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Display of *Neospora caninum* surface protein related sequence 2 on *Rous sarcoma* virus-derived *gag* protein virus-like particles

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ABSTRACT

Virus-like particles (VLPs) displaying antigen have been increasingly recognized as a potential vaccine in the livestock industry. In this study, *Neospora caninum* surface protein related sequence (NcSRS)2 was displayed on the surface of *Rous sarcoma* virus group-antigen protein (RSV-gag) VLPs. Two types of *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmids, encoding RSV-gag and NcSRS2 genes, were co-injected into silkworm larvae to produce VLPs-NcSRS2. At 7 days post-injection, VLPs-NcSRS2 were collected from hemolymph and purified. The antigenicity of the purified protein was confirmed by enzyme-linked immunosorbent assay (ELISA) using neosporosis-positive bovine serum. ELISA revealed that ~0.16 μg rNcSRS2 was displayed per 1 μg VLPs-NcSRS2.To develop an antibody specific for VLPs-NcSRS2, purified VLPs-NcSRS2 were used to immunize mice in a three-dose regimen without adjuvant and the production of antibodies was confirmed in serum samples. By using a silkworm expression system, we demonstrated the display, expression and immunization of neosporosis-targeting membrane proteins, which are vaccine candidates for neosporosis.

Keywords: display technology; *Neospora caninum* surface protein related sequence 2; neosporosis; virus-like particle; silkworm expression system

1. Introduction

Virus-like particles (VLPs) are nanoscale biomaterials that are gaining relevance in nanoscience, owing to their small size and similarity to native virus (Deo et al., 2011). VLPs have properties similar to native viruses, without carrying genetic materials and without transmitting any kind of pathological infection, therefore, they are biologically safe materials. Practically any capsid protein that has self-folding properties can be used to make VLPs. This means that VLPs are more suited for vaccination programs by using them to display antigen and as a drug delivery system (Noad et al., 2003; Roy at al., 2008, 2009).

Rous sarcoma virus (RSV) species belong to the family *Retroviridae*, subfamily *Orthoretrovirinae,* genus *Alpharetrovirus* of single-stranded RNA viruses. Many studies have elucidated the structure of the group antigen protein (gag), which is important in the assembly and packaging of RNA into the virus and subsequent budding from the host cell membrane. The RSV-gag is composed of mature matrix, capsid, and nucleocapsid regions. The protease region is required to package the RNA genome, but is cleaved off later by viral proteases. The RSV-gag antigen codes for a 701-amino-acid protein of 61–75 kDa which dimerizes on the cell membrane to form VLPs of 100–200 nm surrounded by a lipid envelope (Ma et al., 2002). This lipid layer can be used to display membrane-bound proteins on the VLP surface.

Neosporosis is an infectious disease primarily of cattle and dogs, caused by *Neospora caninum*, an obligate intracellular protozoan parasite. *N. caninum* was first identified in dogs in Norway (Bjerkas et al., 1984), and has been found to infect a wide variety of mammals such as cattle, sheep, goats, deer and horses (Baszler et al., 1996; Dubey et al., 1996, 1997; Howe et al., 1997). Dogs are the definitive host of *N. caninum* whereas cattle are usually an intermediate host. Neosporosis causes abortion

57 in dairy cattle, leading to huge economic losses in the worldwide dairy industry
58 (Björkman et al., 1996). Although most studies of *N. caninum* have focused on
59 infections in dairy cattle (Howe et al., 2002), alternative approaches including early
60 detection of neosporosis or vaccination might be possible.
61 Proteins displayed on the surfaces of intracellular pathogens are believed to play
62 critical roles in the infection process (Nishikawa et al., 2002a). The *N. caninum*
63 surface associate antigen (NcSAG)1 and NcSAG1-related sequence (NcSRS)2 have
64 been identified as major surface antigen proteins of *N. caninum* tachyzoites. They are
65 immune-dominant and involved in interactions between tachyzoites and host cells
66 (Nishikawa et al., 2001). In particular, NcSRS2 is a glycosylphosphatidylinositol
67 (GPI)-anchored protein and localized on the plasma membrane through a GPI anchor.
68 The GPI anchor is a glycolipid that is attached to the C terminus of a protein during
69 post-translational modification and is important for the protein to be targeted toward
70 the plasma membrane. The GPI anchoe is composed of a phosphatidylinositol group
71 linked through a carbohydrate-containing linker (glucosamine and mannose
72 glycosidically bound to the inositol residue) and via an ethanolamine phosphate
73 bridge to the C-terminal amino acid of a mature protein. The two fatty acids within
74 the hydrophobic phosphatidylinositol group anchor the protein to the cell membrane.
75 The predominant antigenicity of NcSRS2 has also been demonstrated by recognition
76 by antisera from *Neospora*-infected animals (Nishikawa et al., 2000). These findings
77 highlight the need to pursue aggressively these surface proteins for development of
78 early detection as prospective vaccine candidates. NcSRS2 expressed and purified
79 using the mammalian expression system has shown promise in providing protection,
80 but with low antigenicity, owing to protein refolding difficulties, indicating that

mammalian expression systems are not suitable for the expression of *N. caninum* antigens (Tuo et al., 2011).

The silkworm expression system is able to express proteins efficiently at high levels using *Bombyx mori nucleopolyhedrovirus* (BmNPV), which belongs to the double-stranded DNA virus family *Baculoviridae* (Kato et al., 2010). Recently, by using a BmNPV bacmid-based expression system, it took only 1 month to express and purify protein at the milligram level in silkworm larvae (Dojima et al., 2010). Injecting two or more bacmids coexpresses two or more proteins in their complete functional form, which can then be purified. Silkworm larvae are completely domesticated insects and have been used in the textile industry for many centuries.

Previously, it has been reported that this coexpression system of a membrane protein and RSV-gag in silkworms allows generation of functional protein-displaying RSV VLPs (Tsuji et al., 2011). In the current study, the focus was on the production of macromolecular nanostructures using VLPs-rNcSRS2 in their functional form in silkworms by coexpression of RSV-gag and rNcSRS2. rNcSRS2 expression in insect cells using a baculovirus expression system with all the necessary post-translational modifications including GPI anchorage has been reported (Nishikawa et al., 2002b). In the current study, the feasibility of displaying GPI-anchored protein on macromolecular nanostructures like VLPs in silkworms was reported. Finally, the bioactivity of the functionalized VLPs-rNcSRS2 was tested by ELISA using serum from cattle with neosporosis. In addition, injecting VLPs-rNcSRS2 into BALB/c mice as a model confirmed their use as a vaccine candidate to elicit an immunogenic response. This is also important for large-scale application because vaccines can be made by fusing the robustness of the silkworm expression system and the ease of display of foreign proteins on VLPs.

2. Materials and Methods

2.1. Vectors

Bacmid/RSV-gag-577 and Bacmid/FLAG-NcSRS2 constructions have been reported previously (Deo et al., 2011; Dong et al., 2012). Glycerol stocks were inoculated into 100 ml LB medium (Wako Pure Chemical Industries, Osaka, Japan) and cultured at 37°C for 36 h. The bacmids were isolated and resuspended in PBS (pH 7.5) for injecting them into fifth-instar silkworm larvae.

2.2. Silkworm larvae rearing, feeding and injection

Fifth instars larvae (Ehime Sansyu, Ehime, Japan) were reared on an artificial diet, Silkmate S2 (Nihon Yokohama, Japan), for silkworm larvae in a chamber (MLR-351H, Sanyo, Tokyo, Japan) with 65% humidity at 27°C as described previously (Hiyoshi et al., 2007). Each silkworm was injected with 40 µl recombinant bacmid DNA solutions containing 10 µg BmNPV-gag577 and BmNPV-rNcSRS2 bacmid DNA, respectively, in 10% (v/v) DMREI-C reagent (Invitrogen, Carlsbad, CA, USA) in PBS using a 1-ml syringe. At 7 days post-injection, the hemolymph of the silkworms was harvested in tubes (Falcon, Lincoln Park, NJ, USA) containing 2 mM phenylthiourea to inhibit the hemolymph melanization. These samples were aliquoted into 1-ml Eppendorf tubes and stored at –80°C.

2.3. Purification of VLPs displaying NcSRS2 (VLPs-NcSRS2)

VLPs-NcSRS2 containing hemolymph collected from silkworm larvae were centrifuged at 1000×*g* in a Heraeus Primo R Sorvall Biofuge (Thermo Scientific, Yokohama, Japan) for 3 min using Heraeus 7591 swing bucket rotor to remove debris. The hemolymph was dialyzed with Cellu SepT3 membrane with a 12,000–14,000

molecular weight cut off (Membrane Filtration Products, Seguin, TX, USA) in 1.7 l of 10 mM Tris–HCl (pH 7.5) (Wako) for 3 h at 4°C. The dialyzed hemolymph was centrifuged at 14010×*g* (Kubota, Tokyo, Japan) for 10 min at 4°C to remove any aggregates, and the supernatant was filter-sterilized through a 0.45-μm filter membrane (Merck–Millipore, Billerica, MA, USA). The protein sample was loaded on to a 10 mM Tris–HCl (pH7.5) pre-equilibrated φ26 mm× 6 cm KX26 column (GE Healthcare Bio-Sciences, Uppsala, Sweden) with 30 ml DEAE-TOYOPEARL (TOSOH, Tokyo, Japan). The column was washed with 5 column volumes of 10 mM Tris–HCl buffer containing 125 mM NaCl, and the protein was eluted using 3.3 column volumes of 10 mM Tris–HCl buffer containing 300 mM NaCl (Wako) in a linear gradient. All the fractions were confirmed by western blotting and the fractions showing VLPs-NcSRS2 were pooled and loaded onto 50 mM Tris–HCl (pH 7.5), 150 mM NaCl buffer pre-equilibrated Superdex 200 10/300 column (GE Healthcare), to separate VLPs-NcSRS2 from gag-VLPs and rNcSRS2 by size exclusion chromatography (SEC). The protein was eluted in each 500-μl fraction and those showing VLPs-NcSRS2 were pooled and ultracentrifuged at 122,000×*g* (CS120GXII; Hitachi Koki, Tokyo, Japan) for 3 h at 4°C. The VLP-NcSRS2 pellets were resuspended in 500 μl 50 mM Tris–HCl (pH 7.5) containing 150 mM NaCl buffer and stored at –20°C. The protein concentrations were measured using standard BCA protein estimation kit (Pierce BCA Assay kit, Rockford, IL, USA).

2.4. Western blotting

To detect the expression of VLPs and rNcSRS2, larval hemolymph and fat bodies from silkworm larvae were collected, and one tablet of complete EDTA-free protease inhibitor cocktail was added (Roche Diagnostics, Mannheim, Germany) per

10 ml hemolymph. The samples were diluted using PBS (pH 7.5) and subjected to 10% (w/v) SDS-PAGE using the mini-protean II system (Bio-Rad, Hercules, CA, USA) (Deo et al., 2006). After SDS-PAGE, proteins were blotted on to a PVDF membrane using the Mini Trans-Blot Electrophoretic Transfer cell (Bio-Rad) at 15 V for 1 h. The membranes were probed with mouse anti-DDDDK primary antibody for rNcSRS2 and with anti-RSV-gag primary antibody for gag-577 for 1 h. Secondary antibodies were goat anti-mouse IgG for rNcSRS2 and goat anti-rabbit IgG gag-577. The secondary antibodies were labeled with horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. Specific bands for rNcSRS2 and gag-577 proteins were detected using an ECL prime western blotting reagent pack (GE Healthcare, Amersham, Bucks, UK). The rNcSRS2 and gag-577 proteins bands were analyzed using a Fluor-S/MAX multi-imager (Bio-Rad).

2.5. Transmission electron microscopy (TEM) of VLPs-rNcSRS2

The purified VLPs-rNcSRS2 samples were spotted onto carbon grids (Okenshoji, Tokyo, Japan) and dried at room temperature. Negative staining was performed using 2% (v/v) phosphotungstic acid (Wako) as described previously (Silverman et al., 1969; Vogt et al., 1999). For immunoelectron microscopy, the samples were loaded onto the grids in a similar fashion and the grids were blocked using 4% (w/v) bovine serum albumin (BSA) (Sigma–Aldrich, St Louis, MO, USA) for 1 h and washed with PBS (pH 7.5). The grids were incubated in PBS (pH 7.5) containing mouse anti-neospora H3 Fabs (Dong et al., 2013) at 1:1,000 dilutions specific for the middle region of NcSRS2 for 2 h, and washed with PBS (pH 7.5). Subsequently, the grids were incubated in PBS (pH 7.5) containing 1:20 diluted goat anti-mouse IgG conjugated with 10 nm gold particles (BB International, Cardiff, UK)

for 2 h, and washed with PBS (pH 7.5). Negative staining was performed using 2% (v/v) phosphotungstic acid. Samples were observed at 50,000× magnification (JEM 2100F-TEM; Jeol, Akishima, Japan) operating at 200 kV (Briggs et al., 2006).

2.6. Confirmation of the antigenicity of VLPs-rNcSRS2 using bovine serum

Three micrograms of VLPs-NcSRS2 or gag-VLPs in 100 μl volume was immobilized on each well of an immunoplate (Thermo Scientific, West Palm Beach, FL, USA), overnight at 4°C in triplicate. The plates were blocked with 100 μl/well Ez-block Chemi (ATTO, Tokyo, Japan) for 1 h at room temperature and then washed three times with 200 μl/well PBS (pH 7.5). The plates were incubated for 1 h at room temperature with 100 μl/well positive serum diluted 5,000-fold in PBS-T (PBS with 0.1% Tween-20) and negative serum (provided by Shizuoka Prefecture Tobu Livestock Disease Diagnostic Center, Japan) from infected or uninfected cattle. After incubation, the plates were washed three times with 200 μl/well PBS-T (pH 7.5) and incubated with anti-bovine IgG (H+L) conjugated with HRP (Jackson ImmunoResearch Laboratories, Baltimore, MD, USA) diluted 10,000-fold in PBS-T for 1 h at room temperature. After incubation, the plates were washed three times with 200 μl/well PBS-T (pH 7.5) and proteins were detected using 3,3'-,5,5'-tetramethylbenzidine (TMBZ) (Dojindo, Kumamoto, Japan) solutions by observing the absorbance at 450 nm with a Bio-Rad plate reader (Dong et al., 2012).

2.7. Mouse immunization and confirmation of antigenicity of rNcSRS2 displayed on VLPs using mice sera

A homologous prime-boost three-dose regimen was used to immunize five female BALB/c mice (7 weeks old) with 20 μg purified VLPs-NcSRS2 in 100 μl PBS

(pH 7.5). As a negative control, 100 µl PBS (pH 7.5) was injected alone. Immunization was performed by intramuscular injection after every 2 weeks. Blood was collected and serum isolated at 14 days after the final booster dose. Sera from VLP-NcSRS2 and PBS (pH 7.5) injected samples were labeled as positive and negative sera, respectively, before being aliquoted and stored at –80°C. All experiments were conducted in accordance with the guidelines approved by the Committee on the Ethics of Animal Experiments of Shizuoka University (Permit number: 24-11). One microgram of VLPs-NcSRS2 or VLPs was immobilized on an immunoplate overnight at 4°C in triplicate. The plates were blocked with 100 µl/well Ez-block Chemi (ATTO) for 1 h at room temperature and then washed three times with 200 µl/well PBS (pH 7.5). The plates were incubated with 100 µl/well positive or negative sera diluted 1,000-fold in PBS-T for 1 h at room temperature. After incubation, the plates were washed three times with 200 µl/well PBS-T (pH 7.5) and incubated with anti-mouse IgG conjugated with HRP (GE Healthcare) diluted 2,000-fold in PBS-T for 1 h at room temperature. After incubation, the plates were washed three times with 200 µl/well PBS-T (pH 7.5) and proteins were detected using TMBZ solutions by observing the absorbance at 450 nm using a Bio-Rad plate reader.

2.8. Phospholipase C treatment of VLPs-NcSRS2

Each well of the immunoplate was immobilized with 1 µg VLPs-NcSRS2 and VLPs, respectively, overnight at 4°C in triplicate. The plates were blocked with 100 µl/well Ez-block Chemi (ATTO) for 1 h at room temperature and washed three times with 200 µl/well PBS (pH 7.5). The plates were incubated with 100 µl/well positive and negative sera diluted 1,000-fold in PBS-T for 1 h at room temperature. After incubation, the plates were washed three times with 200 µl/well PBS-T (pH 7.5) and

231 incubated with ECL anti-mouse IgG conjugated with HRP (GE Healthcare) diluted
232 2,000-fold in PBS-T for 1 h at room temperature. After incubation, the plates were
233 washed three times with 200 µl/well PBS-T (pH 7.5) and the presence of the GPI
234 anchor from the lipid layer was confirmed by digestion with 0.1 U
235 phosphatidylinositol-specific phospholipase C (PI-PLC) (Sigma–Aldrich) in 200
236 µl/well PBS (pH 7.5) for 2 h at 27°C. After incubation, the plates were washed three
237 times with 200 µl/well PBS-T (pH 7.5). Proteins were detected using TMBZ solutions
238 by observing the absorbance at 450 nm by plate reader (Dong et al., 2012).
239

240 ## 3. Results and discussion

241 *3.1. Expression and purification of VLPs-NcSRS2*

242 Expression of rNcSRS2 (~50 kDa) was confirmed in hemolymph and fat body
243 samples collected from silkworm larvae co-injected with BmNPV bacmid/RSV-gag-
244 577 and BmNPV bacmid/FLAG-NcSRS2 (Fig. 1A and B). The rNcSRS2 has been
245 reported to be a 42-kDa protein along with a membrane-targeting signal and its own
246 transmembrane region between 35 and 54 amino acids at the N terminus (Nishikawa
247 et al., 2002b). There was an increase of 10 kDa in molecular weight compared to the
248 theoretical molecular weight (Fig. 1A). This increase in molecular weight was due to
249 the presence of the native signal sequence, leading to formation of precursor rNcSRS2
250 and bombyxin signal sequence from *B. mori* for facilitating the secretion of rNcSRS2
251 into silkworm larval hemolymph (Otsuki et. al., 2013). When the native signal
252 sequence was removed from the constructs, the expression levels of rNcSRS2 were
253 low (data not shown), hence new constructs were designed that had the native signal
254 sequence plus the bombyxin signal sequence. In addition, coexpressed gag proteins
255 (25–61 kDa) were also present in both hemolymph and fat body samples by western

256 blotting (Fig. 1B). The gag proteins showed a series of expected size bands from 25 to
257 61 kDa (Fig. 1C). The antibody used to detect gag proteins is specific against the p10
258 region, therefore, a common region in all the different sizes of gag protein is shown
259 due to protease activity in silkworm larvae (Deo et al., 2011).
260 VLPs-NcSRS2 were purified from hemolymph in two steps and the target
261 protein at each step was confirmed by western blotting. The SEC profile clearly
262 showed that VLPs-NcSRS2 were eluted at more than 440 kDa (Fig. 2A, dotted line).
263 The exact mechanism behind the minimum number of gag monomers required to
264 form a macromolecular nanostructure is still unknown. However, the monomer gag
265 (61 kDa) and rNcSRS2 (52 kDa), alone or bound together, could not form large
266 macromolecular structures of 150–200 nm diameter (confirmed by TEM; Section 3.2).
267 One of the possible configurations for forming these VLP-NcSRS2 macromolecular
268 structures requires the assembly of gag monomers and rNcSRS2 protein to form
269 nanoparticles. This may be the reason why the molecular weight of VLPs-NcSRS2
270 was higher than that of the monomers.
271 VLP-NcSRS2 fractions were pooled and concentrated by ultracentrifugation.
272 Approximately 0.85 mg purified VLPs-NcSRS2 was isolated from 30 silkworm
273 larvae. The presence of rNcSRS2 and gag proteins in purified fractions was confirmed
274 by western blotting (Fig. 2B and C).
275
276 *3.2. TEM observation of VLPs-NcSRS2*
277 TEM of purified VLPs-NcSRS2 showed a distinctive bilayer that is usually
278 present on enveloped VLPs (Fig. 3A and B). The VLPs-NcSRS2 were 150–200 nm in
279 diameter, and immuno-TEM of VLPs-NcSRS2 showed the presence of 4–5 rNcSRS2
280 particles per VLP (Fig. 3B). The shape of the VLPs-NcSRS2 was not as smooth as

281 that of the VLPs alone, which might have been due to the presence of rNcSRS2. Our
282 previous study has shown that VLPs are 80–100 nm in diameter (Deo et al., 2011).
283 Based on those data, the apparent increase in diameter of VLPs-NcSRS2 by 50–70
284 nm could be attributed to insertion of the rNcSRS2 protein molecules in between the
285 gag monomers when the macromolecular nanostructure assembly took place. To
286 calculate the amount of rNcSRS2 on VLPs, a standard curve using different amounts
287 was prepared by ELISA. The amount of rNcSRS2 on VLPs-NcSRS2 was estimated to
288 be ~0.16 μg rNcSRS2 on 1 μg VLPs-NcSRS2 of total protein.
289
290 *3.3. Confirmation of antigenicity of rNcSRS2 on VLPs*
291 Antigenicity of rNcSRS2 on VLPs was confirmed by ELISA using neosporosis-
292 positive and -negative cattle sera. The VLP-NcSRS2 samples gave a higher signal for
293 the neosporosis-positive cattle serum compared with the negative serum (Fig. 4A).
294 The signal from the neosporosis-positive serum when compared with the negative
295 serum was almost twofold higher, showing the antigenicity of the displayed rNcSRS2.
296 VLPs alone as a negative control gave a background signal with neosporosis-positive
297 and -negative cattle sera, but it was low due to non-specific binding of serum to VLPs.
298 The purified VLPs-NcSRS2 showed antigenicity, thus, they were injected into
299 BALB/C mice without adjuvants and mouse sera were collected. The signal from the
300 neosporosis-positive serum when compared with the negative serum was almost
301 fivefold higher, showing the antigenicity of the displayed rNcSRS2 (Fig. 4B). VLPs-
302 NcSRS2 have a fusogenic property due to the gag protein that is surrounded by the
303 lipid bilayer. As a result, VLPs-NcSRS2 can fuse easily with the cell membrane and
304 thus facilitate their uptake into antigen-presenting cells by inducing a natural antigen-
305 processing pathway (Gluck et al., 1999). This particular property of VLPs to deliver

antigen with ease to the immune system in a way that mimics a natural pathway is the main reason why VLP-based vaccines stand out, and they have an excellent safety profile.

Serum collected from mice showed specific binding affinity towards purified VLPs-NcSRS2 rather than VLPs alone, and there was a higher signal with mouse positive serum compared with negative serum (Fig. 4B). VLPs alone gave background signals with both positive and negative mouse sera, but the signal was low compared with that of the VLPs-NcSRS2.

3.4. Confirmation of GPI anchorage of rNcSRS2 on VLPs

GPI processing is present in insect cells and has been reported previously but its presence in silkworms is unknown. It has been assumed that similar GPI processing takes place in silkworms, but no direct evidence is available in the literature to support this assumption. Furthermore, no known evidence shows the presence of GPI-anchored protein on VLPs using a bacmid-based expression system in silkworms. The aim of the current study was to show the presence of GPI anchorage and its important features for biofunctionality of rNcSRS2. Purified VLPs-NcSRS2 and VLPs as a negative control were coated onto the immunoplate and treated with PI-PLC, which is known to cleave the phosphoglycerol bond found in GPI-anchored proteins. A marked difference was observed between the wells with and without PI-PLC treatment compared with the negative control. PI-PLC released GPI-linked proteins from the lipid layer surrounding the VLPs-NcSRS2, whereas the wells with no PI-PLC treatment were unaffected (Fig. 5). As a negative control, VLPs alone were also treated with PI-PLC and there was no significant change with or without PI-PLC treatment.

4. Conclusion

Macromolecular nanostructures were produced successfully in silkworm larvae using gag protein to produce VLPs-rNcSRS2. The display system described here allows both the gag and rNcSRS2 proteins to ride the lipid rafts and reach the plasma membrane independent of each other. The assembling of proteins on the plasma membrane surface avoids misfolding or loss of antigenicity for rNcSRS2 compared to the fusion protein expression method. rNcSRS2 displayed on VLPs retained their native structure and underwent all the post-translational modifications, such as GPI anchorage, for their correct folding. The current research demonstrated that a GPI-anchored protein could be displayed successfully on gag-VLPs. The VLPs-NcSRS2 were successful in inducing the immune response in mice without using any adjuvants during vaccination, which avoids undue pain and cost when using live animals.

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Figure legends

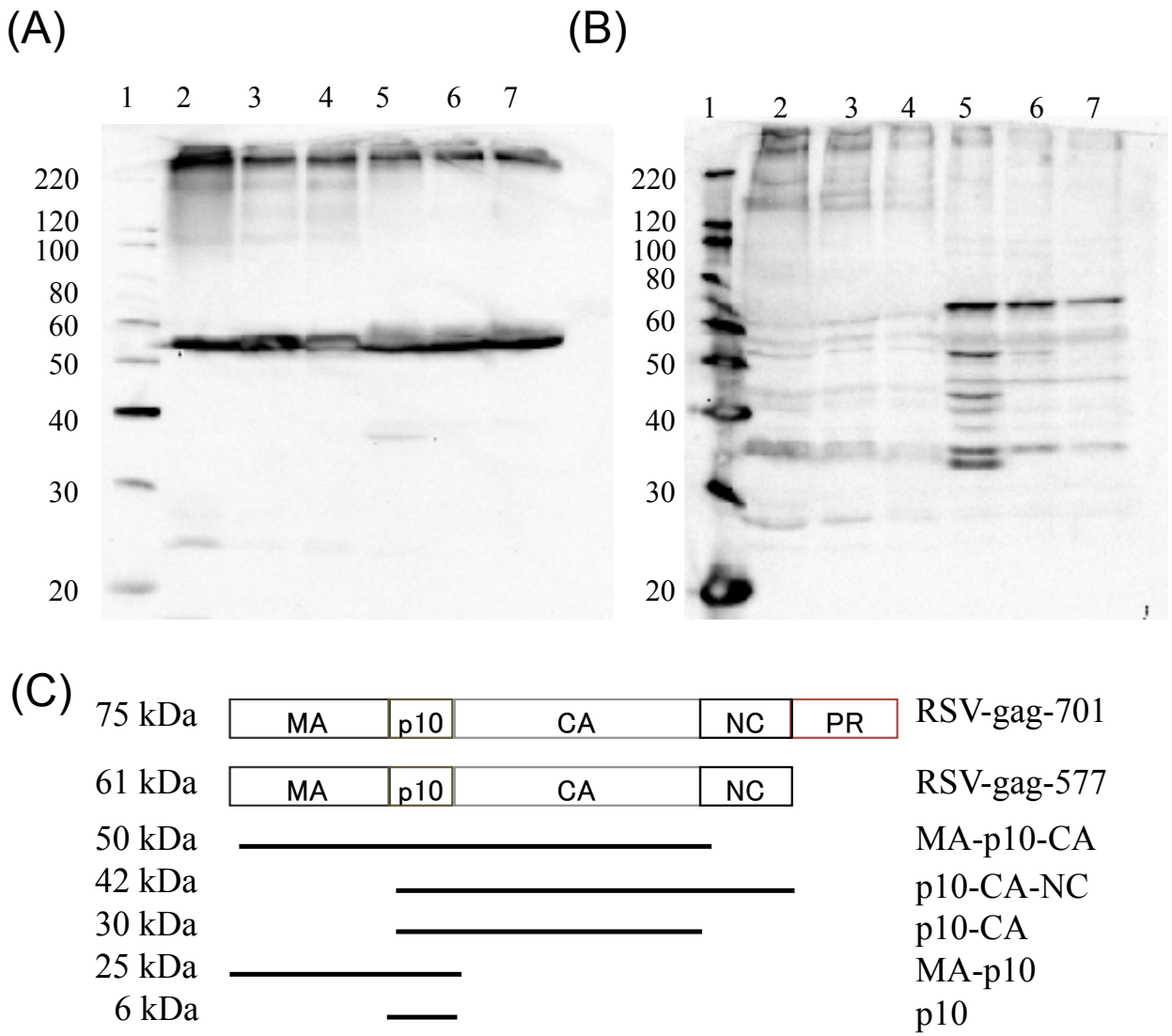
Fig. 1. Coexpression of rNcSRS2 and gag-577 in hemolymph and fat bodies. rNcSRS2 (A) and gag-577 (B) were detected by western blotting using mouse anti-DDDDK primary antibody and rabbit anti-gag primary antibody, respectively. Secondary antibodies used for detection of rNcSRS2 (A) and gag-577 (B) were goat anti-mouse IgG and goat anti-rabbit IgG, respectively. Lane 1: molecular weight markers. Lanes 2, 3 and 4 denote 10-, 20- and 30-times diluted hemolymph samples, respectively. Lanes 5, 6 and 7 denote 10-, 20-, and 30-times diluted fat body samples, respectively. (C) Schematic representation of various regions of RSV-gag protein. MA, CA, NC, and PR denote matrix, capsid, nucleocapsid, and protease regions, respectively.

Fig. 2. (A) Purification of VLPs-NcSRS2 by SEC. The elution profile of VLPs-NcSRS2 eluted from Superdex 200 preparative columns. Thin and thick lines denote molecular marker and VLPs-NcSRS2 profiles, respectively. Section enclosed by the dotted line denotes fractions with VLPs-NcSRS2. SEC high molecular weight marker: thyroglobulin (T) = 669 kDa, ferritin (F) = 440 kDa, aldolase (A) = 150 kDa, conalbumin (C) = 75 kDa, ovalbumin (O) = 43 kDa. (B and C) Western blot analysis of purified VLP-NcSRS2 protein samples. Loaded amount was 0.5 μg per lane and the membranes were probed with mouse anti-DDDDK for NcSRS2 (B) and rabbit anti-gag for VLPs (C). Secondary antibodies were goat anti-mouse IgG for NcSRS2 and goat anti-rabbit IgG for VLPs.

Fig. 3. (A) TEM observation of purified VLPs-NcSRS2 after negative staining. Scale bar is 200 nm. (B) Immunoelectron microscopy was performed using mouse H3 anti-neospora antibody and the grids were negatively stained and observed under TEM. Scale bar is 100 nm.

Fig. 4. Confirmation of antigenicity of rNcSRS2 on VLPs-NcSRS2 by ELISA. Neospora-positive and -negative sera were from cattle (A) and BALB/C mice immunized with VLP-NcSRS2 (B). Black and white bars indicate responses using positive and negative sera, respectively. Data are the mean±SD (*n*=3).

Fig. 5. Confirmation of GPI anchorage of rNcSRS2 on VLPs-NcSRS2 treated with and without PI-PLC by ELISA. Black and white bars indicate VLPs-NcSRS2 and VLP, respectively. Data are the mean±SD (*n*=3).


(A)
1 2 3 4 5 6 7
220
120
100
80
60
50
40
30
20
(B)
1 2 3 4 5 6 7
220
120
100
80
60
50
40
30
20
(C)
75 kDa
MA
p10
CA
NC
PR
RSV-gag-701
61 kDa
MA
p10
CA
NC
RSV-gag-577
50 kDa
MA-p10-CA
42 kDa
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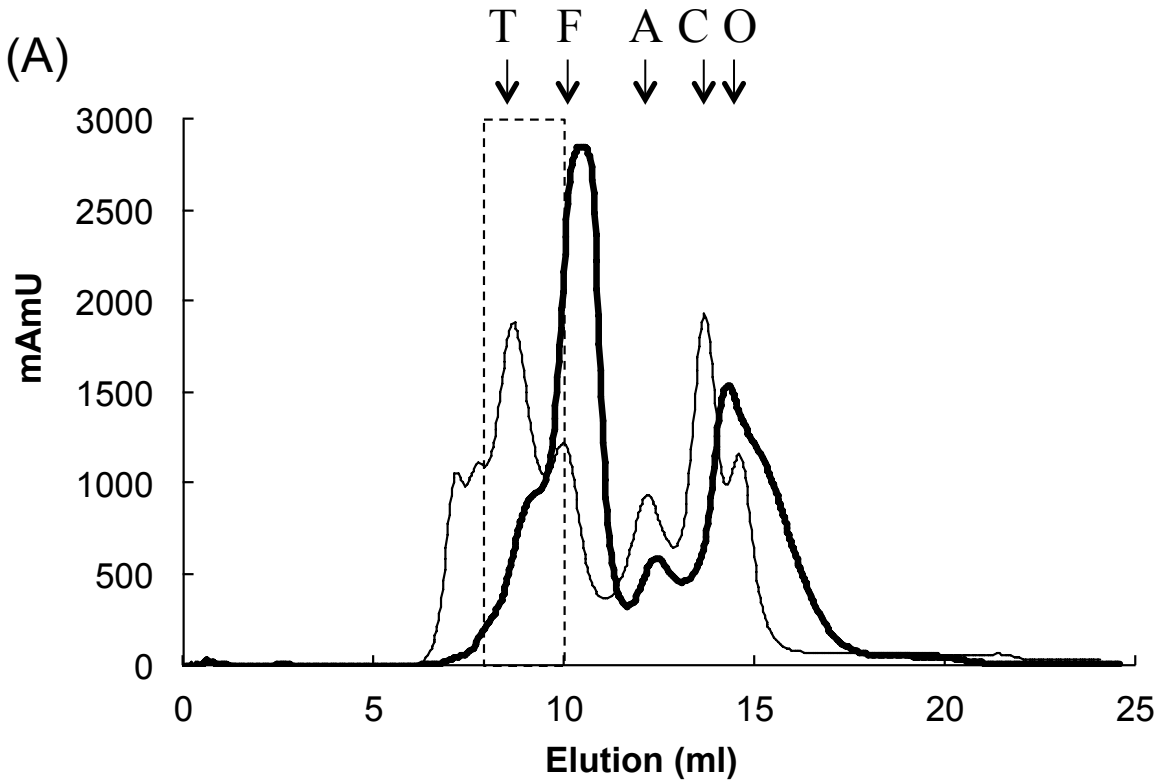
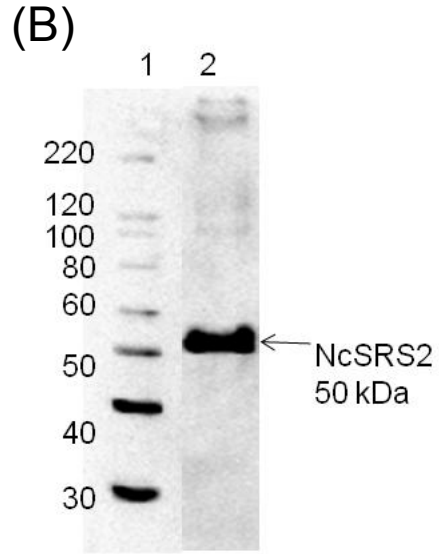
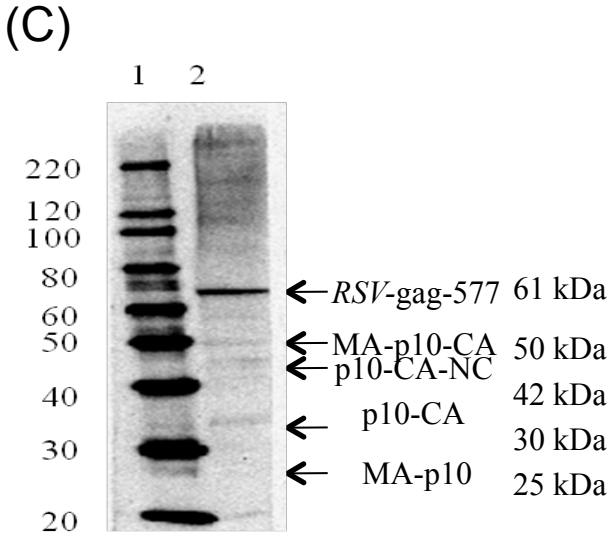
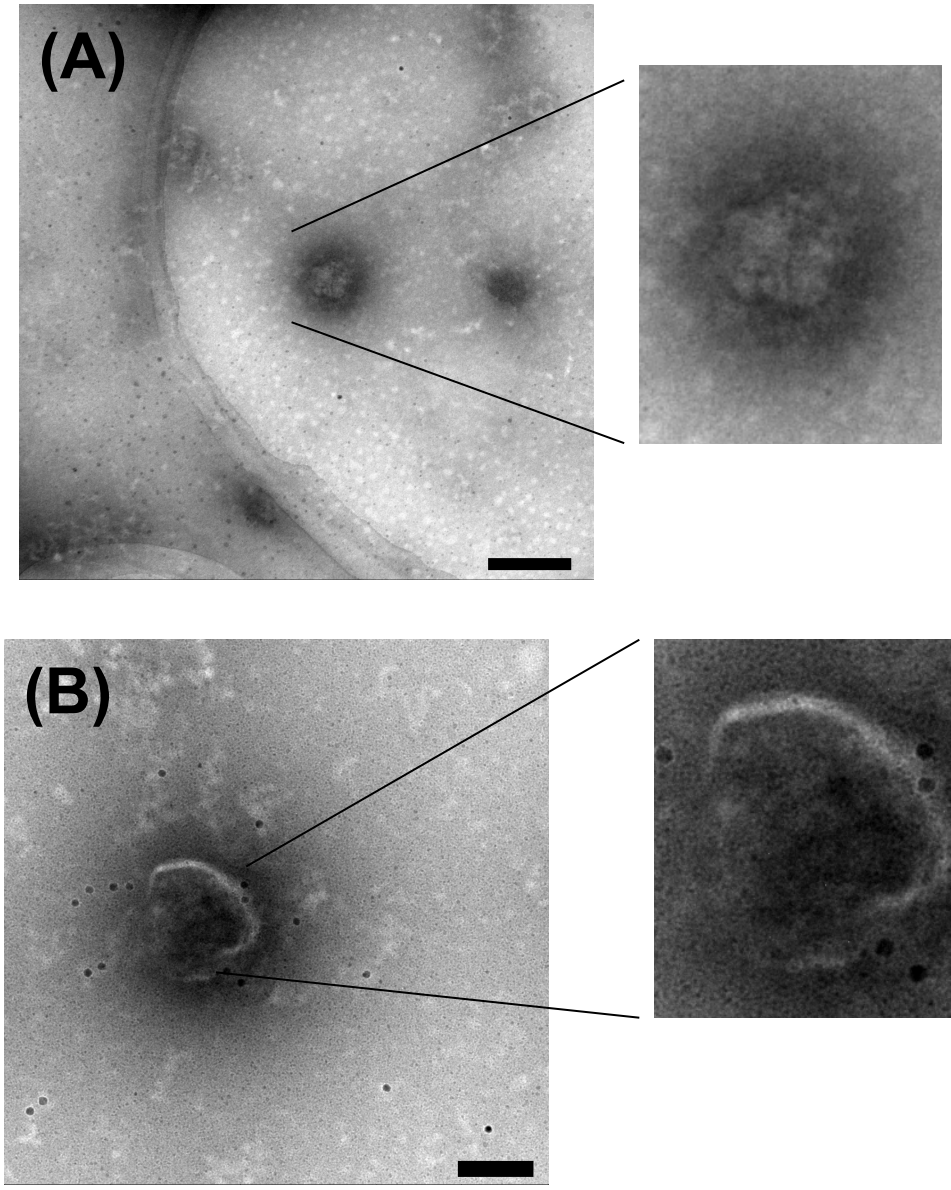
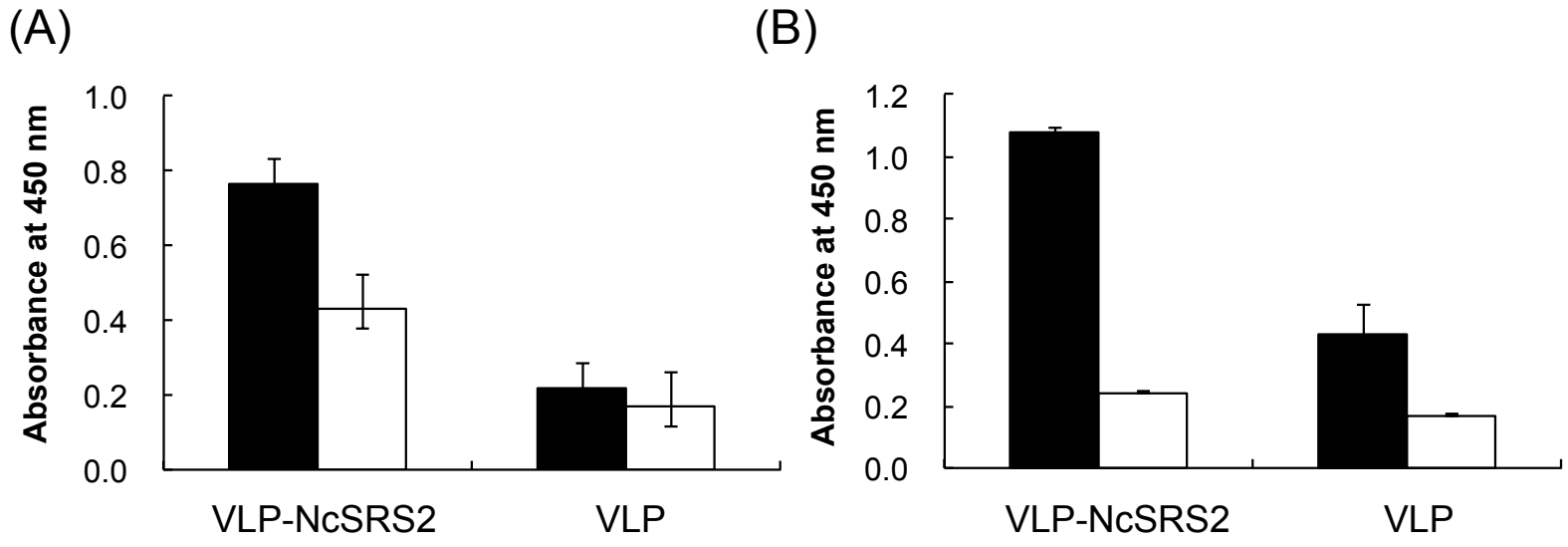
30 kDa
p10-CA
25 kDa
MA-p10
6 kDa
p10

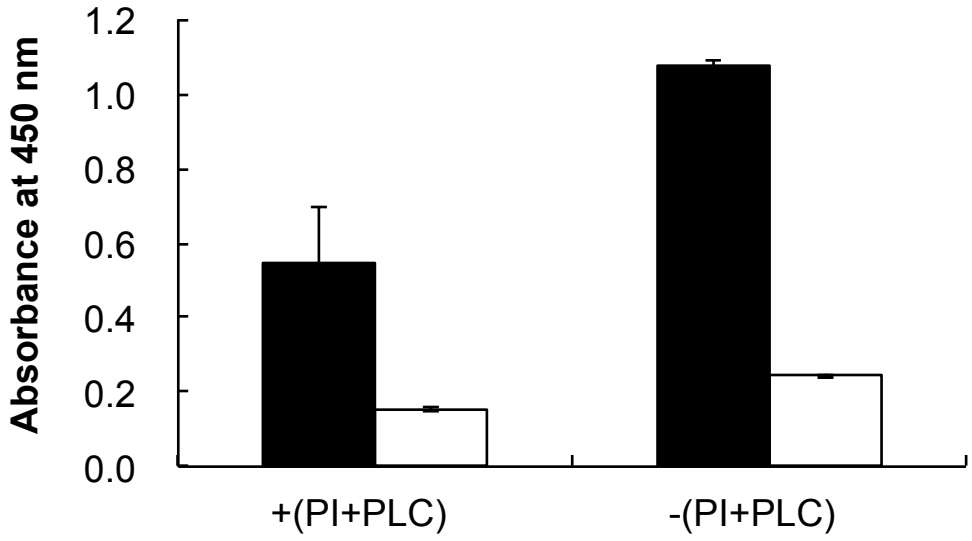

(A)
T F A C O
3000
2500
2000
1500
1000
500
0
mAmU
0 5 10 15 20 25
Elution (ml)


(B)
1 2
220
120
100
80
60
50
40
30
NcSRS2
50 kDa


(C)
1 2
220
120
100
80
60
50
40
30
20
RSV-gag-577 61 kDa
MA-p10-CA 50 kDa
p10-CA-NC 42 kDa
p10-CA 30 kDa
MA-p10 25 kDa


(A)
(B)


(A)
Absorbance at 450 nm
1.0
0.8
0.6
0.4
0.2
0.0
VLP-NcSRS2
VLP
(B)
Absorbance at 450 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
VLP-NcSRS2
VLP


Absorbance at 450 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
+(PI+PLC)
-(PI+PLC)