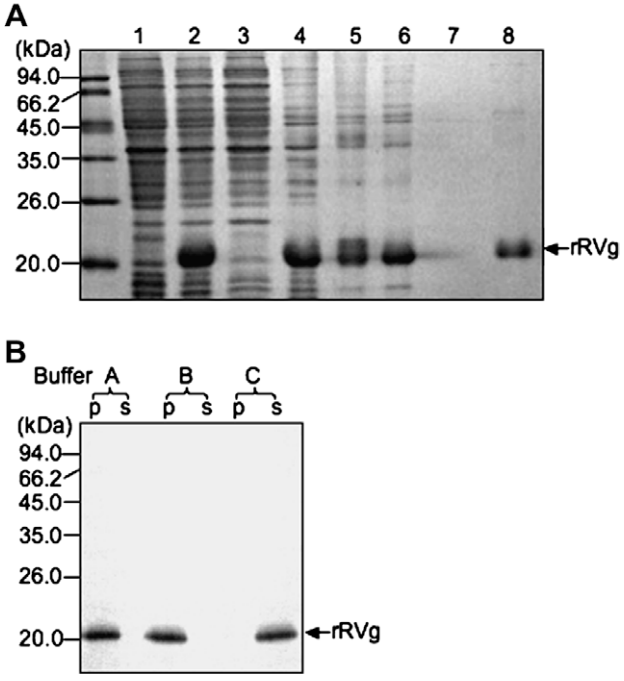

Fig. 3. (A) Analysis of the expression and purification of rRVg. Bacterial lysates and purified rRVg were separated on a 15% SDS–PAGE gel and stained with Coomassie blue R250. Lane 1, uninduced bacterial cells; lane 2, bacterial cells induced with isopropyl-β-D-thiogalactopyranoside (IPTG); lane 3, supernatant from the sonicated IPTG-induced bacterial cells; lane 4, pellet from the sonicated IPTG-induced bacterial cells; lane 5, pellet washed with 2% sodium deoxycholate; lane 6, 6 M-urea-solubilized protein; lane 7, flow-through of the His Bind Resin column; lane 8, rRVg eluted from the His Bind Resin column. (B) Solubilization of the purified rRVg. The purified rRVg was dialyzed against buffers A, B or C, respectively. The precipitates (p) and supernatants (s) were separated on 15% SDS–PAGE gel and stained with Coomassie blue R250.

Table 2
Purification of rRVg from 500 mL culture.

Fraction	Total protein[a] (mg)	Yield[b] (mg)	Purity[b] (%)	Recovery (%)
Cell lysate	321	132	41	100
Pellet after sonication	156	81	52	61.4
Pellet after washing[c]	110	72	66	54.5
rRVg in 6 M urea[d]	92	63	68	47.7
Eluted rRVg[e]	23	21	91	15.9
Dialyzed rRVg[f]	20	19.6	98	14.8

[a] Total protein was determined by BCA protein assay kit (Pierce Biotechnology).
[b] Yield and purity were calculated from densitometric scanning of stained gels.
[c] Insoluble pellet washed with 2% sodium deoxycholate.
[d] rRVg inclusion bodies solubilized in 6 M urea buffer.
[e] rRVg eluted from His Bind Resin.
[f] rRVg dialyzed against buffer C.

in a Western blot analysis. The purified rRVg and an unrelated recombinant protein, His-tagged influenza virus M1 protein (unpublished data), were probed with anti-His antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then re-probed with the rabies-immune serum or a rabies-negative serum control. The His-tagged M1 was used as a negative control to exclude the possibility that the rabies-immune serum reacted with His tag. As shown in Fig. 4, the rabies-immune serum (positive serum) specifically recognized a distinct 20.1 kDa protein corresponding to rRVg. In contrast, the rabies-negative serum control (negative serum) did not react with rRVg.

Competition of neutralization activity of rabies-immune serum

Since the rRVg was recognized by the rabies-immune serum in the Western blot analysis (Fig. 4), we determined whether the antibodies present in the rabies-immune serum reacting with rRVg were neutralizing to RV. The neutralizing activity of the rabies-immune serum was evaluated in the presence of varying amounts of rRVg from 0.01 to 3.2 μM. As shown in Fig. 5, the neutralizing titer of the rabies-immune serum was significantly reduced in the presence of rRVg (0.8–3.2 μM) compared with that in the absence of rRVg ($p < 0.01$). These results suggested that the rRVg was recognized by rabies-neutralizing antibodies.

Discussion

The objective of our study was to produce and immuno-characterize a recombinant protein comprising the multi-neutralization

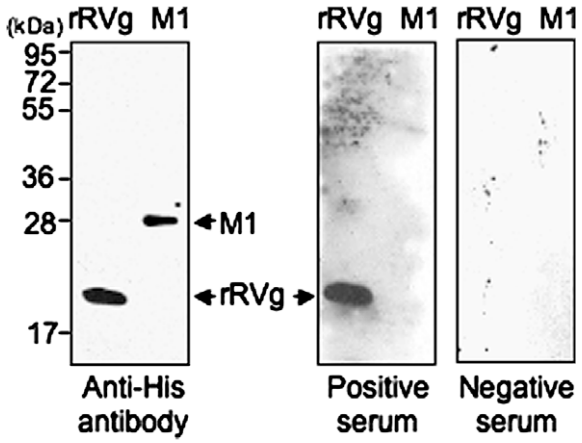

Fig. 4. Analysis of the reactivity of rRVg. The purified rRVg and His-tagged M1 (influenza virus M1 protein) were electrotransferred to an Immobilon-P membrane after SDS–PAGE, probed with anti-His antibody, then re-probed with rabies-immune serum (positive serum) or rabies-negative serum control (negative serum).

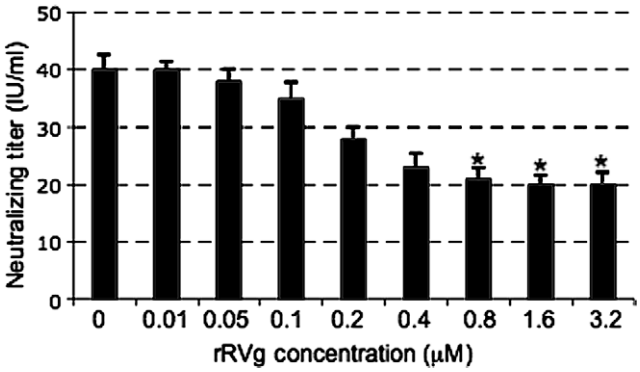

Fig. 5. Competition of the neutralizing activity of the rabies-immune serum by rRVg. The neutralizing titers (IU/mL) were determined in the presence of varying amounts of rRVg. Data are present as means ± standard error from 3 experiments. Asterisks indicate statistical significance ($p < 0.01$) by Duncan's multiple range test.

sites of the RV G protein. The G protein is a major antigenic stimulus of host immune system during infection and vaccination, specifically inducing neutralizing antibodies [4]. Using an *E. coli* expression system to generate large amounts of individual proteins potentially provides an economic source of antigens to replace live virus [13]. Although glycosylation is important for the conformational structure of the G protein antigenic sites, the linear neutralization sites expressed in *E. coli*, which lacks the ability to perform glycosylation modification, has been reported to be antigenic and recognized by rabies-neutralizing antibodies [9]. Therefore, we decided to use an *E. coli* expression system to produce RV G protein.

In order to increase its antigenicity, we excised the gene fragments that do not encode the linear neutralization sites from the G protein gene, and spliced together in tandem three gene fragments that encode polypeptides (corresponding to amino acid residues 19–60, 181–219, and 300–458) comprising the linear neutralization sites of the G protein (Fig. 1). The engineered fragment of the G protein gene was expressed in *E. coli* and its expression product, designated rRVg, was purified (Fig. 3A). Since one of our interests was the generation of rRVg as a diagnostic antigen adequate for detecting RV-neutralizing antibodies. The antigenicity of the rRVg was analyzed by both Western blot and competitive neutralization assay. The purified rRVg was recognized by the rabies-immune serum (Fig. 4) and able to significantly reduce the neutralizing activity of the rabies-immune serum (Fig. 5), showing good antigenicity. In the competitive neutralization assay, nearly half of the neutralizing titer still remained in the presence of 0.8–3.2 μM rRVg (Fig. 5). This is probably due to the nature of rabies-immune serum, which contains a variety of neutralizing antibodies recognizing different types of neutralization sites. The neutralization sites of G protein are classified into conformational sites and linear, non-conformational sites [6,7]. Presumably, the rRVg reacted with the neutralizing antibodies that recognize the linear neutralization sites, whereas the remained neutralizing titer was for conformational sites.

Although it is convenient, safe, and cheap to use recombinant *E. coli* DNA technology as a source of antigen, high-level protein expression frequently results in the formation of inclusion bodies. Several expression strategies – such as co-expression of molecular chaperones, use of thioredoxin-deficient host strains, and use of highly soluble polypeptides as fusion partners – have been developed to solve this problem. However, the success of these strategies appears to be protein specific [14,15]. In addition, purification of the soluble target protein from the *E. coli* homogenate is not an easy task because the homogenate consists of the vast majority of the total cellular proteins. Taking advantage of the properties of inclusion

bodies – including high yields, easy purification, and resistance to proteolytic degradation [14] – we expressed the rRVg as inclusion bodies after failing to express it as soluble protein (Fig. 3A). By using centrifugation and affinity chromatography, we purified the rRVg efficiently and with a high degree of purity (Fig. 3A).

Because the purification of rRVg from *E. coli* was performed under denaturing conditions (6 M urea), removal of the urea from the purified rRVg was attempted by dialysis method. Dialysis against conventional dialysis buffers (buffer A, 0.01 M PBS pH 7.2; buffer B, 0.05 M Tris–HCl pH 8.0) resulted in protein precipitation (Fig. 3B). Extreme pH conditions frequently lead to the solubilization of inclusion bodies [14]. Because we wished to use rRVg as a diagnostic antigen in ELISA, we dialyzed the purified rRVg against an alkaline buffer of 0.05 M Na_2CO_3 pH 9.6 (buffer C), which is frequently used as a coating buffer in ELISA. Nearly all of the purified rRVg was recovered in a soluble state (Fig. 3B, Table 2). The rRVg remained soluble even when stored at 4 °C or −20 °C and showed good reactivity with rabies-immune serum (Figs. 4 and 5).

Rabies vaccines induce predominantly antibodies to internal viral proteins [10], however, the current rabies-ELISA kit uses whole virus as the diagnostic antigen, which may lead to erroneous interpretation of the immune state of vaccinated host. Several studies have evaluated the potential of heterologously expressed G protein as a diagnostic antigen for monitoring RV-neutralizing antibodies [9,10]. In the present study, rRVg was able to react with rabies-immune serum and reduce its neutralizing activity, thus showing potential as a diagnostic antigen for detecting the neutralizing antibodies of RV. More detailed investigation of the immunological properties of rRVg is underway in our laboratory.

Acknowledgments

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