

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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1 Production of *Rous sarcoma* virus-like particles displaying
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5 Yoshitaka Tsuji^a, Vipin Kumar Deo^b, Tatsuya Kato^a, and Enoch Y Park^{a,b*}

6

7 ^a*Laboratory of Biotechnology, Department of Applied Biological Chemistry, Faculty of*

8 *Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan*

9 ^b*Laboratory of Biotechnology, Integrated Bioscience Section, Graduate School of*

10 *Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka*

11 *422-8529, Japan*

* Corresponding author at: Laboratory of Biotechnology, Integrated Bioscience Section, Graduate School of Science and Technology, Shizuoka University, 836 Ohya Suruga-ku, Shizuoka 422-8529, Japan. Tel.: +81 54 238 4887; fax: +81 54 238 4887. E-mail address: acypark@ipc.shizuoka.ac.jp (E.Y. Park).

12

13 **ABSTRACT**

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

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

34

35 **1. Introduction**

36

37 Virus-like particles (VLPs) are formed by self-assembling virus-derived structural
38 proteins. VLPs are empty shells and do not have virus genome DNA or RNA, therefore
39 VLPs are not infectious. Moreover, VLPs can induce strong cellular and humoral
40 responses as direct immunogens (Grgacic et al., 2006). For this property, VLPs have
41 been utilized for virus vaccines (Roldão et al., 2010). Human papillomavirus vaccine
42 (HIV), Gardasil and Cervarix, which were produced in *Saccharomyces cerevisiae* and
43 insect cells respectively, have received approvals for marketing (Garland et al., 2007).
44 The HIV VLPs composed of HIV-1 Pr55Gag have been developed for an AIDS
45 vaccine (Ludwig and Wagner, 2007). Otherwise, VLPs have been utilized as scaffolds
46 in nano-structural technology, especially, nanowires and nanobiopolymers (Mao et al.,
47 2004).

48 VLPs have been produced in *Escherichia coli*, yeasts, insect cells, plants and
49 mammalian cells. Especially, Gag proteins of enveloped viruses, for example, HIV,
50 *Rous sarcoma virus* (RSV) have all of the necessary domains for VLP assembly, and
51 VLPs can bud extracellularly from cells when Gag is solely expressed in insect and
52 mammalian cells (Ludwig and Wagner, 2007). RSV belongs to the avian retrovirus
53 family and RSV Gag protein, which self-assembles to VLPs, is proteolytically
54 processed by RSV protease to matrix (MA), p2 (p2a,b), capsid (CA), nucleocapsid
55 (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.

77

78 **2. Materials and Methods**

79

80 *2.1. Construction of recombinant BmNPV bacmid*

81

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

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

98 hPRR was amplified by PCR using EcoRI_{bx}SFLAG-F
99 (CACCGAATTCATGATACTCCTTGCTATTGCATTAATGTT
100 GTCAACAGTAATGTGGGTGTCAACACAAACCCCGCCCGACTACAAGGACG
101 ACGACGACAA) and SphI-hPRR-R
102 (TCAGCATGCTCAATCCATTCGAATCTTCTGGTTT). BmNPV CP⁺/GFP_{uv}-hPRR
103 bacmid DNA (Du et al., 2008) was used as a template. To obtain hPRR gene connected
104 with GP64 signal peptide sequence from BmNPV, hPRR was amplified by PCR using
105 EcoRI_{gp64S1-60}-F
106 (CACTGCAACGCGCAAATGAAAACGGGTCCGTACAAAATTA AAAACTTGGA
107 CATTACCCCGCCCGACTACAAGGACGACGACGACAA) and SphI-hPRR-R.
108 Then hPRR gene connected with whole GP64 signal peptide sequence was amplified
109 using gp64S60-114FLAG-F
110 (CACCGAATTCATGGTAGGCGCTATTGTTTTATACGTGCTTTTGGCGGCGCA
111 TTCTGCCTTTGCGGCGGAGCACTGCAACGCGCAAATGAA) and SphI-hPRR-R.
112 Each amplified gene was inserted into pENTR/D/TOPO by topoisomerase reaction.
113 hPRR connected with bx and gp signal peptides were designated as bx-hPRR and
114 gp-hPRR, respectively. Each gene was inserted into pDEST8, pDB3 and pDB5 by
115 Gateway system (Invitrogen). pDB3 and pDB5 was constructed previously to enhance
116 the protein expression under the control of modified polyhedron promoter (Manohar et
117 al., 2010). pDB3 and pDB5 have 3 and 5 repeated burst sequence in the region of
118 polyhedrin promoter, respectively. Total six plasmids (pDEST/bx-hPRR,

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

122

123 2.2 *Expression and sample preparation*

124

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

135

136 2.3 *Purification of VLPs from silkworm larvae*

137

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

140 was laid on 25% (w/v) sucrose cushion. This mixture was centrifuged at $80000 \times g$ for
141 90 min at $4^{\circ}C$ and the pellet was suspended with PBS (pH 6.2). This suspension was
142 sonicated to dissolve the pellet and this suspension was laid on 25-60% (w/v) sucrose
143 density gradient. This suspension was centrifuged at $96000 \times g$ for 3 h at $4^{\circ}C$. A white
144 band was recovered carefully and diluted with PBS (pH6.2). This diluent was
145 centrifuged at $96000 \times g$ for 90 min at $4^{\circ}C$. The pellet was suspended with a small
146 volume of PBS (pH 6.2) and this suspension was used as a purified VLP sample.

147

148 2.4. *SDS-PAGE and protein analysis*

149

150 Samples were subjected to SDS-PAGE on 10 or 12% (w/v) polyacrylamide gel
151 with the Mini-protean II system (Bio-Rad Co. Ltd, Hercules, CA, USA). Total proteins
152 on SDS-PAGE gel was detected with Coomassie Brilliant blue (CBB) R-250. In the
153 case of Western Blot, proteins in gels were blotted onto a polyvinylidene fluoride
154 (PVDF) membrane using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad,
155 Hercules, CA, USA). After being blocked in 5% skim milk in Tris-buffered saline
156 containing 0.1% (v/v) Tween 20 (TBST), the membrane was incubated in either
157 1:10000 diluted mouse anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA)
158 or 1:5000 diluted rabbit anti-RSV Gag CA antibody (gifted by Dr. Vogt) for 1 hour.
159 The membrane was washed with TBST, and then incubated in 1:20,000 diluted either
160 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).

167

168 2.5. *ELISA*

169

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

182 four times followed by HRP reaction. 100 μ l of substrate [0.1-mg/ml
183 3,3',5,5'-tetramethylbenzidine(TMBZ) in 100-mM sodium acetate, pH 6.0, with 0.2%
184 (v/v) of 30% hydrogen peroxide] was added to each well and left at room temperature
185 for blue-color development. The reaction was stopped by the addition of 50 μ l of 1N
186 H₂SO₄ solution. The developed color was measured at optical densities (ODs) of 450
187 nm and 655 nm. The value of OD₆₅₅ minus OD₄₅₀ was used as a measure of the
188 amount of hPRR.

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

202 2.6. *Immunoelectron microscopy*

203 Purified VLPs were immobilized on the carbon grid (Okenshoji, japan) and
204 blocked with 4% (w/v) BSA in PBS (pH 7.4). This grid was soaked in either mouse
205 anti-FLAG M2 antibody diluted by 30 fold with 1% (w/v) BSA in PBS or rabbit
206 polyclonal anti-GP64 antibody (Dojima et al., 2010) diluted by 500 fold with 1% (w/v)
207 BSA in PBS for 2 h. After washing with PBS, grid was soaked in either 10 nm gold
208 conjugated goat polyclonal anti-mouse IgG+IgM (H+L) (British BioCell International,
209 Cardiff, UK) diluted by 25 fold with 1%(w/v) BSA in PBS or 10 nm gold conjugated
210 goat polyclonal anti-rabbit IgG+IgM (H+L) (British BioCell International) diluted by
211 25 fold with 1%(w/v) BSA in PBS for 1 h. After washing with PBS, grid was stained
212 with 2% (v/v) phosphotungstic acid. VLPs were observed by transmission electron
213 microscope (TEM, JEM-2100F (JEOL Ltd., Tokyo, Japan)) at 200 kV.

214

215 **3. Results**

216

217 *3.1. Expression of RSV Gag protein in silkworms*

218

219 RSV Gag protein has several domains including N-terminal matrix domain (MA),
220 proline-rich late (L) domain (p2), spherical shape determinant (p10), two capsid
221 sub-domain (CA), RNA binding nucleocapsid domain and virus protease (PR). In
222 insect cells, PR deletion allowed to express normal spherical VLPs (Johnson et al.,
223 2001). In this study, two type of RSV gag protein (full-length Gag: amino acid 1-701,

224 Δ PR 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, Δ PR 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 Δ PR Gag using three types of BmNPV bacmid but Δ PR 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.

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

253

254 3.3 *Co-expression of RSV Gag and hPRR in silkworm larvae*

255

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

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

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

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

289

290 *3.4. Binding of hPRR on the surface of VLPs to human prorenin.*

291

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

297

298 **4. Discussion**

299

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

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

321 hPRR expression in fat body was higher using gp signal peptide under the control
322 of normal polyhedrin promoter than bx signal peptide, but vice versa with regard to
323 hPRR incorporation level into RSV VLPs. It has been reported, HIV envelope protein
324 (Env) was more efficiently incorporated into HIV Gag VLPs using mellitin signal
325 peptide than its native and AcMNPV GP64 signal peptides (Wang et al., 2007).

326 Normally, most of signal peptides are cleaved from expressed proteins in endoplasmic
327 reticulum by signal peptidase and expressed proteins are folded and transported to
328 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

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

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

367 In this study RSV Gag deleted PR domain was expressed in silkworm larvae and
368 VLPs composed of RSV Gag were purified from hemolymph. hPRR was displayed on
369 the surface of RSV Gag VLP when hPRR and RSV Gag were co-expressed in
370 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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384

385

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478 **Figure legends**

479

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

485

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

496

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

499 Grey bar: GFP_{uv}-hPRR expression, open bars: gp-hPRR expression, black bars:
500 bx-hPRR expression. polh indicates polyhedrin promoter. GFP_{uv}-hPRR was expressed
501 using polyhedrin promoter. Detailed methods are described in Materials and [Methods](#).
502

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

510

511 **Fig. 5.** Comparison of hPRR level on the surface of RSV Gag VLPs by ELISA.
512 Purified VLPs were immobilized onto the ELISA plate and hPRR was detected by
513 HRP reaction using mouse anti-FLAG M2 antibody and HRP conjugated anti-mouse
514 IgG antibody. Detailed methods are described in Materials and methods. Open circles:
515 RSV Gag VLPs, black circles: RSV Gag VLPs from GFP_{uv}-hPRR- and RSV
516 Gag-co-expressing silkworms, squares: RSV Gag VLPs from gp-hPRR- and RSV
517 Gag-co-expressing silkworms, triangles: RSV Gag VLPs from bx-hPRR- and RSV
518 Gag-co-expressing silkworms. gp-hPRR and bx-hPRR was expressed using DB3
519 promoter.

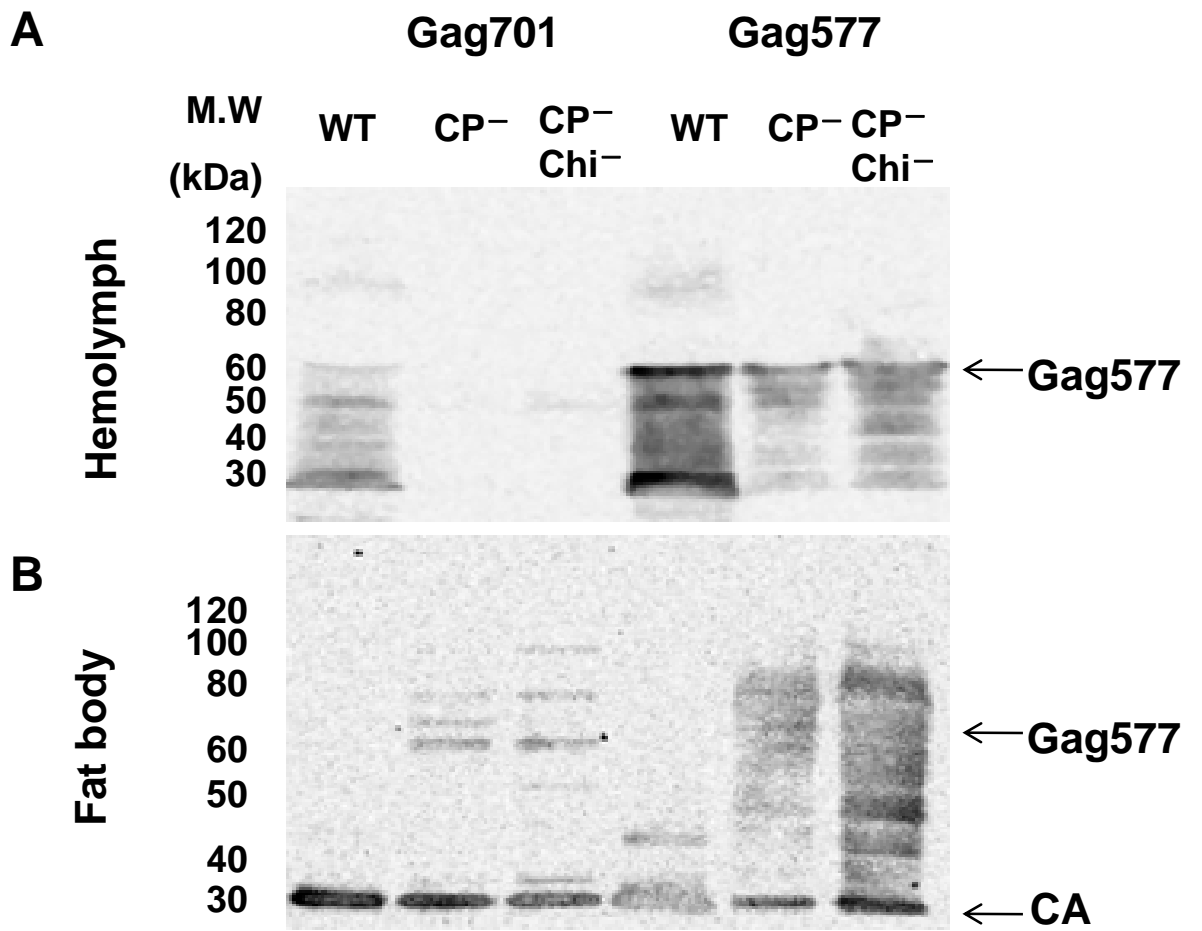
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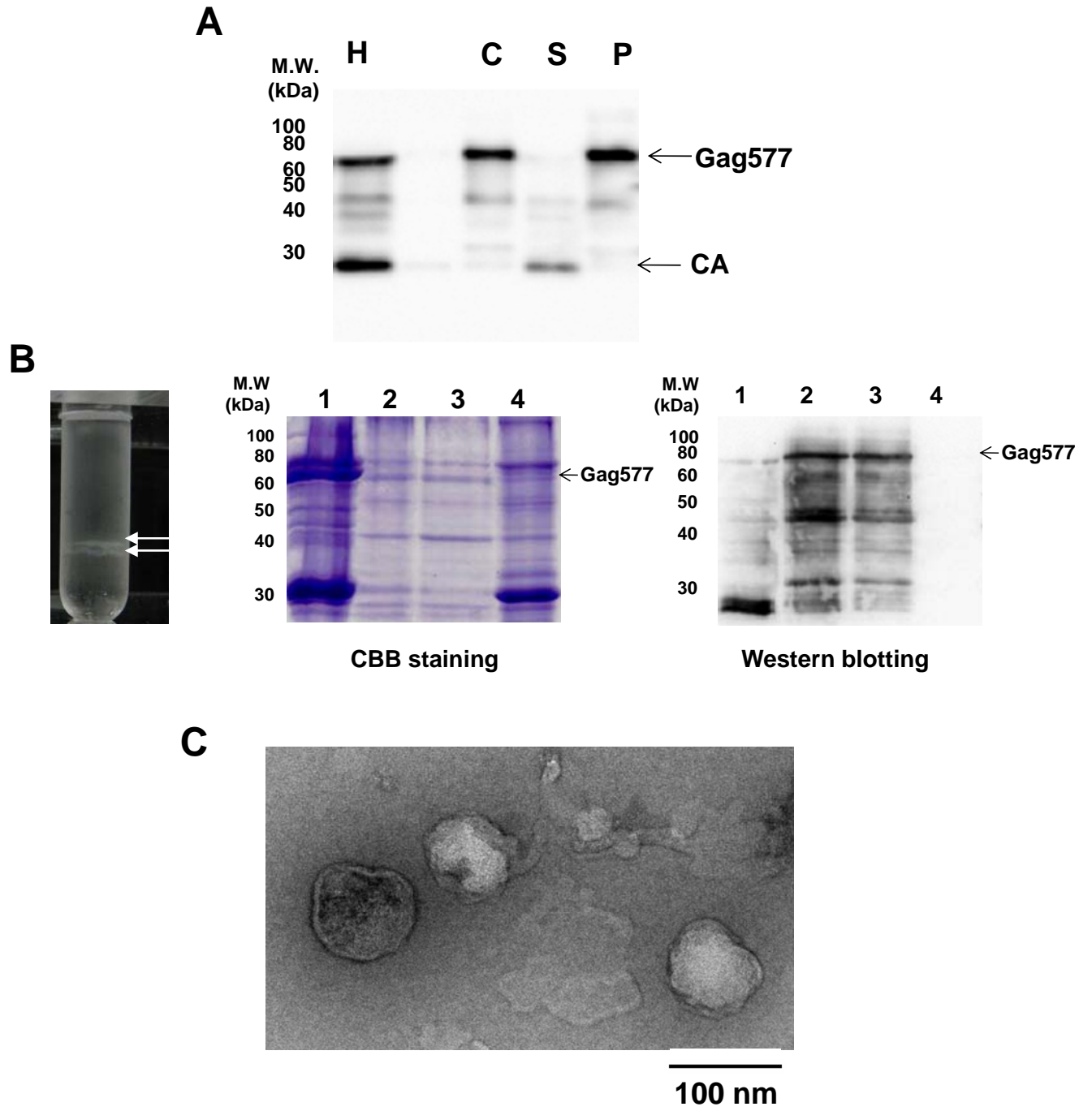
521 **Fig. 6.** Immunoelectron microscopy of RSV Gag VLP displaying bx-hPRR. (A)
522 Images of VLPs using mouse anti-FLAG M2 antibody as a primary antibody. (B) An
523 image of VLPs using rabbit anti-BmNPV GP64 antibody as a primary antibody.
524 Detailed methods are described in Materials and methods.

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.





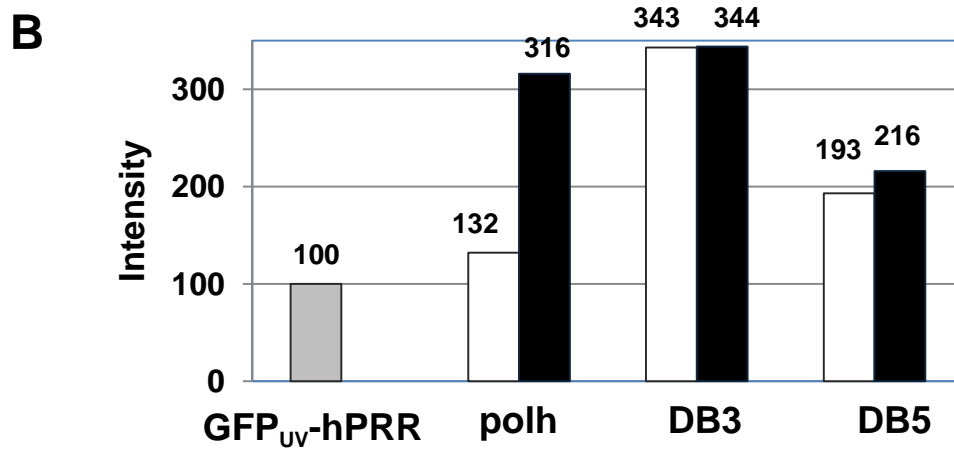
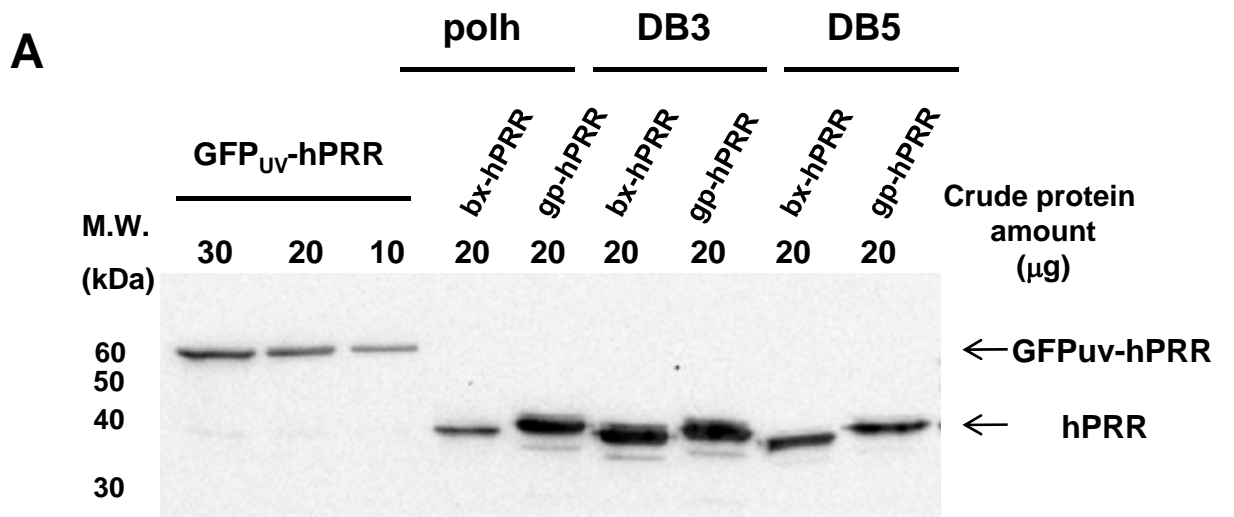


Fig. 4. Tsuji et al.

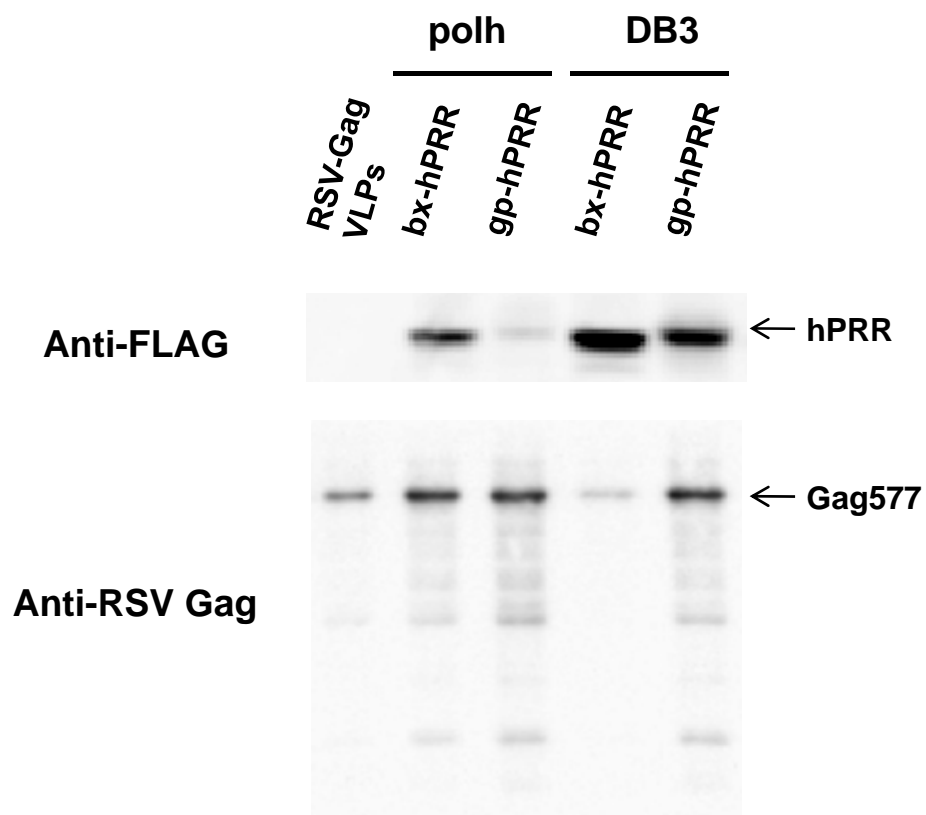
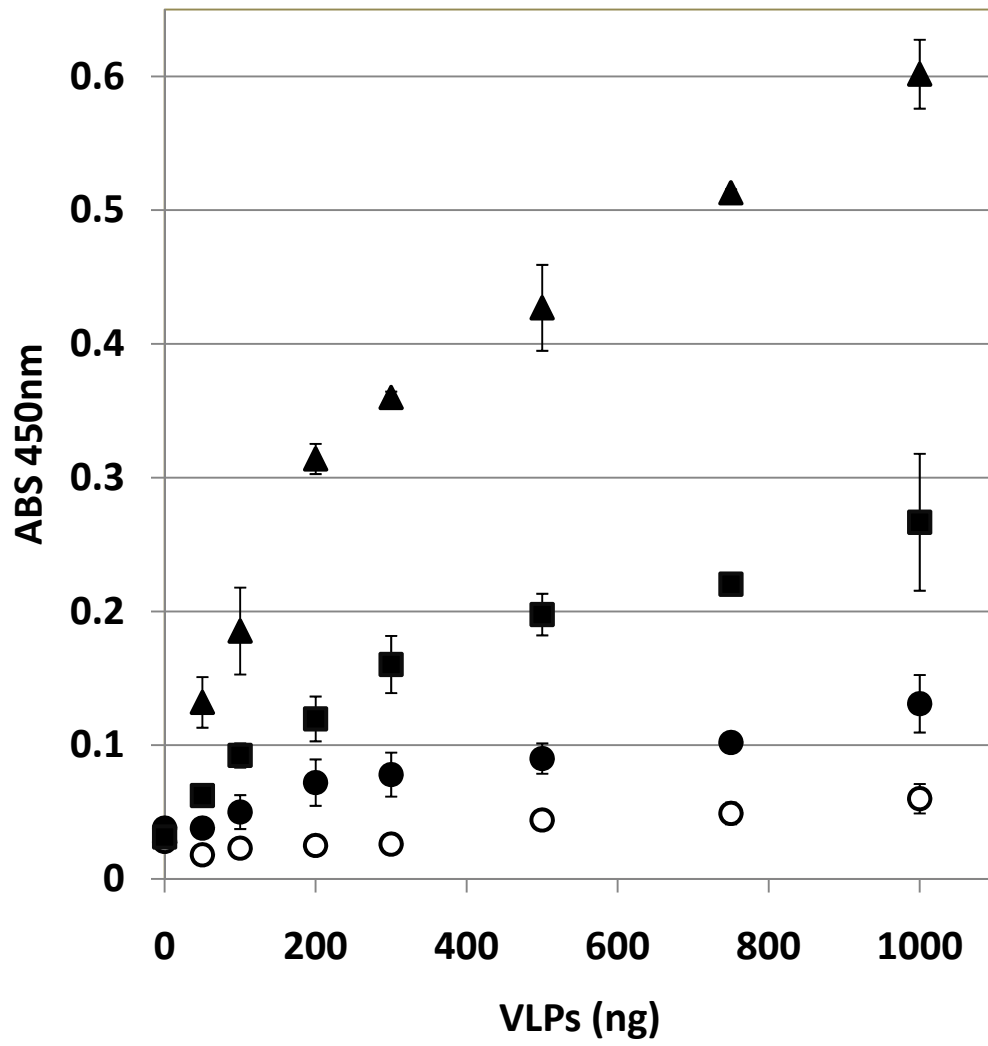
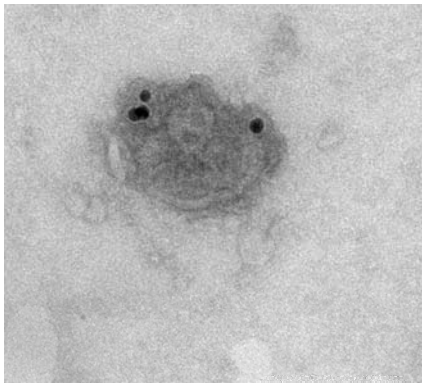


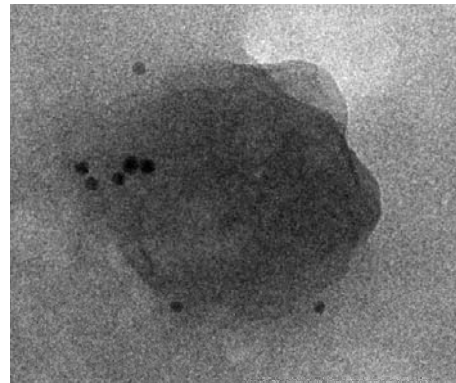
Fig. 5. Tsuji et al.



A. Anti-FLAG

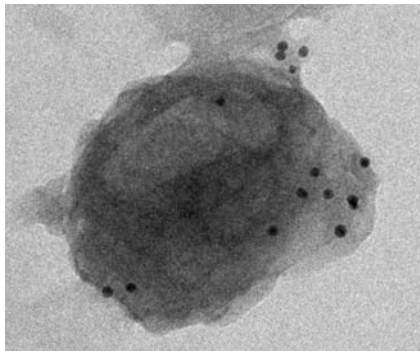


100nm



50nm

B. Anti-gp64



50nm

Fig. 7. Tsuji et al.

