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Preparation of divalent antigen-displaying enveloped virus-like particles using a single recombinant *Bombyx mori* nucleopolyhedrovirus bacmid in silkworms

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Abstract

Silkworms have been used as a host for the production of recombinant proteins in a baculovirus expression system using *Bombyx mori* nucleopolyhedrovirus (BmNPV). To coexpress several recombinant proteins, a silkworm must be coinfected with several recombinant BmNPVs, which requires a difficult DNA manipulation procedure. In this study, we constructed recombinant BmNPVs containing three expression cassettes, Rous sarcoma virus (RSV) Gag protein, surface antigen 1 of *Neospora caninum* (NcSAG1) and SAG1-related sequence 2 of *N. caninum* (NcSRS2), by Gibson assembly and the Bac-to-Bac system, designated BmNPV/SAG-SRS-Gag and BmNPV/SAG-Gag-SRS. BmNPV/SAG-SRS-Gag was expressed in silkworms and characterized. NcSAG1 and NcSRS2 were purified with RSV Gag proteins using sucrose density gradient centrifugation and affinity chromatography. RSV Gag formed virus-like particles (RSV-LPs) at a diameter of 20–30 nm based on transmission electron microscopy (TEM). Immuno-TEM analysis showed that both NcSAG1 and NcSRS2 were displayed on the surface of the RSV-LPs. These results indicate that RSV-LPs displaying two different kinds of proteins were produced in the hemolymph of silkworm larvae by the single polycistronic strategy. This expression platform is efficient for generating multiantigen-displaying VLPs and facilitates the development of vaccines against infectious diseases.

Keywords: BmNPV, coexpression, polycistronic, virus-like particle, *Neospora caninum*

1. Introduction

Viral structural proteins form virus-like particles (VLPs) without any assembly steps during the protein expression process regardless of the presence of the viral envelope (Zeltins, 2013). VLPs mimic viruses in terms of their structure. However, since they do not have any viral genetic materials, VLPs have been applied to vaccine platforms and drug delivery strategies as antigens and carriers (Charlton Hume et al., 2019).

To form intact VLPs, several structural proteins that resemble virions morphologically must be simultaneously expressed. For example, triple-layered rotavirus-like particles can be generated by coexpression of VP2, VP6 and VP7 in insect cells (Shoja et al., 2015). Thus, to mimic virus particles, several proteins can be expressed by a coexpression strategy, such as matrix 1 protein, hemagglutinin and neuraminidase in influenza VLPs (Kang et al., 2009). Additionally, the coexpression of structural and other proteins can generate hybrid VLPs containing full-length proteins from distinct viruses and recombinant protein-displaying VLPs (Choi et al., 2018; Ludwig and Wagner, 2007).

Insect cells can produce many kinds of VLPs by the baculovirus expression system and stably transformed expression system (Lee et al., 2011; Shoja et al., 2015; Sokolenko et al., 2012). The baculovirus expression system is the first choice to produce recombinant proteins in insect cells because of its ease of use and rapidity. To coexpress several recombinant proteins in the baculovirus expression system, normally, several kinds of recombinant baculoviruses have to infect insect cells simultaneously (coinfection strategy). This method causes an imbalanced expression of the recombinant proteins due to incomplete coinfection, which leads to the formation of incomplete VLPs (Sokolenko et al., 2012).

Alternatively, to achieve the simultaneous coexpression of recombinant proteins in the baculovirus expression system, a recombinant baculovirus containing several protein expression cassettes (a single polycistronic strategy) has been used (Latham and Galarza, 2001; Pushko et al., 2005). In this case, only one recombinant baculovirus is used to infect insect cells, which leads to a more homogeneous expression of recombinant proteins than in the case of the coinfection strategy (Shanks and Lomonossoff et al., 2000). However, the construction of this type of recombinant baculovirus is very tedious and time-consuming. Recently, several gene cloning techniques have been developed to replace the conventional DNA ligase-based cloning method. A MultiBac system that is based on recombination technology using different recombinases facilitates the expression of many kinds of protein complexes, including VLPs (Pelosse et al., 2017). The uracil-specific excision reagent (USER) method with the MultiBac system has also been applied for the expression of protein complexes (Zhang et al., 2016). The biGBac system, based on the Gibson assembly principle, achieved the simultaneous expression of 17 subunits of human anaphase promoting complex/cyclosome using a single recombinant baculovirus (Gibson et al., 2009; Weissmann et al., 2016). This single polycistronic strategy is also very useful for the expression of protein complexes in insect cells.

Previous studies have employed *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) as a vector in insect cells, while *Bombyx mori* nucleopolyhedrovirus (BmNPV) has been used in silkworm. Silkworm larvae and pupae are favorable hosts for the large-scale production of recombinant proteins due to their high protein production capacity (Kato et al., 2010; Motohashi et al., 2005; Usami et al., 2010), including producing VLPs (Masuda et al., 2018; Minkner et al., 2018; Watanabe et al., 2017; Xu et al., 2019). In this study, antigen-displaying enveloped VLPs were

expressed in silkworm larvae using a single recombinant BmNPV containing three protein expression cassettes. A single polycistronic strategy was also suitable for the expression of intact VLPs displaying these antigens in silkworm larvae compared to the coinfection strategy.

2. Materials and methods

2.1. Cell culture and silkworm rearing

Bm5 cells were maintained in Sf-900 II medium (Thermo Fisher Scientific K. K., Tokyo, Japan) supplemented with antibiotic-antimycotic solution (Thermo Fisher Scientific K. K.) and 10% FBS (Thermo Fisher Scientific K. K.) at 27°C. The fourth-instar silkworm larvae were purchased from Ehime Sansyu (Ehime, Japan). Silkworm larvae were reared on an artificial diet, Silkmate 2S (Nosan, Yokohama, Japan), at 25°C.

2.2. Construction of recombinant baculoviruses

The recombinant BmNPV containing the Rous sarcoma virus Gag protein expression cassette (BmNPV/Gag) was previously constructed (Deo et al., 2011). To express surface antigen 1 of *Neospora caninum* (NcSAG1) fused with the C-terminal domain of GP64 from BmNPV (BmGP64), BmNPV/SAG1-GP64TM constructed previously was used (Kato et al., 2015). To express SAG1-related sequence 2 of *N. caninum* (NcSRS2) fused with the C-terminal domain of GP64 from BmNPV (BmGP64), BmNPV/SRS2-GP64TM, which was constructed previously, was also used (Kato et al., 2015).

To construct a single polycistronic BmNPV containing Gag, NcSAG1 and NcSRS2

114 expression cassettes, the expression cassettes were first amplified by PCR using three
115 primer sets {set 1 (α-1-F and β-1-R), set 2 (β-2-F and γ-2-R), set 3 (γ-3-F and ω-4-R)}
116 (Table 1). Additionally, a linear pFastbac1 vector (Thermo Fisher Scientific K. K.) was
117 prepared by PCR using primer set 4 (ω-pFB-F and α-pFB-R) (Table 1). These DNA
118 fragments (100 ng each) were assembled by Gibson assembly (Gibson et al., 2009;
119 Gibson 2011). Then, pFB/SAG-SRS-Gag and pFB/SAG-Gag-SRS were constructed (Fig.
120 1). These plasmids were individually transformed into *Escherichia coli* BmDH10Bac
121 (Motohashi et al., 2005), and BmNPV/SAG-SRS-Gag and BmNPV/SAG-Gag-SRS
122 bacmids were extracted from white colonies.
123 These recombinant BmNPV bacmids (10 μg DNA containing the helper plasmid)
124 were injected into silkworm larvae using chitosan (Kato et al., 2016). After 6–7 days,
125 hemolymph was collected from recombinant BmNPV bacmid-injected silkworm larvae.
126 Virus titers in the hemolymph were determined according to a protocol reported
127 previously (Kato et al., 2009). Briefly, the genomic DNA was extracted from hemolymph
128 by High Pure Viral Nucleic Acid Kit (Roche Diagnostics K. K., Tokyo, Japan) according
129 to the manufacturer's protocol. Quantitative PCR (qPCR) was carried out using
130 THUNDERBIRD SYBR qPCR Mix（TOYOBO, Otsu, Japan）with Bm-ie1 F and Bm-
131 ie1 R primers (Table 1). Mx3000P system (Stratagene, La Jolla, CA, USA) and MxPro
132 software (Stratagene) was used for the analysis.
133
134 *2.3. Antigen-displaying VLPs in silkworm larvae*
135 Using the coinfection strategy, three recombinant BmNPVs (BmNPV/Gag,
136 BmNPV/SAG1 and BmNPV/SRS2) with the same titer (1×10^8 pfu) were injected into
137 silkworm larvae. Using the single polycistronic strategy, BmNPV/SAG-SRS-Gag or

BmNPV/SAG-Gag-SRS was injected into silkworm larvae. After 4 days, the hemolymph and fat body were collected. 1-Phenyl-2-thiourea (200 mM) was added to the hemolymph at 2 mM, and 0.1 g of the fat body was suspended in 1 mL of Tris-buffered saline (pH 7.6) containing 0.1% Triton X-100. The fat body was disrupted by sonication, and the homogenate was centrifuged at 10,000 × g. The supernatant was used as an extract of the fat body.

For the purification of VLPs, the collected hemolymph was centrifuged at 10,000 × g. The resulting supernatant (4 mL) was centrifuged on a 20% sucrose cushion at 122,000 × g. The pellet was suspended in phosphate-buffered saline (PBS, pH 7.4) by sonication. The suspension was subjected to 20–60% sucrose density gradient centrifugation at 122,000 × g for 3 h. The fractions containing VLPs were subjected to anti-DDDDK tag affinity chromatography. Approximately 100 μL of anti-DDDDK tag purification gel (Medical & Biological Laboratories, Nagoya, Japan), which was equilibrated with PBS, was added into the fractions and incubated at 4°C with gentle stirring for 1 h. The gel was washed with PBS, and proteins were eluted with 0.1 M glycine-HCl (pH 3.0) in each fraction (100 μL). Immediate neutralization was carried out by the addition of 1 M Tris-HCl (pH 9.0).

Genomes of recombinant BmNPVs were extracted by a High Pure Viral Nucleic Acid Kit (Roche Diagnostics K. K., Tokyo, Japan) according to the manufacturer's protocol. To detect the genomes of recombinant BmNPVs, a part of the *ie-1* gene of BmNPV was amplified by PCR. PCR was performed with the ie-1 primer set Bm-ie1 F and Bm-ie1 R (Table 1) using the extracted recombinant BmNPV DNA as a template.

2.4. SDS-PAGE and western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to separate proteins using 12% acrylamide. After SDS-PAGE, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). The membrane was blocked in 5% skim milk in TBST (pH 7.6), followed by incubation with 10000-fold-diluted mouse anti-DDDDK tag monoclonal antibody (Medical & Biological Laboratories) for NcSAG1 and NcSRS2 detection or 1000-fold-diluted mouse anti-Gag serum for RSV Gag detection. As a secondary antibody, 10000-fold-diluted horseradish peroxidase (HRP)-linked goat anti-mouse IgG (Medical & Biological Laboratories) was used. Detection of proteins was performed using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA). Protein bands were detected on a Fluor-S MAX Multi-Imager (Bio-Rad).

2.5. Transmission electron microscopy (TEM)

Purified samples were loaded on a TEM grid (649, Nisshin EM, Tokyo, Japan) and stained with 2% phosphotungstic acid. Particles were observed by JEM-2100F (JEOL, Tokyo, Japan) operated at 100 kV. For immuno-TEM, TEM grid-loaded samples were blocked with 1% BSA, followed by washing with PBS 3 times. Then, the grid was incubated with 100-fold-diluted mouse anti-DDDDK tag monoclonal antibody (MEDICAL & BIOLOGICAL LABORATORIES). As a secondary antibody, 100-fold-diluted anti-IgG＋IgM (H＋L), Mouse, Goat-Poly, Gold 10 nm, EM (EMGAF10, BBI, Solutions, Crumlin, UK) was used. Staining with phosphotungstic acid was carried out before observation by TEM.

3. Results and discussion

3.1. Construction of recombinant BmNPV

Recombinant BmNPVs BmNPV/Gag, BmNPV/NcSAG1 and BmNPV/NcSRS2 (Fig. 1A–C) was previously constructed for the expression of RSV Gag protein, NcSAG1, and NcSRS2 in silkworms, respectively (Deo et al., 2011; Kato et al., 2015). The signal sequence of NcSAG1 or NcSRS2 at the N-terminus was deleted, and the signal sequence of bombyxin from *B. mori* was fused at the N-terminus instead. Additionally, the transmembrane and cytoplasmic domains of GP64 from BmNPV were also fused with NcSAG1 and NcSRS2 at the C-terminus for the display of NcSAG1 and NcSRS2 on the RSV-LPs. Using these recombinant BmNPVs, NcSAG1 and NcSRS2-displaying RSV-LPs were prepared by coinfection strategy (Xu et al., 2019). In this study, we attempted to construct a single recombinant BmNPV containing three expression cassettes by Gibson assembly (Gibson et al., 2009). We constructed two recombinant BmNPVs (BmNPV/SAG-SRS-Gag and BmNPV/SAG-Gag-SRS2) containing the three expression cassettes described in Fig. 1D and E.

3.2. Expression of RSV-LPs

To prepare NcSAG1 and NcSRS2-displaying RSV-LPs, these three proteins were coexpressed in silkworm larvae. Three different recombinant BmNPVs (BmNPV/Gag, BmNPV/NcSAG1, and BmNPV/NcSRS2) were coinjected to express the three proteins. RSV Gag (61 kDa) was observed in both the hemolymph and fat body, but NcSAG1 and NcSRS2 were observed only in the fat body (Fig. 2A). This result suggested that most

NcSAG1 and NcSRS2 proteins were expressed in the fat body and that a small amount may be displayed on the RSV-LPs. Estimated molecular weight of NcSAG1 and NcSRS2 was 36 and 40 kDa, respectively (Kato et al., 2015). However, NcSAG1 was detected at between 40 and 50 kDa in the fat body by western blot as several bands and NcSRS2 was also detected at between 50 and 60 kDa as a single band. Now this discrepancy of each molecular weight is unknown, but some post-translational modification including *N*-glycosylation might be carried out. Several bands of NcSAG1 detected in the fat body might be caused by some post-translational modification.

In addition to coinfection of three recombinant BmNPVs, the three proteins were coexpressed in silkworm larvae using single recombinant BmNPV, BmNPV/SAG-SRS-Gag or BmNPV/SAG-Gag-SRS2 (Fig. 2B). The expression pattern of each protein by BmNPV/SAG-SRS-Gag or BmNPV/SAG-Gag-SRS2 was almost the same as that in the coinfection of the three recombinant BmNPVs. However, the expression level of each protein using BmNPV/SAG-SRS-Gag (lane 2 of Fig. 2A and B) was higher than that obtained using the coinfection strategy with the three recombinant BmNPVs. This result suggests that the reduction in the burden of multiple recombinant BmNPV infections by the use of the single polycistronic strategy may lead to the improvement of the expression level of recombinant proteins. BmNPV/SAG-Gag-SRS2 also produced a larger amount of NcSAG1 compared to the coinfection strategy, but only a faint band of NcSRS2 was detected compared to that observed with BmNPV/SAG-SRS-Gag. This result suggests that the order of the three expression cassettes may have affected their expression levels. Using an anti-Gag antibody, several proteins were detected below the full-length Gag protein (Fig. 2A). These protein bands may be produced by the processing of expressed Gag protein because Gag protein is processed into a protein matrix consisting of p2, p10

234 and capsid domains in insect cells (Deo et al., 2011).

235 RSV-LPs collected from the hemolymph expressed using the single polycistronic
236 strategy were concentrated and purified using sucrose density gradient centrifugation (Fig.
237 3). Using BmNPV/SAG-SRS-Gag, NcSAG1 and NcSRS2 were detected with Gag
238 protein in the same fractions (fractions 4–7), even though these two proteins were not
239 detected in the hemolymph (Fig. 2B). These results suggested that NcSAG1 and NcSRS2
240 were displayed on the RSV-LPs. However, NcSRS2 was hardly observed for
241 BmNPV/SAG-Gag-SRS. This result corresponded to the expression level of each protein
242 in Fig. 2B. To confirm the display on the RSV-LPs, RSV-LPs in fractions 4–7 were
243 purified by affinity chromatography. The three proteins were detected in the same elution
244 fractions using BmNPV/SAG-SRS-Gag (Fig. 4A). However, more NcSAG1 was detected
245 than NcSRS2, which was observed as only a faint band. This result indicates that
246 NcSAG1 and NcSRS2 are displayed on the RSV-LPs but not at the same amounts. This
247 result corresponded to the expression level of each protein in Figs. 2 and 3. In our previous
248 study, NcSAG1 and NcSRS2 were displayed on BmNPV particles (Kato et al., 2015). To
249 investigate the possibility of NcSAG1 and NcSRS2 being displayed on the BmNPV
250 particles, detection of BmNPV genomic DNA was carried out in the elution fractions by
251 PCR to amplify a part of the ie-1 gene. Amplified DNA fragments were detected in
252 fraction 1, but NcSAG1 and NcSRS2 were not detected in fraction 1. On the other hand,
253 in fractions 2–4, no amplified DNA fragments were detected, and the three recombinant
254 proteins were detected using BmNPV/SAG-SRS-Gag. This result indicates that NcSAG1
255 and NcSRS2 were displayed on the RSV-LPs. From 1 mL of hemolymph, 3.0 and 2.7 μg
256 of RSV-LPs were prepared using BmNPV/SAG-SRS-Gag and BmNPV/SAG-Gag-SRS,
257 respectively.

The morphology of RSV-LPs purified by affinity chromatography was investigated by TEM. Particles were 20–30 nm in diameter (Fig. 5A), and this size is smaller than that in previous reports (Deo et al., 2011; Kato et al., 2015). In this study, RSV-LPs were sonicated to be suspended after precipitation from the hemolymph by ultracentrifugation. In addition, acidic buffer was used to elute RSV-LPs from an anti-DDDDK tag purification gel. These steps may cause the difference in the particle size between this study and previous studies. To confirm the display of NcSAG1 and NcSRS2 on the surface of RSV-LPs, immuno-TEM was carried out. Gold particles were observed on the surface of the RSV particles (Fig. 5B and C), indicating that NcSAG1 and NcSRS2 were displayed on the surface of RSV-LPs because both NcSAG1 and NcSRS2 were fused with the FLAG tag.

We previously reported that NcSAG1 and NcSRS2-displaying RSV-LPs, which were prepared in silkworms by the coinfection strategy using three different recombinant BmNPVs, were not sufficient to prevent *N. caninum* infection in mice (Xu et al., 2019). Optimization of each recombinant baculovirus, which is often difficult, has to be carried out to balance the expression of each protein when multiple proteins are coexpressed by the use of multiple recombinant baculoviruses (Mena et al., 2007). In silkworm larvae and pupae, the single polycistronic strategy using a single recombinant baculovirus is better for the coexpression of recombinant proteins to produce protein complexes (Liu et al., 2020; Yao et al., 2012; Zhou et al., 2011). In this study, we achieved the coexpression of RSV Gag protein, NcSAG1 and NcSRS2 using the single polycistronic strategy in silkworm larvae to prepare divalent antigen (NcSAG1 and NcSRS2)-displaying RSV-LPs. Compared to the coinfection strategy, the single polycistronic strategy facilitated improved expression of multiantigen-displaying RSV-LPs in silkworm larvae. This

platform can be utilized for the preparation of antigen-displaying VLPs, regardless of the kind of VLPs (enveloped and nonenveloped) and will be helpful for the development of vaccines against infectious diseases.

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390 **Table 1.** Used primers

Name	5’-3’
α-1-F	AACGCTCTATGGTCTAAAGATTTACTCCGGAATATTAATAG
β-1-R	AAACGTGCAATAGTATCCAGTTTTAGATTTCACTTATCTGG
β-2-F	AAACTGGATACTATTGCACGTTTACTCCGGAATATTAATAG
γ-2-R	AAACATCAGGCATCATTAGGTTTTAGATTTCACTTATCTGG
γ-3-F	AAACCTAATGATGCCTGATGTTTACTCCGGAATATTAATAG
ω-3-R	AAACTAAGCTATGTGAACCGTTTTAGATTTCACTTATCTGG
ω-pFB-F	AAACACTGACATTGACTTGGTTTCCCGGTCCGAAGCGCGCG
α-pFB-R	AAATCTTTAGACCATAGAGCGTTCTATTAATATTCCGGAGT
Bm-ie1 F	CCCGTAACGGACCTTGTGCTT
Bm-ie1 R	TTATCGAGATTTATTTACATACAACAAG

391

392

393 **Figure legends**

394

395 **Fig. 1.** Constructs of recombinant BmNPVs used in this study. P_{PH} and SV40 pA indicate
396 the polyhedrin promoter and simian virus 40 polyadenylation sequence, respectively. α,
397 β, γ, ω indicate linker sequences for Gibson assembly (Weissmann et al., 2016). A,
398 BmNPV/Gag; B, BmNPV/SAG1; C, BmNPV/SRS2; D, BmNPV/SAG-SRS-Gag; E,
399 BmNPV/SAG-Gag-SRS.

400

401 **Fig. 2.** Western blot of expressed RSV Gag protein, NcSAG1, and NcSRS2 in silkworm
402 larvae by the coinfection and single polycistronic strategies. In the case of the coinfection
403 strategy, BmNPV/Gag, BmNPV/NcSAG1 and BmNPV/NcSRS2 were coinjected into
404 silkworm larvae at 1:1:1. In the case of the single polycistronic strategy, BmNPV/SAG-
405 SRS-Gag or BmNPV/SAG-Gag-SRS2 was injected into silkworm larvae. Hemolymph
406 and fat bodies were collected at 4 days post injection, and each sample was detected with
407 anti-Gag antibody (A) and anti-DDDDK (B). 1: Mock, 2: BmNPV/SAG-SRS-Gag, 3:
408 BmNPV/SAG-Gag-SRS, 4, 5: the coinfection strategy (the three recombinant BmNPVs
409 were mixed). Asterisks show protein bands detected by each antibody and # shows non-
410 specific bands.

411

412 **Fig. 3.** Sucrose density gradient centrifugation of hemolymph from silkworm larvae
413 injected with BmNPV/SAG-SRS-Gag (A) or BmNPV/SAG-Gag-SRS2 (B). The
414 hemolymph was centrifuged on a 25% sucrose cushion at 122,000 × g and resuspended
415 in PBS by sonication. The suspension was subjected to 25-60% sucrose density gradient
416 centrifugation. Each fraction was collected, and each protein was detected by western blot.

417 Asterisks show the bands detected by each antibody in fraction 4 to 7. RSV-LPs were
418 purified from collected fractions (fraction 4 to 7).
419
420 **Fig. 4.** Purification of NcSAG1 and NcSRS2-displaying RSV-LPs from the fractions of
421 sucrose density gradient centrifugation. The hemolymph was centrifuged on a 25%
422 sucrose cushion at 122,000 × g and suspended in PBS by sonication. The suspension was
423 subjected to 25–60% sucrose density gradient centrifugation. Fractions containing RSV
424 Gag protein, NcSAG1 and NcSRS2 were collected, and NcSAG1 and NcSRS2-
425 displaying RSV-LPs were purified by anti-DDDDK tag purification gel. The elution was
426 carried out with 0.1 M glycine-HCl buffer (pH 3.0). BmNPV/SAG-SRS-Gag (A) and
427 BmNPV/SAG-Gag-SRS2 (B) were used for the coexpression of the three recombinant
428 proteins, and the same volume of hemolymph was used for purification using both
429 recombinant BmNPV. Asterisks show the bands detected by each antibody in elution
430 fractions.
431
432 **Fig. 5.** Morphology of NcSAG1 and NcSRS2-displaying RSV-LPs purified by affinity
433 chromatography. A TEM image (A) and Immuno-TEM (B) of NcSAG1 and NcSRS2-
434 displaying RSV-LPs. (C) Magnified immuno-TEM image. Arrows indicate secondary
435 antibody-conjugated gold nanoparticles.

Fig. 1. Kato et al.

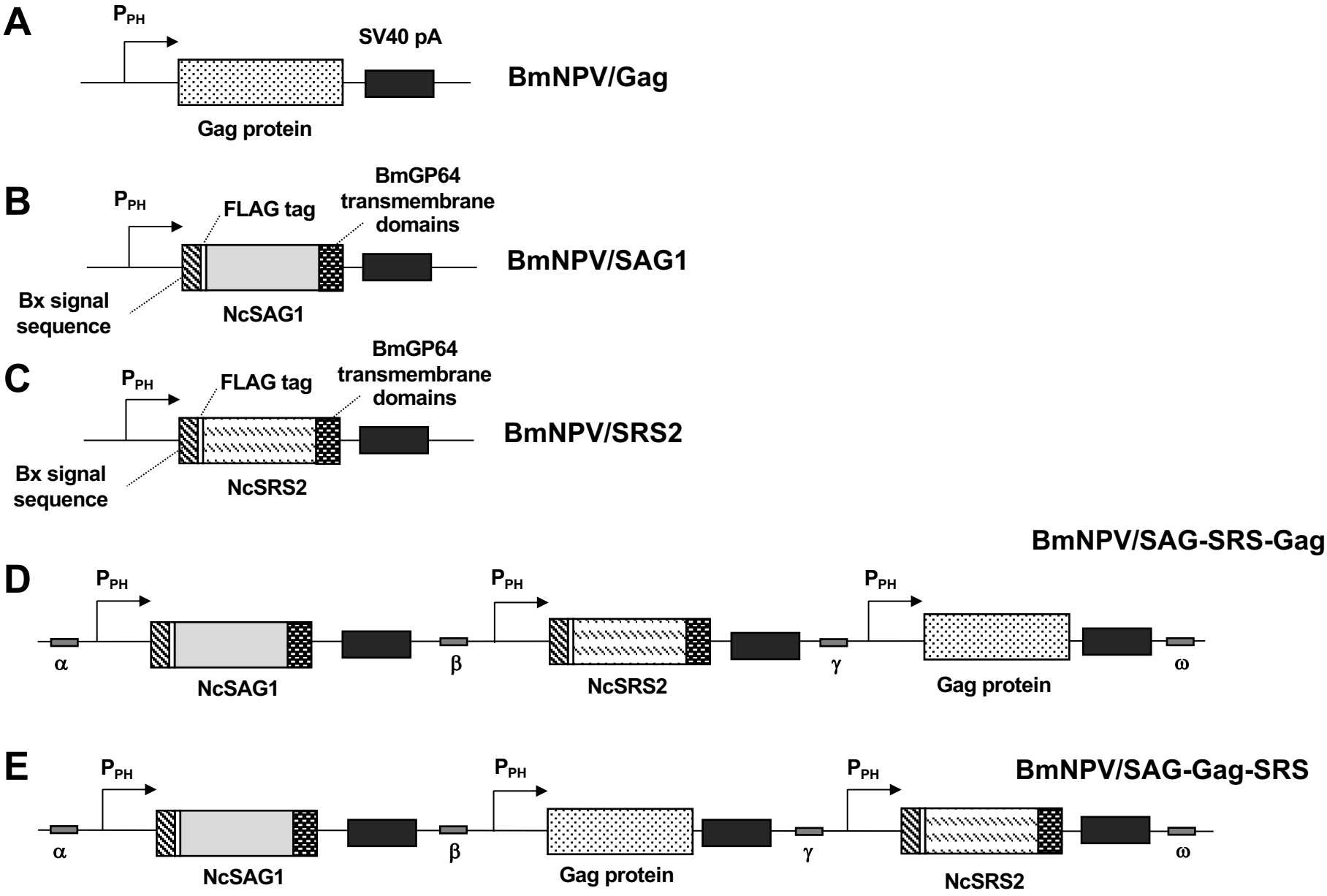

Fig. 2. Kato et al.

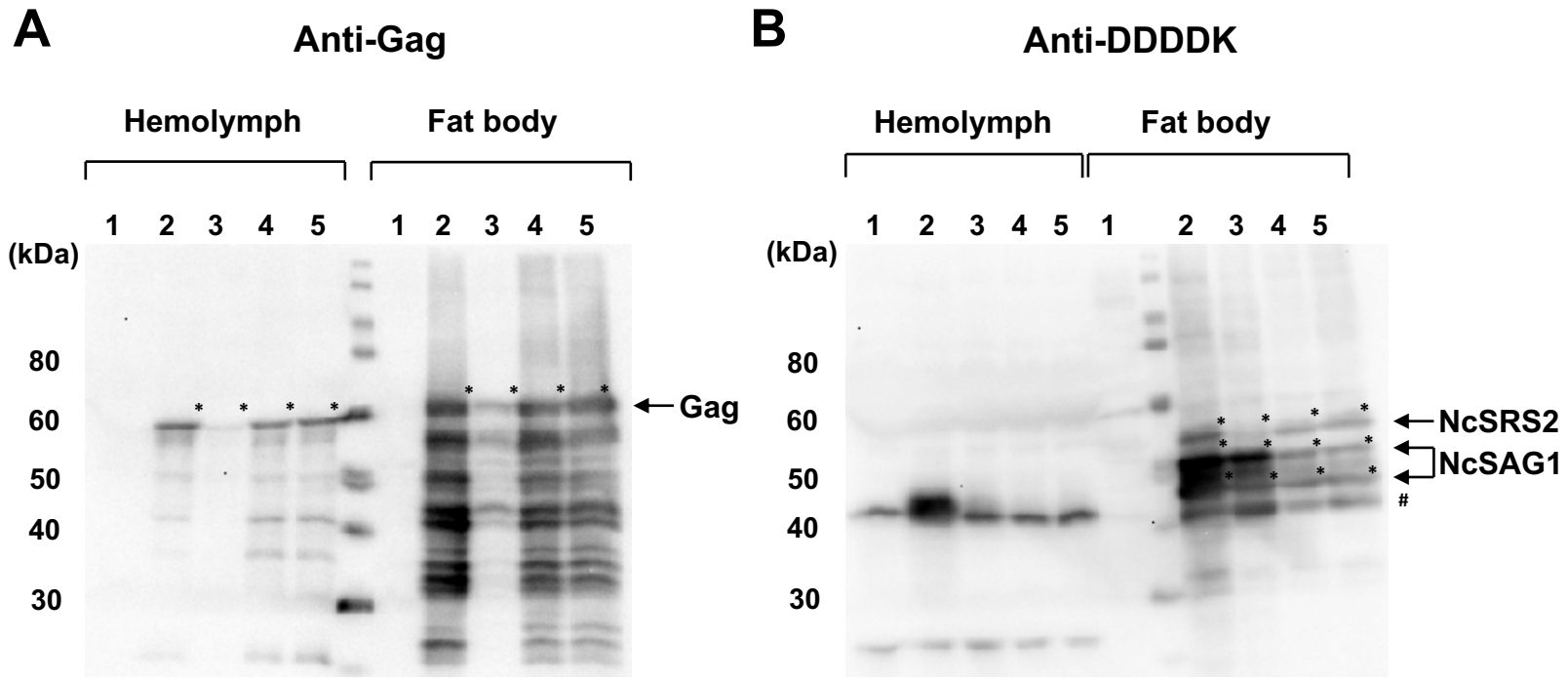

Fig. 3. Kato et al.

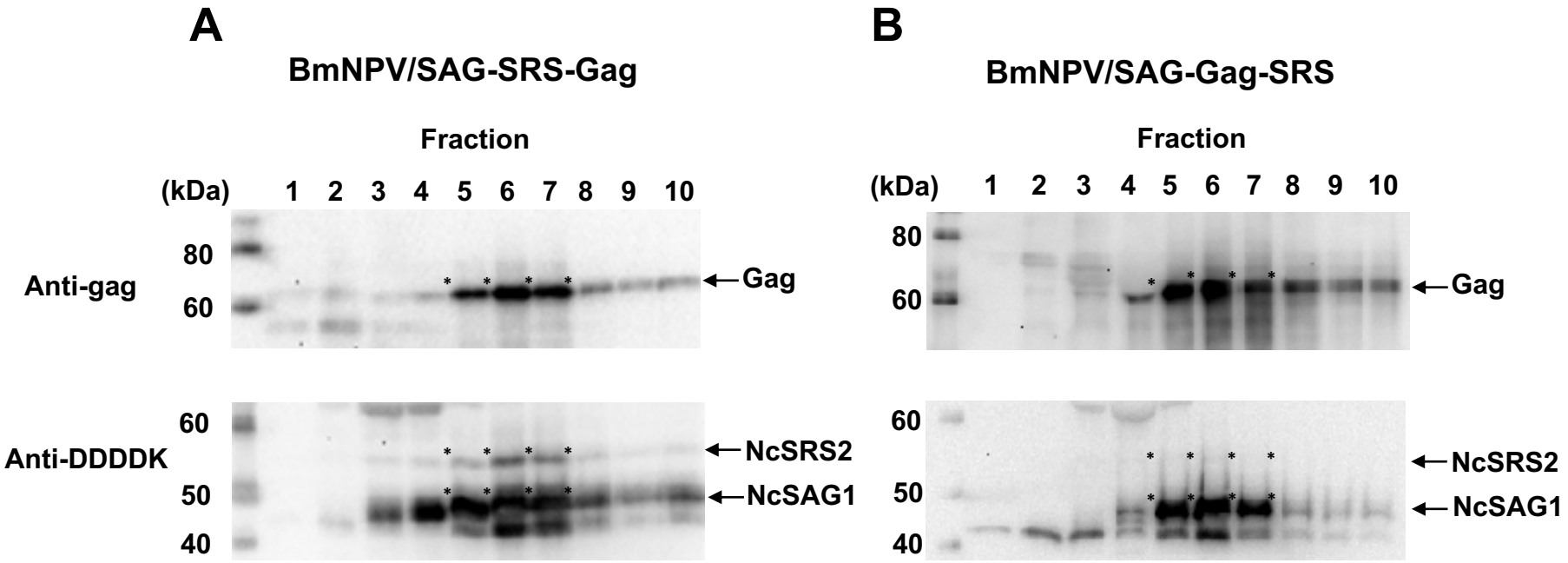

Fig. 4. Kato et al.

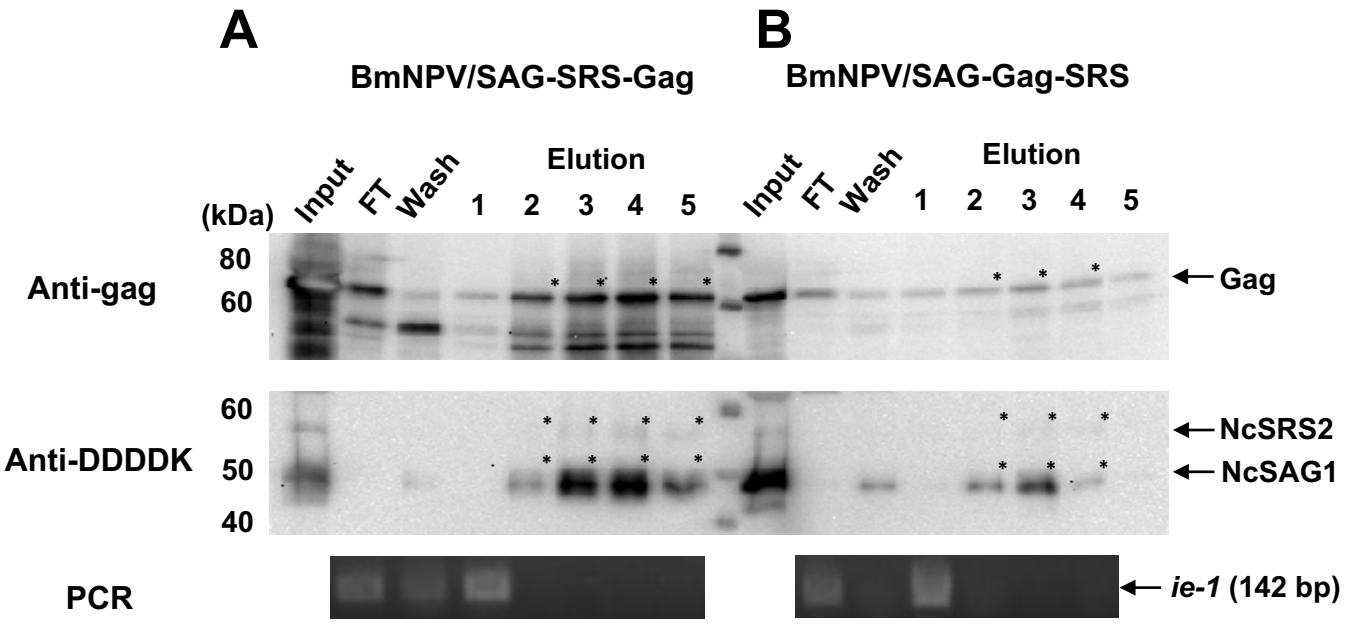

Fig. 5. Kato et al.