Production of Rous sarcoma virus-like particles displaying human transmembrane protein in silkworm larvae and its application to ligand-receptor binding assay

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ABSTRACT

Two types of *Rous sarcoma* virus (RSV) group-antigen protein (Gag) virus like particles (VLPs), full-length Gag (Gag701) and RSV protease domain (PR)-deleted mutant (Gag577) were expressed in silkworm larvae. Gag 577 was secreted into hemolymph efficiently using wild type bacmid (WT), cysteine protease-deficient bacmid (CP^-), cysteine protease and chitinase-deficient bacmid (CP^-Chi^-) bacmids, but comparatively Gag 701 secretion levels were low. VLPs were purified on 10%-60% (v/v) sucrose density gradient by ultracentrifugation and their structures confirmed under electron microscope. When hPRR and RSV Gag577 were co-expressed in silkworm larvae, human prorenin receptor (hPRR) was displayed on the surface of RSV VLPs, which was detected by western blotting and immunoelectron microscopy. Moreover, binding of hPRR localized on the surface of VLPs to human prorenin was confirmed by ELISA. These results indicate that active hPRR was displayed on the surface of RSV VLPs, which can be utilized for drug discovery of hPRR blockers to prevent nephropathy. Moreover, this transmembrane protein display system using RSV Gag in silkworm larvae is applicable to expression of intact transmembrane proteins and binding assay of transmembrane proteins to its ligands, especially for transmembrane proteins which cannot be purified from membrane fractions in active states.

Keywords: *Rous sarcoma* virus, Gag, Virus-like-particles, Surface display, Human prorenin receptor, Silkworm

1. Introduction

Virus-like particles (VLPs) are formed by self-assembling virus-derived structural proteins. VLPs are empty shells and do not have virus genome DNA or RNA, therefore VLPs are not infectious. Moreover, VLPs can induce strong cellular and humoral responses as direct immunogens (Grgacic et al., 2006). For this property, VLPs have been utilized for virus vaccines (Roldão et al., 2010). Human papillomavirus vaccine (HIV), Gardasil and Cervarix, which were produced in *Saccharomyces cerevisiae* and insect cells respectively, have received approvals for marketing (Garland et al., 2007). The HIV VLPs composed of HIV-1 Pr55Gag have been developed for an AIDS vaccine (Ludwig and Wagner, 2007). Otherwise, VLPs have been utilized as scaffolds in nano-structural technology, especially, nanowires and nanobiopolymers (Mao et al., 2004).

VLPs have been produced in *Escherichia coli*, yeasts, insect cells, plants and mammalian cells. Especially, Gag proteins of enveloped viruses, for example, HIV, *Rous sarcoma* virus (RSV) have all of the necessary domains for VLP assembly, and VLPs can bud extracellularly from cells when Gag is solely expressed in insect and mammalian cells (Ludwig and Wagner, 2007). RSV belongs to the avian retrovirus family and RSV Gag protein, which self-assembles to VLPs, is proteolytically processed by RSV protease to matrix (MA), p2 (p2a,b), capsid (CA), nucleocapsid (NC) and protease (PR). When full-length RSV Gag protein was expressed in insect

56 cells, high level of gag protein was observed (Mayo et al., 2002). However, unlike HIV
57 Gag expression, RSV VLPs were not efficiently secreted into culture medium (Johnson
58 et al., 2001). PR domain-deleted Gag mutant formed VLP correctly and extracellularly
59 secreted from cells.
60 Baculoviruses also belongs to enveloped viruses and can bud from infected cell
61 into extracellular fractions. Like HIV and RSV, baculoviruses are surrounded by a
62 lipid bilayer from host cellular membrane. Some transmembrane proteins expressed on
63 the surface of host cells can be displayed on the surface of baculoviruses together with
64 baculovirus major envelope glycoprotein GP64 when baculoviruses bud from host
65 cells (Grabherr et al., 2001; He et al., 2009; Makela and Oker-Blom, 2006). This
66 display system is very useful for some applications, antibody production, screening
67 and cloning of binding partners of transmembrane proteins and antigens and receptor
68 binding assay (Kakutani et al. 2011; Sakihama et al. 2008).
69 In this study, RSV Gag was expressed in silkworm larvae and VLPs derived from
70 RSV Gag were purified from hemolymph by sucrose density gradient centrifugation.
71 Moreover, human prorenin receptor (hPRR), which has a signal peptide at its
72 N-terminus, an extracellular domain, a single transmembrane domain and a short
73 cytoplasmic domain and is a multi-functional protein (Nguyen 2011), was
74 co-expressed together with RSV Gag in silkworm larvae. We investigated the
75 possibility of displaying hPRR on the surface of RSV VLPs and improving the
76 incorporation of hPRR into RSVs using different signal peptides and promoters.

2. Materials and Methods

2.1. Construction of recombinant BmNPV bacmid

Full-length RSV gag gene (2106 bp) was amplified by PCR using RSV-Gag-F (CACCATGGAAGCCGTCATAAAGGTG) and RSV-Gag701-R (CTATAAATTTGTCAAGCGGAG) primers. ΔPR gag gene or gag577 (1734 bp) was amplified by PCR using RSV-Gag-F and RSV-Gag577-R (TTACGAGACGGCAGGTGGCTCAGG) primers. In both cases, pRep(A), which is purchased from ATCC (ATCC number: 87702), was used as a template. Each amplified gene was inserted into pENTR/D/TOPO (Invitrogen, San Diego, CA) by topoisomerase reaction. Using these plasmids, full-length RSV gag and ΔPR gag genes were inserted into pDEST8 (Invitrogen), respectively and pDEST-Gag701 and pDEST-Gag577 were constructed. These plasmids were transformed into *E. coli* BmDH10Bac (Motohashi et al., 2005), *E. coli* BmDH10Bac CP^- (Hiyoshi et al., 2007) and *E. coli* BmDH10Bac CP^- Chi^- (Park et al., 2008), respectively. Blue-white selection was performed to identify colonies containing the recombinant bacmid. Recombinant BmNPV bacmids were extracted from white transformants and insertion of each Gag gene was confirmed by PCR.

To obtain hPRR gene with bomyxin (bx) signal peptide sequence at its N-terminus,

hPRR was amplified by PCR using EcoRIbxSFLAG-F (CACCGAATTCATGATACTCCTTGCTATTGCATTAATGTT GTCAACAGTAATGTGGGTGTCAACACAAACCCCGCCCGACTACAAGGACG ACGACGACAA) and SphI-hPRR-R (TCAGCATGCTCAATCCATTCGAATCTTCTGGTTT). BmNPV CP^-/GFP_{uv}-hPRR bacmid DNA (Du et al., 2008) was used as a template. To obtain hPRR gene connected with GP64 signal peptide sequence from BmNPV, hPRR was amplified by PCR using EcoRIgp64S1-60-F (CACTGCAACGCGCAAATGAAAACGGGTCCGTACAAAATTAAAAACTTGGA CATTACCCCGCCCGACTACAAGGACGACGACGACAA) and SphI-hPRR-R. Then hPRR gene connected with whole GP64 signal peptide sequence was amplified using gp64S60-114FLAG-F (CACCGAATTCATGGTAGGCGCTATTGTTTTATACGTGCTTTTGGCGGCGCA TTCTGCCTTTGCGGCGGAGCACTGCAACGCGCAAATGAA) and SphI-hPRR-R. Each amplified gene was inserted into pENTR/D/TOPO by topoisomerase reaction. hPRR connected with bx and gp signal peptides were designated as bx-hPRR and gp-hPRR, respectively. Each gene was inserted into pDEST8, pDB3 and pDB5 by Gateway system (Invitrogen). pDB3 and pDB5 was constructed previously to enhance the protein expression under the control of modified polyhedron promoter (Manohar et al., 2010). pDB3 and pDB5 have 3 and 5 repeated burst sequence in the region of polyhedrin promoter, respectively. Total six plasmids (pDEST/bx-hPRR,

pDB3/bx-hPRR, pDB5/bx-hPRR, pDEST/gp-hPRR, pDB3/gp-hPRR, pDB5/gp-hPRR) were constructed. Each plasmid was transformed *E. coli* BmDH10Bac CP^- and recombinant bacmids containing hPRR cDNA was extracted from its transformants.

2.2 Expression and sample preparation

A recombinant bacmid containing Gag cDNA (10 μg) was mixed with one-tenth of DMRIE-C (Invitrogen) and incubated at room temperature for over 45 min. When Gag protein and hPRR were co-expressed, 5 μg of each recombinant bacmid containing each gene was mixed with DMRIE-C. Fifty microliter of this mixture was injected into a silkworm larva. Injected silkworm larvae were reared for 5-7 days and hemolymph was recovered. Collected hemolymph was centrifuged at 2400 × g for 10 min at 4°C to remove hemocytes. The supernatant was used as a hemolymph sample. In the case of fat body, 100 mg fat body was suspended with 1 ml of TBS buffer containing 0.1 % (v/v) Triton X-100 and sonicated. The homogenate was used as a fat body sample.

2.3. Purification of VLPs from silkworm larvae

VLPs were purified from hemolymph by sucrose density gradient centrifugation. Two milliliter of hemolymph was mixed with 2 ml of PBS (pH 6.2) and this mixture

was laid on 25% (w/v) sucrose cushion. This mixture was centrifuged at 80000 × g for 90 min at 4°C and the pellet was suspended with PBS (pH 6.2). This suspension was sonicated to dissolve the pellet and this suspension was laid on 25-60% (w/v) sucrose density gradient. This suspension was centrifuged at 96000 × g for 3 h at 4°C. A white band was recovered carefully and diluted with PBS (pH6.2). This diluent was centrifuged at 96000 × g for 90 min at 4°C. The pellet was suspended with a small volume of PBS (pH 6.2) and this suspension was used as a purified VLP sample.

2.4. SDS-PAGE and protein analysis

Samples were subjected to SDS-PAGE on 10 or 12% (w/v) polyacrylamide gel with the Mini-protean II system (Bio-Rad Co. Ltd, Hercules, CA, USA). Total proteins on SDS-PAGE gel was detected with Coomassie Brilliant blue (CBB) R-250. In the case of Western Blot, proteins in gels were blotted onto a polyvinylidene fluoride (PVDF) membrane using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). After being blocked in 5% skim milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST), the membrane was incubated in either 1:10000 diluted mouse anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) or 1:5000 diluted rabbit anti-RSV Gag CA antibody (gifted by Dr. Vogt) for 1 hour. The membrane was washed with TBST, and then incubated in 1:20,000 diluted either anti-mouse or anti-rabbit labeled with horseradish peroxidase (HRP) (GE Healthcare

161 Japan, Tokyo, Japan) for 1 hour. Detection was performed using ECL Plus Western
162 blotting reagent (GE Healthcare Japan). Specific bands were detected using a
163 Fluor-S/MAX multi-imager (Bio-Rad). Protein band intensity was analyzed by
164 Quantity One software (Bio-Rad).

165 Protein concentration was determined by BCA protein assay kit (Thermo Fisher
166 Scientific K. K., Yokohama, Japan).

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168 *2.5. ELISA*

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170 VLPs were incubated in a 96 wells plate at 4°C with 0.1M carbonate buffer (pH
171 9.6) for 16 hours to be immobilized on a plate. Wells were washed with PBS (pH 7.4)
172 containing 0.01% (v/v) Tween 20 (PBST) four times. Blocking buffer (PBST
173 containing 3% (w/v) skimmed milk) was added into each well and plates were
174 incubated at room temperature for 1 hour. Wells were washed with PBS (pH 7.4)
175 containing 0.01% (v/v) Tween 20 (PBST) four times. Mouse anti-FLAG M2 antibody
176 diluted by 1000 fold with Can Get Signal Solution I (TOYOBO, Osaka, Japan) was
177 added into each well and plates were incubated at room temperature with moderately
178 stirring for 1 hour. After wells were washed with PBST four times, HRP-conjugated
179 anti-mouse IgG antibody (GE Healthcare Japan) diluted by 5000 fold with Can Get
180 Signal Solution II (TOYOBO) was added into each well and plates were incubated at
181 room temperature with moderately stirring for 1 hour. Wells were washed with PBST

four times followed by HRP reaction. 100 μl of substrate [0.1-mg/ml 3,3′,5,5′-tetramethylbenzidine(TMBZ) in 100-mM sodium acetate, pH 6.0, with 0.2% (v/v) of 30% hydrogen peroxide] was added to each well and left at room temperature for blue-color development. The reaction was stopped by the addition of 50 μl of 1N H_2SO_4 solution. The developed color was measured at optical densities (ODs) of 450 nm and 655 nm. The value of OD655 minus OD450 was used as a measure of the amount of hPRR.

In prorenin-hPRR binding detection experiment, VLPs were incubated in a 96 wells plate at 4°C with 50 mM phosphate buffer (pH 7.4) for 16 hours to be immobilized on a plate. Wells were washed with PBS (pH 7.4) containing 0.01% (v/v) Tween 20 (PBST) four times. Blocking buffer (PBST containing 3% (w/v) BSA) was added into each well and plates were incubated at room temperature for 1 hour. Wells were washed with PBST four times. One hundred nanogram of human prorenin (Cayman Chem., MI, USA) were added into each well and this plate was incubated at room temperature with stirring moderately for 3 hours. Wells were washed with PBST four times. Mouse anti-human prorenin antibody (Innovative Res., MI, USA) diluted by 1000 fold with Can Get Signal Solution I (TOYOBO) was added into each well and plates were incubated at room temperature with moderately stirring for 1 hour. After wells were washed with PBST four times, detection of hPRR binding to human prorenin was the same method as hPRR detection.

2.6. Immunoelectron microscopy

Purified VLPs were immobilized on the carbon grid (Okenshoji, japan) and blocked with 4% (w/v) BSA in PBS (pH 7.4). This grid was soaked in either mouse anti-FLAG M2 antibody diluted by 30 fold with 1% (w/v) BSA in PBS or rabbit polyclonal anti-GP64 antibody (Dojima et al., 2010) diluted by 500 fold with 1% (w/v) BSA in PBS for 2 h. After washing with PBS, grid was soaked in either 10 nm gold conjugated goat polyclonal anti-mouse IgG+IgM (H+L) (British BioCell International, Cardiff, UK) diluted by 25 fold with 1%(w/v) BSA in PBS or 10 nm gold conjugated goat polyclonal anti-rabbit IgG+IgM (H+L) (British BioCell International) diluted by 25 fold with 1%(w/v) BSA in PBS for 1 h. After washing with PBS, grid was stained with 2% (v/v) phosphotungstic acid. VLPs were observed by transmission electron microscope (TEM, JEM-2100F (JEOL Ltd., Tokyo, Japan)) at 200 kV.

3. Results

3.1. Expression of RSV Gag protein in silkworms

RSV Gag protein has several domains including N-terminal matrix domain (MA), proline-rich late (L) domain (p2), spherical shape determinant (p10), two capsid sub-domain (CA), RNA binding nucleocapsid domain and virus protease (PR). In insect cells, PR deletion allowed to express normal spherical VLPs (Johnson et al., 2001). In this study, two type of RSV gag protein (full-length Gag: amino acid 1-701,

224 ΔPR Gag: amino acid 1-577) was expressed in silkworm larvae using wild type
225 BmNPV bacmid (WT), cysteine protease-deficient BmNPV bacmid (CP^-) and cysteine
226 protease- and chitinase-deficient BmNPV bacmid (CP^- Chi^-). Its expression pattern in
227 hemolymph is shown in Fig.1. Full-length Gag was expressed in hemolymph using
228 WT bacmid, but no bands were observed in hemolymph using CP^- and CP^- Chi^-
229 bacmids respectively. However, proteins in hemolymph of WT sample contained
230 approximately 60, 50 and 30 kDa of expected size. Full length Gag (75 kDa) could not
231 be observed. Likewise, ΔPR Gag was expressed using three types of BmNPV bacmid.
232 A protein band with estimated molecular weight (61 kDa) was observed with some low
233 molecular weight bands. In fat body, some protein bands were observed in all samples
234 using six bacmids, indicating that RSV Gag protein was expressed in all the cases of
235 full-length Gag and ΔPR Gag using three types of BmNPV bacmid but ΔPR Gag was
236 secreted into hemolymph more efficiently than full-length Gag. These results were
237 reaffirmed by Johnson et al. (Johnson et al., 2001). WT bacmid was adopted for further
238 experiment because RSV Gag protein was secreted most efficiently.
239
240 *3.2. Purification of RSV Gag VLPs from hemolymph*
241
242 RSV Gag expressed was purified from hemolymph by sucrose density gradient
243 centrifugation. RSV Gag expressed was concentrated by ultracentrifugation (Fig. 2A)
244 and RSV Gag (61 kDa) was brought down into the pellet from the supernatant.

However, the band below 30 kDa remained in the supernatant. Molecular weight of this band corresponded to that of CA domain (27 kDa). After sucrose density gradient centrifugation, two white bands appeared (Fig. 2B) and were recovered separately. These samples had the same protein band pattern in view of CBB staining and western blotting (Fig. 2B). Little distorted particles were observed in these samples (Fig. 2C), indicating that RSV Gag formed VLPs in hemolymph. Calculating the recovery ratio of RSV Gag VLPs by densitometry based on the result of western blotting, these VLPs were purified with 5.4 % of recovery ratio (Data not shown).

3.3 Co-expression of RSV Gag and hPRR in silkworm larvae

RSV Gag and hPRR were co-expressed in silkworm larvae by injection of the mixture two BmNPV bacmids at similar concentrations. In previous study, active hPRR fused with GFP_{uv} could be expressed in silkworm larvae (Du et al., 2008). Hemolymph and fat body were collected from injected larvae and hPRR expression was checked by western blotting (Fig. 3). Judging from the densitometric band intensity of the bands on the western blot membranes, hPRR expression level using DB3 promoter was the highest among the three types of promoters. Using DB3 promoter, hPRR expression level was the same regardless of kinds of signal peptides. hPRR expression level is higher under the polyhedrin promoter using gp signal peptide than that using bx signal peptide. DB3 promoter was used in further experiments.

Incorporation level of hPRR onto RSV Gag VLPs was compared under the control of polyhedrin and DB3 promoters (Fig. 4). Higher expression level of hPRR was observed in purified VLP fraction using DB3 promoter than that using polyhedrin promoter in the both cases of bx and gp64 signal peptides. The expression of bx-hPRR was the highest in all purified VLP samples. bx signal peptide allowed to enhance the incorporation of hPRR in purified VLPs compared to gp signal peptide.

Moreover, to compare the incorporation level of hPRR into RSV Gag VLPs, hPRR in purified VLP fractions was detected by ELISA (Fig. 5). bx-hPRR in a VLP fraction showed the highest absorbance in all four samples (VLPs, GFP_{uv}-hPRR in VLPs, bx-hPRR in VLPs, gp-hPRR in VLPs), indicating that bx-hPRR was more efficiently incorporated into RSV Gag VLPs than gp-hPRR (Fig. 4). This ELISA utilized intact VLPs immobilized onto the plate and then hPRR was detected using primary and secondary antibodies. hPRR can be detected in this ELISA only when hPRR is localized on the surface of VLPs. Considering this, hPRR could be displayed on the surface of RSV Gag VLPs in this system. To confirm hPRR display on the surface of VLPs, immunoelectron microscopy was performed using VLPs incorporating bx-hPRR (Fig. 6). Gold particles conjugated to the anti-FLAG specific for FLAG tagged hPRR were used to confirm the presence on the surface of VLPs together with GP64 (Fig. 6). It was confirmed that hPRR could be displayed on the surface of RSV Gag VLPs when RSV Gag and hPRR was co-expressed in silkworm larvae. Higher expression of GP64 was observed on the surface of VLPs compared to

hPRR, indicating that GP64 was more efficiently displayed on the surface of RSV Gag VLPs than hPRR.

3.4. Binding of hPRR on the surface of VLPs to human prorenin.

Activity of RSV VLPs displaying hPRR on its surface, expressed in silkworm larvae using bx signal peptide under the control of DB3 promoter, was assayed by ELISA and confirmed by binding of hPRR to human prorenin. Compared to normal VLPs, higher absorbance was detected using VLPs displaying hPRR (Fig. 7), indicating that hPRR on VLPs' surface was active and able to bind to human prorenin.

4. Discussion

Various VLPs have been produced in *E. coli*, yeasts, insect (cells), plants, mammalian cells and have been used as vaccines, for antibody production and nanocarriers for gene targeting (Ludwig and Wagner, 2007; Tan et al., 2011). In this study, VLPs of *Rous sarcoma* virus were produced in silkworm larvae by the expression of RSV Gag with protease (PR) domain deleted and hPRR was pseudo typed on the VLPs surface. The hPRR is involved in hypertension, cardiovascular and renal disease and organ damage and binds to both prorenin and renin (Barakumar and Jagadeesh 2011; Nguyen 2011; Welkinson-Berka et al., 2011). In this study, the hPRR

was displayed on VLPs' surface when RSV Gag and hPRR were co-expressed in silkworm larvae. It was previously reported that hPRR was displayed on the surface of BmNPV a baculovirus belonging to the family of enveloped double stranded DNA viruses in silkworm larvae (Kato et al., 2009; 2011). When baculoviruses bud from the surface of infected cells, some transmembrane proteins can be incorporated onto its envelope and then transmembrane proteins can be displayed on the surface of baculoviruses (Grabherr et al., 2001; He et al., 2009; Makela and Oker-Blom, 2006). Similar to this baculovirus surface display, active hPRR could be displayed on the surface of RSV Gag VLPs. BmNPV GP64 was also displayed on its surface, suggesting that RSV Gag VLPs bud from cells somehow with the similar route to baculovirus. The advantage of using VLPs display system is that it is free from any active virus carrying any genetic material. Thus the VLPs display system is safer for biological applications in human without the risk of causing any viral infections.

hPRR expression in fat body was higher using gp signal peptide under the control of normal polyhedrin promoter than bx signal peptide, but vice versa with regard to hPRR incorporation level into RSV VLPs. It has been reported, HIV envelope protein (Env) was more efficiently incorporated into HIV Gag VLPs using mellitin signal peptide than its native and AcMNPV GP64 signal peptides (Wang et al., 2007). Normally, most of signal peptides are cleaved from expressed proteins in endoplasmic reticulum by signal peptidase and expressed proteins are folded and transported to Golgi apparatus. It is a well-established theory that signal peptides do not work during

329 the incorporation of transmembrane proteins into VLPs. But from our results we can
330 speculate that in the case of transmembrane proteins, signal peptides might contribute
331 to the efficient anchoring to membrane.

332 It has been already reported that substitution of HIV Env transmembrane and
333 cytoplasmic domains with that of mouse mammary tumor virus (MMTV) envelope
334 glycoprotein or AcMNPV GP64 enhanced the incorporation of Env into HIV Gag
335 VLPs (Wang et al., 2007), indicating that transmembrane and cytoplasmic domains of
336 viral glycoproteins play an important role in its incorporation onto VLPs. Env has a
337 long cytoplasmic domain (over 162 amino acids). On the contrary, cytoplasmic
338 domains of MMTV glycoprotein and AcMNPV GP64 are composed of 43 and 7 amino
339 acids, respectively. So it can be concluded that the long cytoplasmic domain may cause
340 low level of incorporation of HIV Env onto VLPs. hPRR has a short cytoplasmic
341 domain (19 amino acids). This short cytoplasmic domain may be favored and hPRR be
342 displayed with more efficiency on the surface of RSV Gag VLPs. Moreover, it is
343 possible that hPRR incorporation level to RSV VLPs can be improved by substitution
344 of the transmembrane and cytoplasmic domains.

345 Gag577 was expressed and formed VLPs in hemolymph, but Gag701 did not form
346 VLPs with efficiency. Especially, higher level of Gag577 was detected using WT
347 bacmid in hemolymph than using CP^- and CP^-Chi^- bacmids. WT bacmid has
348 baculovirus cysteine protease gene and its gene is deleted in CP^- and CP^-Chi^- bacmids.
349 Normally, high level expression of recombinant proteins is achieved by using cysteine

protease-deficient baculovirus compared to using wild type baculovirus. So it can be concluded that, with respect to RSV gag expression, cysteine protease may enhance RSV gag expression or VLP formation because full-length Gag (Gag701) has aspartic protease domain and this protease cleaves expressed Gag protein to make Gag protein mature (Katz and Skalka, 1994; Xiang et al., 1997). However, full length Gag protein was neither processed nor purified in this study.

Surface of VLPs purified from hemolymph was more uneven compared to ones produced in insect cells (Ako-Adjei et al., 2005). This might be owing to BmNPV having specificity for the hemocytes and buds from hemocytes. As a result the budded BmNPV infects fat body cells (Katsuma et al., 2008). Since recombinant proteins are mainly expressed in fat body, the RSV Gag VLPs were expressed in fat body. On the other hand, in most of cultured insect cells originating from insect ovaries the VLPs surface morphology is smooth. This difference of host cell origin accounts for the difference of surface morphology between VLPs from hemolymph and cultured insect cells. Active hPRR was observed on the surface of VLPs from hemolymph, indicating that the surface environment on the surface of VLPs from hemolymph may be not so different from that of cultured insect cells in view of expressed protein stability.

In this study RSV Gag deleted PR domain was expressed in silkworm larvae and VLPs composed of RSV Gag were purified from hemolymph. hPRR was displayed on the surface of RSV Gag VLP when hPRR and RSV Gag were co-expressed in silkworm larvae. Moreover, hPRR on its surface can bind to human prorenin,

371 indicating that hPRR was displayed as an active form. It is possible that various
372 transmembrane proteins can be displayed on the surface of RSV Gag VLPs using this
373 system and this system is applicable to binding assay of transmembrane proteins or
374 antigens to its ligands, especially for transmembrane proteins which cannot be purified
375 from membrane fractions as active forms.
376
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Figure legends

Fig. 1. Expression of Gag701 and Gag577 in hemolymph and fat body. Silkworm larvae were reared for 7.5 days after injecting each recombinant bacmid and hemolymph and fat body were harvested. Each Gag expression was analyzed by western blotting. Sample preparation and western blotting methods are described in Materials and methods.

Fig. 2. Purification of RSV Gag VLP from hemolymph. (A) Western blotting analysis of each fraction during purification. H: hemolymph, C: concentrated VLP fraction (pellet) by centrifugation from hemolymph, S: supernatant after VLP concentration step, P: VLPs purified by sucrose density gradient centrifugation. (B) Purification of VLPs by sucrose density gradient centrifugation. After sucrose density gradient centrifugation two bands appeared. 1: hemolymph, 2: upper band after sucrose density gradient centrifugation, 3: lower band after sucrose density gradient centrifugation, 4: BmNPV purified by sucrose density gradient centrifugation. (C) TEM observation of purified RSV Gag VLPs. Mixture of upper and lower bands in (B) was used as purified VLPs.

Fig. 3. Expression of GFP_{uv}-hPRR, bx-hPRR and gp-hPRR in fat body. (A) Western blotting analysis (B) Densitometry analysis of each hPRR expression based on (A).

Grey bar: GFP_{uv}-hPRR expression, open bars: gp-hPRR expression, black bars: bx-hPRR expression. polh indicates polyhedrin promoter. GFP_{uv}-hPRR was expressed using polyhedrin promoter. Detailed methods are described in Materials and Methods.

Fig. 4. Western blotting analysis of incorporation level of each hPRR into RSV Gag VLPs. RSV Gag VLPs were purified by sucrose density gradient centrifugation from hemolymph when hPRR and RSV Gag577 were co-expressed in silkworm larvae. RSV Gag 577 and each hPRR were detected by western blotting using purified VLPs. For detection of hPRR using mouse anti-FLAG M2 antibody, 10 μg protein of purified VLPs was applied to SDS-PAGE. For detection of Gag577 using rabbit anti-RSV Gag antibody, 1 μg protein of VLPs was applied to SDS-PAGE.

Fig. 5. Comparison of hPRR level on the surface of RSV Gag VLPs by ELISA. Purified VLPs were immobilized onto the ELISA plate and hPRR was detected by HRP reaction using mouse anti-FLAG M2 antibody and HRP conjugated anti-mouse IgG antibody. Detailed methods are described in Materials and methods. Open circles: RSV Gag VLPs, black circles: RSV Gag VLPs from GFPuv-hPRR- and RSV Gag-co-expressing silkworms, squares: RSV Gag VLPs from gp-hPRR- and RSV Gag-co-expressing silkworms, triangles: RSV Gag VLPs from bx-hPRR- and RSV Gag-co-expressing silkworms. gp-hPRR and bx-hPRR was expressed using DB3 promoter.

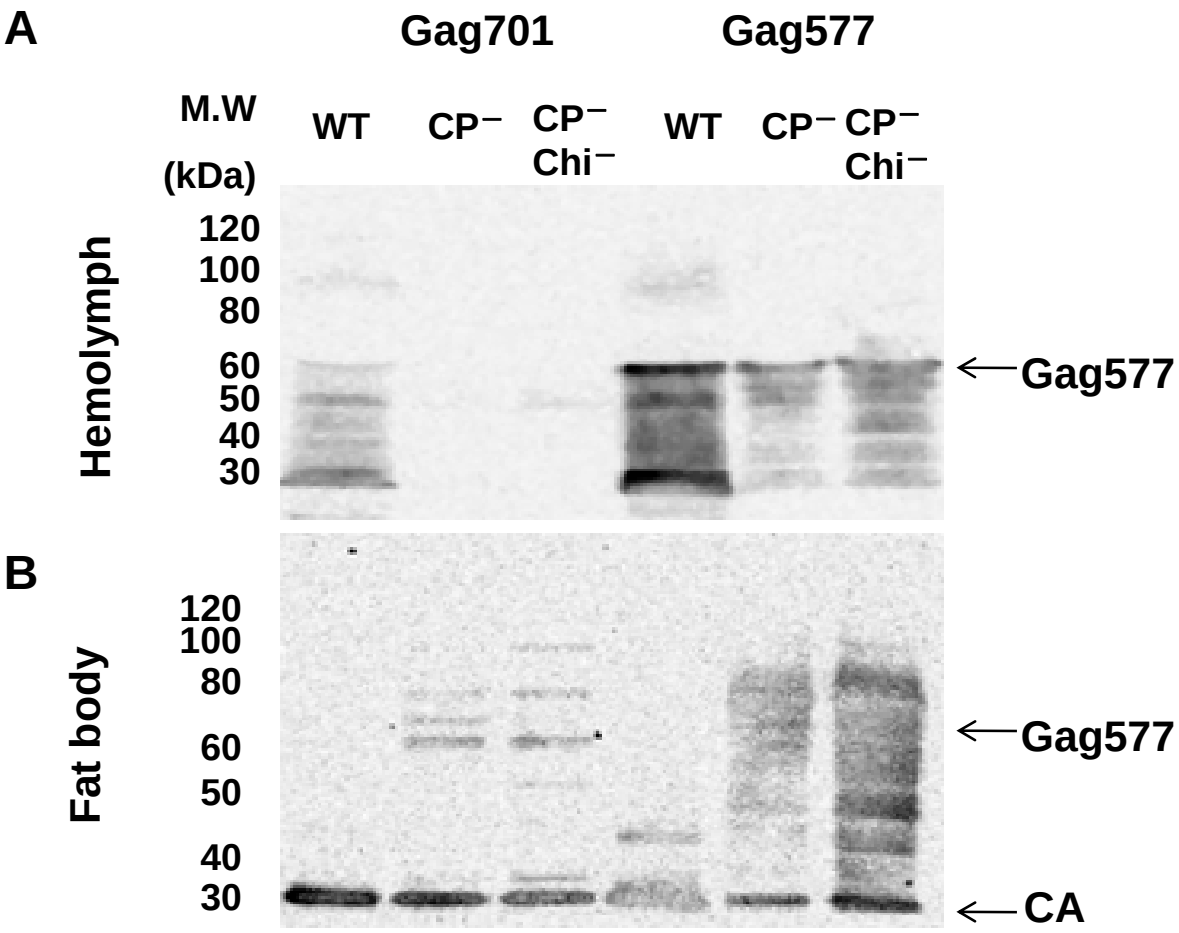
520

521 **Fig. 6.** Immunoelectron microscopy of RSV Gag VLP displaying bx-hPRR. (A)
522 Images of VLPs using mouse anti-FLAG M2 antibody as a primary antibody. (B) An
523 image of VLPs using rabbit anti-BmNPV GP64 antibody as a primary antibody.
524 Detailed methods are described in Materials and methods.

525

526 **Fig. 7.** Binding assay of hPRR on the surface of RSV Gag VLPs by ELISA using
527 purified RSV Gag VLPs. RSV Gag VLPs displaying hPRR were purified from
528 hemolymph by sucrose density gradient centrifugation after bx-hPRR and RSV
529 Gag577 were co-expressed in silkworm larvae. Human prorenin was added into each
530 well and the plate was incubated at room temperature after purified VLPs were
531 immobilized onto the ELISA plate. Human prorenin binding to hPRR were detected by
532 HRP reaction using mouse anti-human prorenin antibody and HRP-conjugated
533 anti-mouse IgG antibody. Detailed methods are described in Materials and methods.
534 Open bars: RSV Gag VLPs, black bars: RSV Gag VLPs displaying hPRR.

Fig. 1. Tsuji et al.



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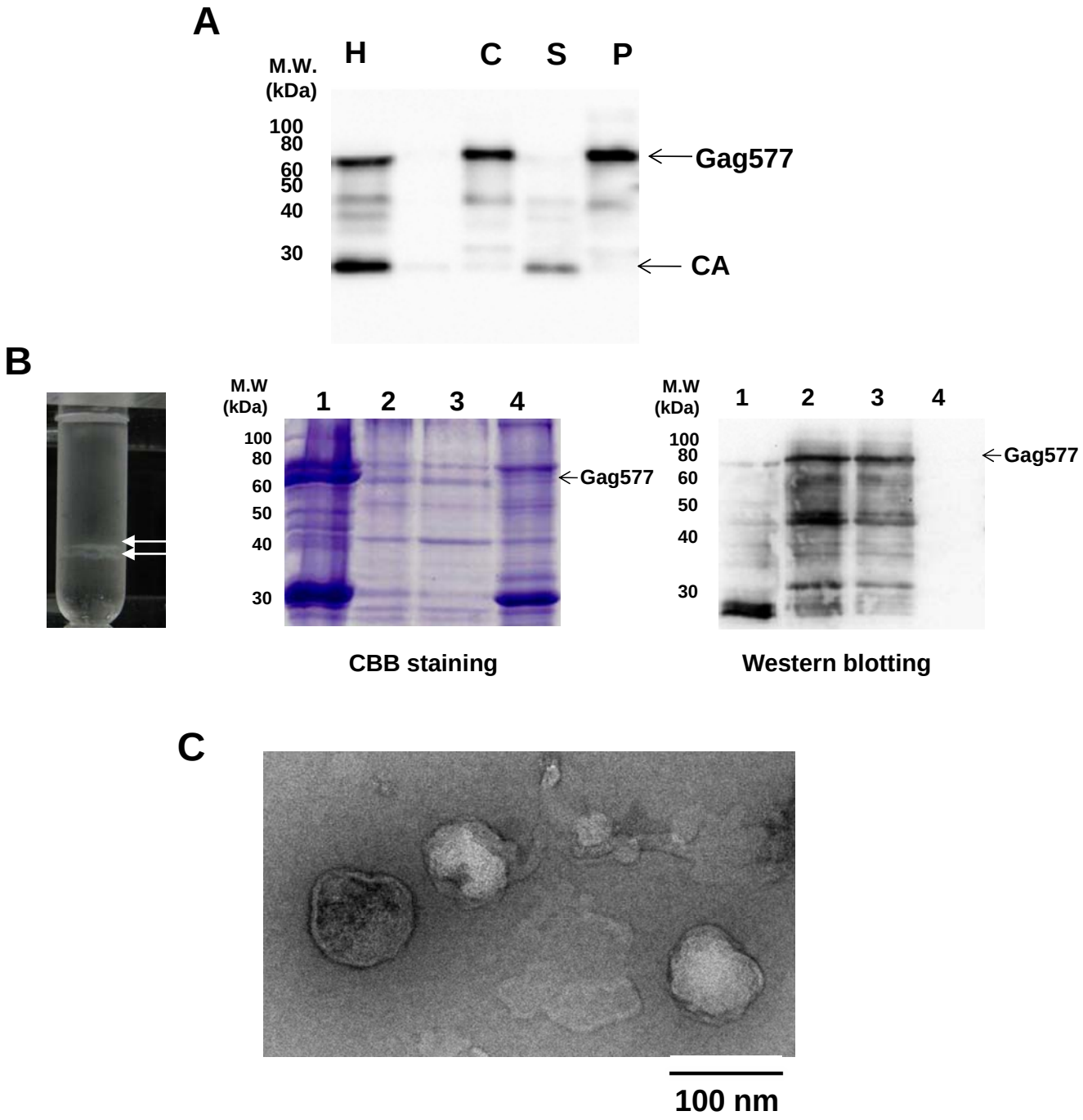

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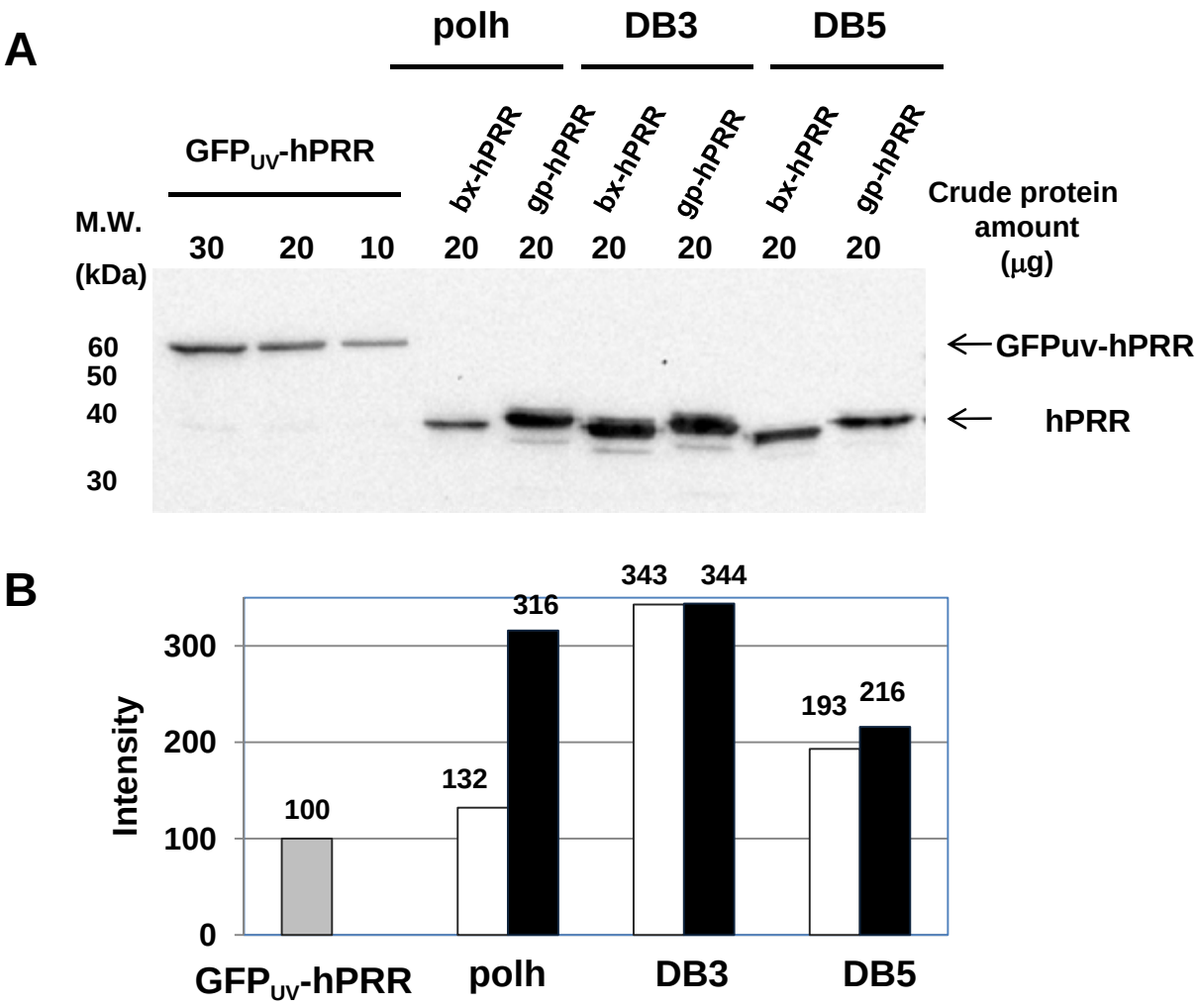

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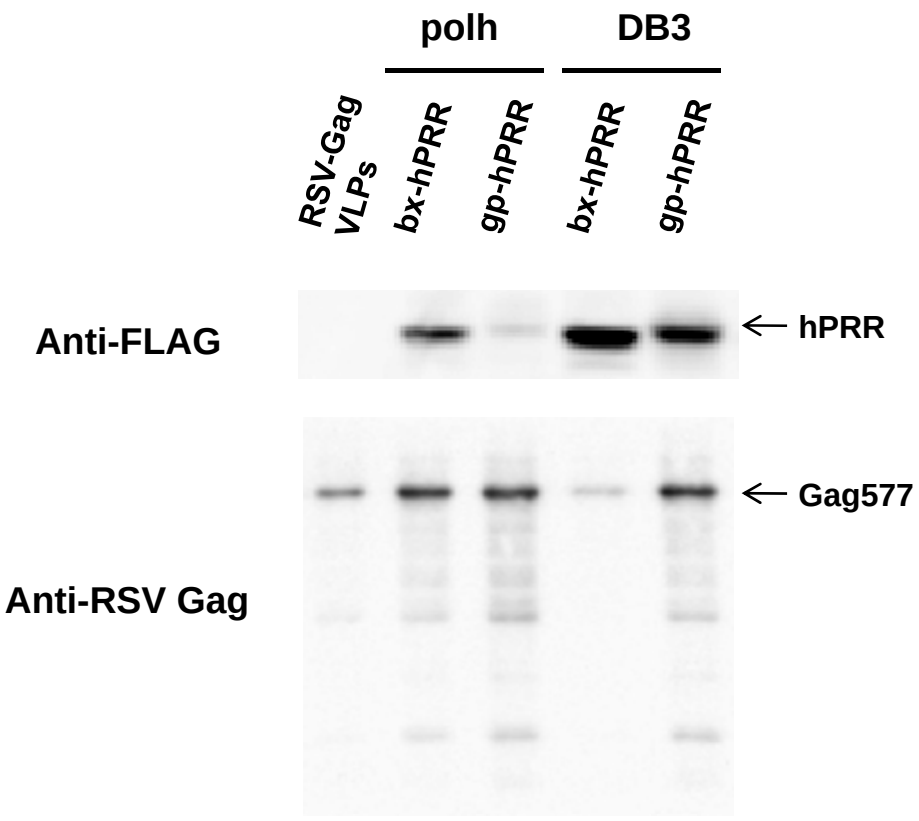

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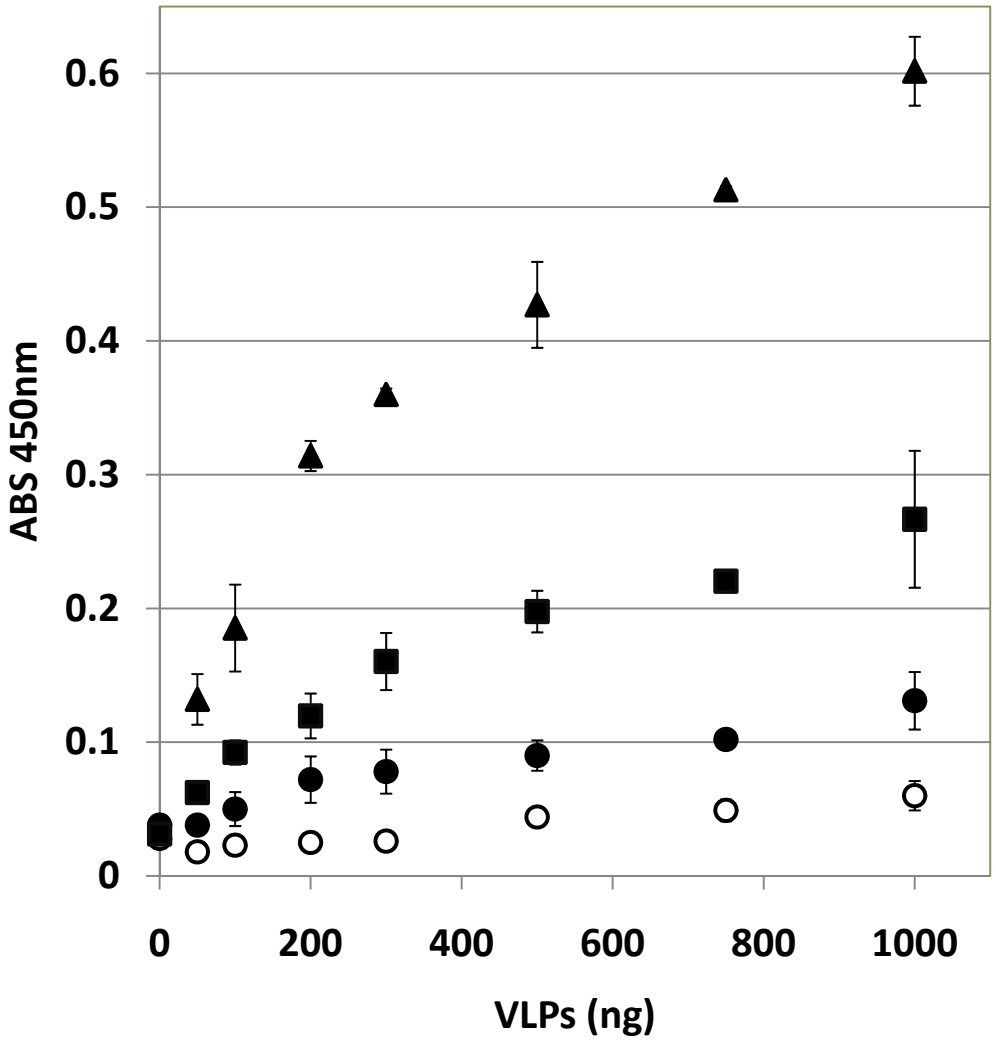
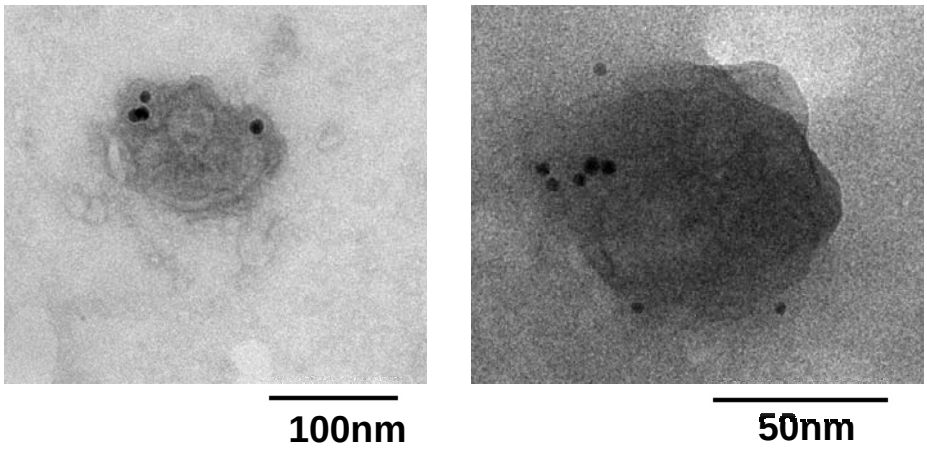


Fig. 6. Tsuji et al.

A. Anti-FLAG



B. Anti-gp64

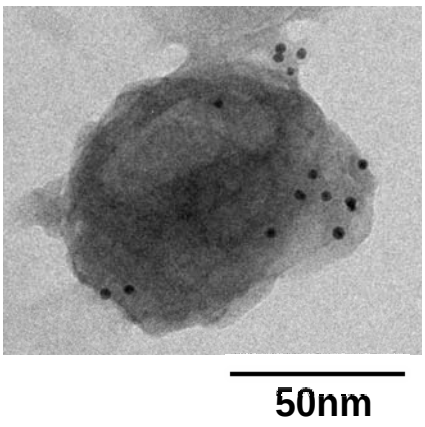


Fig. 7. Tsuji et al.