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Abbreviations: BmNPV, *Bombyx mori* nucleopolyhedrovirus; DDS, Drug delivery system; ER, Endoplasmic reticulum; QD525, Quantum dot 525nm; RSV-gag, *Rous sarcoma* virus-group antigen; TEM, Transmission electron microscope; VLPs, Virus like particles

16 **Abstract**

17 *Rous sarcoma* virus group antigen protein based virus like particles (VLPs) are well
18 known for their structural integrity and ease of handling. VLPs are playing an important
19 role in drug delivery system as they can be manipulated with ease. Therefore, in this
20 study a new method was established for expressing *Rous sarcoma* virus group antigen
21 protein based VLPs in silkworm larvae and establishing stably expressing insect cell
22 lines. These VLPs have been isolated using step sucrose gradient of 10%-60% (v/v) by
23 ultracentrifugation method and their spherical structure has been confirmed under
24 transmission electron microscope. The spherical morphology is similar in both the
25 silkworm larvae and in stably expressing cell lines. Silkworm larvae are better suited for
26 producing *Rous sarcoma* virus group antigen protein based VLPs on large scale
27 approximately 8.2 fold higher than the stable cell lines. These VLPs shall provide a new
28 method for large scale application in vaccine development and drug delivery system.

29 **Keywords:** *Rous sarcoma* virus group antigen protein, Virus like particle, *Bombyx mori*

30 nucleopolyhedrovirus bacmid, Silkworm, Insect cell

31

1. Introduction

Rous sarcoma virus (RSV) species belongs to the family *Retroviridae*, subfamily *Orthoretrovirinae,* genus *Alpharetrovirus* of single stranded RNA virus was one of the first found to cause cancer in chickens. Lot of study has been reported to elucidate the structure of group antigen protein (gag) which is important in the assembly and packaging of the RNA into the virus and subsequent pinching from the host's cell membrane. It's composed of mature proteins matrix (MA), capsid (CA) and nucleocapsid (NC) proteins required to package the RNA genome and later being cleaved off by viral proteases (Ma and Vogt, 2002). RSV-gag codes for 701 amino acid length protein which dimerize on the cellular membrane to form the VLPs of 30-120 kDa range. These researches have become more relevant owing to the conserved nature of the protein found in other virus belonging to the retroviridae family.

Retrovirus based vaccines have the potential of becoming the next generation DDS. Retroviruses are famous for their ingenuity to enter the host cell utilizing the viron which have evolved the art of crossing the host plasma membrane barrier efficiently (Waelti and Gluck, 1998). Up till now mainly viral based gene therapy has been focus of research and improvement but a yet not well established field wherein only the virion or the viral envelope only is used as a container to transport therapeutics to a cell or

tissues has not been exploited efficiently(Kondo et al., 2008). VLPs are capsid proteins minus the genetic material of the virus; in short they are hollow viral protein cages. These capsid proteins from almost any virus minus the genetic material when expressed in an expression system have the innate ability to fold and self-assemble as VLPs. This property has been of great interest from the vaccine development aspect (Roy and Noad, 2008). Neutralizing a threat from an epidemic virus like influenza is very important. They have the ability to mask their surface proteins like hemagglutinin (HA) very quickly and resurface with a vengeance with very high mortality rate (Fanning and Taubenberger, 1999). Presenting these active components of the virus for developing antibodies or vaccines for treatment and detection is of prime importance. Concerns regarding the safety of recombinant viruses have prompted increasing interest in viral systems carrying no genetic material; VLPs present an appropriate opportunity to be used as a DDS since it carries no genetic material.

Insect cell serves as a good host and an equally better expression system to express different proteins for commercial and research applications utilizing baculovirus (Dojima et al., 2009). Up till now many researchers have used baculovirus expression system to express virus like particles (VLPs) and using it is well known to produce vaccine delivery systems (Keller et al., 2008; Noad and Roy, 2003; Wills et al., 1989).

68 Recently silkworms have evolved as an efficient expression system able to express
69 proteins at high levels using *Bombyx mori* nucleopolyhedrovirus (BmNPV) species
70 belonging to family *Baculoviridae* (Kato et al., 2010) of the family of double stranded
71 DNA virus. It takes almost one month to express and purify any protein at milligram
72 level using BmNPV bacmid based expression system in silkworm larvae (Dojima et al.
73 2010). Injecting two or more bacmids with two or more sub-unit proteins can be
74 co-expressed and allowed to form complete functional proteins which can then be
75 purified. Silkworm larvae are completely domesticated insects and they have been in
76 use for many centuries without affecting humans.

77 In the current study the focus is on the need to establish a system comprising of
78 stable insect cell line and silkworm based expression system to express a complete and
79 RSV-gag minus the PR region of approximately 75 kDa and 61 kDa theoretical
80 molecular weight, respectively. The short RSV-gag-577 codes for 577 amino acids and
81 its ability to form VLPs is well documented (Joshi and Vogt, 2000). The ability of
82 silkworms to express the VLPs and serve as a more potent system to express VLPs in
83 future compared to other bacterial systems (Yu et al., 2001). This is also important for a
84 large scale application of VLPs as vaccine/DDS can be made by fusing robustness of
85 insect cell expression system and VLPs.

2. Materials and methods

2.1. Cell line, medium, silkworm larvae and culture

Tn-5B1-4 cells from *Trichoplusia ni* were purchased from Invitrogen (San Diego, CA, USA) and grown in both 25 cm^2 tissue culture flasks (Falcon) and 100 ml flasks. Suspension cultures were carried out in 100 ml flask with a working volume of 15 ml in Express five serum free medium (SFM) (Invitrogen) supplemented with 1% (v/v) antibiotic–antimycotic (Invitrogen) (Deo and Park, 2006). A scale up of the suspension culture was done under similar condition of culture using 500 ml flask with a culture volume of 100 ml. Fifth instars larvae (Ehime Sansyu Co. Ltd., Ehime, Japan) were reared on an artificial diet Silkmate S2 (Nihon Yokohama, Japan) for silkworms in a chamber (MLR-351H, Sanyo, Tokyo, Japan) with 65% humidity at 27°C.

2.2. Construction of vectors

pIB/V5-His-Dest Gateway vector was used for stable expression in insect cell expression system (Invitrogen, Carlsbad, CA, USA). The *RSV*-gag cDNA was PCR amplified using pRep(A) (ATCC catalogue number 87702) as a template. The *RSV-gag*-577 insert was PCR amplified using primers; Forward primer: 5′-CACC ATGGAAGCCGTCATAAAGGTG -3′; reverse primer: 5′-TTA CGA GAC GGC AGG

103 TGG CTC AGG-3′. The *RSV-gag*-701 insert was PCR amplified using primers;
104 Forward primer: 5′-CACC ATGGAAGCCGTCATAAAGGTG-3′; reverse primer:
105 5′-CTA TAA ATT TGT CAA GCG GAG-3′. PCR products were inserted into the entry
106 vector, pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA) to give
107 pENTR/RSV-gag-701 and pENTR/RSV-gag-577 respectively. The sequences of all the
108 PCR fragments inserted into pENTR/D-TOPO were confirmed by dideoxynucleotide
109 chain terminating sequence (Sanger et al., 1977) using Thermo Sequenase Cycle
110 Sequencing kit (USB, Cleveland, Ohio, USA). pENTR/RSV-gag-577 was used to
111 prepare the expression plasmid, pIB/V5-His-RSV-gag-577, by performing LR reaction
112 as per the protocol of stable cell line technology (Invitrogen).
113 For expression of RSV-gag in silkworm larvae BmNPV bacmid was used.
114 *RSV-gag*-701 and *RSV-gag*-577 from pENTR/RSV-gag-701 and pENTR/RSV-gag-577,
115 respectively, were transferred to pDEST8 by LR reaction according to the protocol to
116 make pDEST8/RSV-gag-701 and pDEST8/RSV-gag-577 respectively, which was used
117 to transform the DH10 BmNPV *Chi*$^{-}$-*CP*$^{-}$ competent cells to make recombinant bacmids
118 (Deo et al., 2006). White colonies of recombinant bacmids carrying the *RSV-gag*-701
119 and *RSV-gag*-577 were isolated and resulting bacmids were designated as BmNPV
120 bacmid/RSV-gag-701 and BmNPV bacmid/RSV-gag-577, respectively, and inoculated

into 3 ml LB medium along with the antibiotics used for screening. After culturing them in LB medium with the antibiotics for 36 hours the BmNPV bacmids were isolated and confirmed by using standard M13 primers. Positive transformants were re-inoculated in 100 ml LB medium and isolated in large amount for injecting them into fifth-instar silkworm larvae.

2.3. Injecting silkworms

Each Silkworm was injected with 40 µl recombinant bacmid DNA solutions containing 10 µg of BmNPV bacmid DNA, 10% (v/v) DMREI-C reagent (Invitrogen) in PBS using 1 ml syringes. Post injection 7^{th} day the silkworm's hemolymph was harvested in falcon tubes containing 2 mM phenyl thiourea to inhibit the melanization. These samples were then aliquoted into 1 ml eppendorf tubes and stored at – 80ºC.

2.4. Serial screening of stable cell lines

Tn-5B1-4 cells at 1×10^4 cells per well were seeded into the 6 well plates and allowed to grow overnight in the incubator. The cells were washed with serum free medium and then 1 ml containing vectors and lipofectin mixed as per the kit protocol (Invitrogen) were overlaid. The cells were then incubated for 4 hours in the incubator with mild shaking in an interval of 30 minutes. After 4 hours the medium was removed

138 and replaced with fresh Express Five SFM. These cells were incubated and allowed to
139 grow for 2 days. After 2 days Express five SFM containing 80 µg/ml Blasticidin
140 (Invitrogen) was added and the medium was replaced every 3rd day. The foci of resistant
141 polyclonal stable cell lines formed were picked up carefully maintaining its
142 homogeneity for each transformed cell line and were seeded into 96-well plates (Falcon,
143 Lincoln Park, NJ, USA) separately, subsequently (Deo and Park, 2006). After 1 week,
144 confluent grown cells were seeded into 24-well plates of 2 cm^2 surface area (Falcon).
145 After 2 weeks, confluent grown cells were selected and their supernatant was removed
146 and exchanged with fresh medium. The supernatant was used for western-blot analysis
147 and the positive cell lines were selected and further seeded into six-well plates of 9.6
148 cm^2 surface area (Falcon).

149 *2.5. Genomic DNA extraction and confirmation by PCR*

150 Genomic DNA was extracted from the transformed cells using a FlexiGene DNA
151 kit (Qiagen, Valencia, CA, USA). For analyzing the genomic *RSV-gag* cDNA, a PCR
152 was performed using the primers used for amplifying the cDNA earlier under similar
153 conditions. PCR product was resolved on 0.75 % (w/v) agarose gel by electrophoresis.

154 *2.6. Western-blot analysis*

155 To detect the expression of recombinant protein, culture supernatants of stable cell
156 lines and larval hemolymph and fat body collected from silkworm larvae, respectively
157 were subjected to 12% (w/v) SDS-poly-acrylamide gel electrophoresis (SDS-PAGE)
158 using the mini-protean II system (Bio-Rad) (Deo and Park, 2006). After SDS-PAGE,
159 proteins were blotted on to a PVDF membrane using the Mini Trans-Blot
160 Electrophoretic Transfer cell (Bio-Rad). The membranes were probes with
161 anti-RSV-gag primary antibody solution for 1 h (A kind gift from Dr. Vogt) and
162 secondary goat anti-rabbit IgG antibody labeled with horse-radish peroxidase (HRP) for
163 1 h. Using ECL plus western blotting reagent pack (Amersham Biosci.) specific bands
164 were detected. Those bands were analyzed using a Fluor-S/MAX multi-imager
165 (Bio-Rad).

166 The protein concentrations were estimated using standard BCA protein estimation
167 protocol from kit (Pierce BCA Assay kit, Japan).

168 *2.7. Sucrose gradient and purifications*

169 VLPs containing supernatant collected from stable cell line cultures and silkworm
170 larvae were centrifuged at 1000×g in a Sorvall Biofuge (Primo R, Heraeus, Germany)
171 for 3 min using Heraeus 7591 swing bucket rotor to remove any cells or debris. The
172 clear supernatant was spun in a micro ultracentrifuge (Himac CS120GX, Hitachi Koki,

Tokyo, Japan) at 171,000×g at 4ºC for 30 min using S52ST swing bucket rotor (Hitachi Koki) to pellet the VLPs. The pellets were resuspended in 200 µl of STE (100 mM NaCl, 10 mM TrisHCl pH 7.5, 1 mM EDTA) buffer. This was overlaid on a step sucrose gradient of 4.5 ml of 10%-60% (w/w) prepared in STE. Gradients were spun at 244,000×g at 4ºC for 2 h using S52ST swing bucket rotor (Hitachi Koki). The resulting gradients were collected in 7 fractions (fraction 1 to 4 of 500 µl and fraction 5 to 7 of 1 ml). Each fraction was further concentrated and buffer was exchanged using Microsep spin 10K columns (Pall, NY, USA). VLPs containing fractions were pooled and stored at 4ºC.

2.8. Visualization of VLPs under transmission electron microscope (TEM) and confocal laser scanning microscope

Stable cell lines were cultured for 3 days and around 1×10^4 cells were seeded on cover slips (2 cm×2 cm) and incubated at 27^{o}C overnight. The cells were washed with fresh medium and incubated with Quantum dot 525 conjugated anti-RSV-gag antibodies prepared as per kit protocol (Invitrogen) for 2 hours (Kampani et al., 2007). The cells were washed once more and then viewed under confocal laser scanning microscope (LSM 700, Carl Zeiss, Oberkochen, Germany). Zen LE software available on Carl Zeiss website was used for simulating the surface expression of RSV-gag based VLPs.

191 Samples prepared were diluted 1:500 in STE buffer. The samples were then
192 spotted upon the carbon grid (Okenshoji, Tokyo, Japan) and dried at room temperature.
193 Negative staining was performed using 4% phospho tungstenic acid (Silverman and
194 Glick, 1969; Vogt and Simon, 1999). Samples were observed at 50,000× magnifications
195 on TEM (JEM 2100F, JOEL, Tokyo, Japan) operating at 200 kV (Briggs et al., 2006).

196 **3. Results**

197 *3.1. Expression of RSV-gag based VLPs in stably expressing insect cells*

198 RSV-gag based VLPs and its components have molecular weight in the region of
199 6 – 75 kDa (Fig. 1A). Many clones were screened for complete RSV-gag-701 and
200 RSV-gag-577 for expression of RSV-gag based VLPs and their genomic DNAs were
201 isolated. No good clones where found for the complete RSV-gag-701. Using primers
202 specific for *RSV-gag*-577 cDNA their presence was confirmed in D6 clone (Fig. 1B).
203 D6 clone was then grown in suspension culture and the expression of RSV-gag based
204 VLPs was confirmed using supernatants by western blotting (Fig. 2A). It was found that
205 the expression was the highest on 2nd after passaging for D6 clone in the supernatant
206 (Data not shown). Clone D6 was scaled up and the supernatant was collected for
207 isolation of VLPs by step sucrose gradient method. The various fractions collected were

208 analyzed and it was found that fraction number 5 was the best. Fraction number 5
209 contains RSV-gag-577 and p10-CA which were more homogenous VLPs based upon
210 the theoretical molecular weight (Fig. 2B).

211 *3.2. Expression of RSV-gag based VLPs in silkworm larvae*

212 BmNPV bacmid/RSV-gag-701 and BmNPV bacmid/RSV-gag-577 were injected
213 into the silkworms, respectively, and the larval hemolymph and fat bodies were
214 collected post-injection 7^{th} day. There was no band present for RSV-gag-701 in the
215 larval hemolymph and fat bodies collected (Data not shown). Thus only samples
216 collected from BmNPV bacmid/RSV-gag-577 infected silkworms were used for
217 isolation of VLPs. VLPs produced in silkworms were mostly present in hemolymph and
218 isolated in similar manner using step sucrose gradient by ultracentrifugation. After
219 ultracentrifugation two distinctive layers were observed (Fig. 3A). Both the layers were
220 eluted carefully and checked by western blotting and SDS-PAGE, respectively (Fig. 3B
221 and C) to confirm the RSV-gag protein and the purity of the samples.

222 *3.3. Images of VLPs from silkworm and stably expressing cell lines*

223 In order to understand the VLPs assembly on the plasma membrane D6 clone was
224 selected and the cells were prepared for observation using confocal laser microscopy.

225 The cells were stained with ER marker (a of Fig. 4A). Using anti-RSV-gag conjugated
226 with Qdot525nm the cells showed sharp fluorescence on their surface (b of Fig. 4A).
227 VLPs accumulated on the surface can be distinctively observed. The overlap of QD525:
228 Anti-RSV-gag provided the proof that the VLPs were transported to the plasma
229 membrane (d of Fig. 4A). Using Zen LE software for 3D morphology in the Carl Zeiss
230 LSM 700 microscope, pictures were collected in a time stacked manner which was used
231 as a data for simulating the expression of VLPs conjugated to QD525 (Fig. 4B).
232 VLPs purified from the step sucrose gradient method from both D6 clone and
233 silkworm hemolymph were analyzed under TEM using phospho tungstenic acid as a
234 negative stain. The samples from D6 and silkworm hemolymph showed a smooth
235 spherical shaped VLP resembling the native form (Fig.4C and D). The bilayer was
236 distinct and clearly visible owing to the contrast surrounding the dense core. The VLPs
237 purified from fraction number 5 of stably expressing cell lines and from the silkworms
238 showed approximately less than 100 nm (Fig. 4C and D). The VLPs purified from
239 stably expressing cell line D6 were spherical and this proves the establishment of stably
240 expressing RSV-gag based VLPs cell line (Fig. 4C).

241 **4. Discussion**

242 The molecular weight of the mature RSV-gag-577 based VLPs is little higher than

243 expected due to post-translational modifications in insect cells. Further glycosylation
244 analysis is required to elucidate this pattern. In western blotting antibody raised against
245 p10 region of RSV-gag was used, this region is very important for VLPs (Kenney et al.,
246 2008; Scheifele et al., 2007). From results it was found that since all the varying sizes of
247 VLPs have p10 region in common hence the antibodies attach to these regions which is
248 why various sizes of bands for VLPs is observed.

249 No significant differences in the molecular weight and the intensity of the bands
250 were observed although there were two distinct layers after ultracentrifugation in
251 hemolymph from silkworms. This might probably be due to some hydrophobic
252 intracellular protein contaminants sticking to the VLPs thereby causing change in
253 density as proteins are separated based upon their density in sucrose gradient. The
254 protein concentrations from the different fractions were estimated (Table 1) and the
255 yield was determined. The purified VLPs from lower layer of silkworm larval
256 hemolymph are approximately 8.2 fold higher than that of 5^{th} fraction from D6 stable
257 cell line. In larval hemolymph lot of proteins are present in very high concentration
258 compared to expressed VLPs hence the yield is low. This is due to the infection
259 rupturing the internal organs and releasing their contents into the hemolymph. On the
260 other hand in stable cell lines only supernatant is collected as VLPs are secreted out by

forming vesicles at the outer periphery of the cell boundary thereby very less amount of cellular proteins are present giving more overall yield (Nandhagopal et al., 2004). To further enhance the yield and quality of the VLPs tagged proteins carrying the plasma membrane targeting signal can be displayed on the surface and using tag specific columns further improvement can be achieved.

From the results it's clear that VLPs aggregate on the plasma membrane and it also supports the theory that the VLPs accumulate on the plasma membrane where they are self assembled to form the VLPs (Dalton et al., 2005; Johnson et al., 2005; Krishna et al., 1998). When sufficient number of gag monomers have accumulated then owing to the decrease in surface tension causes pinching of the VLPs from the surface of the plasma membrane. Because of this phenomenon the VLPs can be easily collected from the supernatant and larval hemolymph without the use of any procedure leading to disruption of cells. Using the stable cell lines the pinching method of VLPs was confirmed. The assembly and pinching of VLPs is dependent upon the cells ability to express the core proteins which in turn is also dependent upon the culture conditions. These variables have yet not been explored and from the data presented in current study a hypothesis can be proposed that the VLPs form different sizes of VLPs and hence the presence of different bands in purified samples. The structural morphology was similar

279 to native RSV particles (Keller et al., 2008) and better than bacterial expression systems
280 (Joshi and Vogt, 2000). The RSV-gag based VLPs purified from silkworms had smooth
281 spherical morphology and the distinctive bilayer of the lipid was very well preserved
282 (Fig. 4D). The protein core is stained differentially in comparison with lipid bilayer as
283 phospho tungstenic acid has more specificity for protein. The results from electron
284 microscopy have revealed that there are more number of approximately 50-100 nm
285 diameter RSV-gag based VLPs although there are many bands in western blotting. This
286 proves that the path of VLPs formation is similar, but the rate of pinching was fast or
287 slow which gave the VLPs the size (Ako-Adjei et al., 2005; Campbell and Vogt, 1997).

288 **5. Conclusion**

289 In conclusion, VLPs were expressed in silkworms and stable cell lines to establish a
290 VLPs purification system. It was clear that the silkworm based expression system owing
291 to its scale of production and ease of handling makes the expression system very
292 reliable. Further studies are required to improve the purification of VLPs in order to
293 obtain a fixed size and molecular weight VLPs using some tags. This shall benefit in
294 making VLPs a prominent candidate for vaccine platform and drug delivery systems.

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References

Ako-Adjei, D., Johnson, M.C., Vogt, V.M., 2005. The retroviral capsid domain dictates virion size, morphology, and coassembly of gag into virus-like particles. J Virol 79, 13463-72.

Briggs, J.A., Johnson, M.C., Simon, M.N., Fuller, S.D., Vogt, V.M., 2006. Cryo-electron microscopy reveals conserved and divergent features of gag packing in immature particles of Rous sarcoma virus and human immunodeficiency virus. J Mol Biol 355, 157-68.

Campbell, S., Vogt, V.M., 1997. In vitro assembly of virus-like particles with Rous sarcoma virus Gag deletion mutants: identification of the p10 domain as a morphological determinant in the formation of spherical particles. J Virol 71, 4425-35.

312 Dalton, A.K., Murray, P.S., Murray, D., Vogt, V.M., 2005. Biochemical characterization
313 of rous sarcoma virus MA protein interaction with membranes. J Virol 79,
314 6227-38.
315 Deo, V.K., Hiyoshi, M., Park, E.Y., 2006. Construction of hybrib Autographa californica
316 nuclear polyhedrosis bacmid by modification of p143 helicase. J Virol Methods
317 134, 212-6.
318 Deo, V.K., Park, E.Y., 2006. Multiple co-transfection and co-expression of human
319 beta-1,3-N-acetylglucosaminyltransferase with human calreticulin chaperone
320 cDNA in a single step in insect cells. Biotechnol Appl Biochem 43, 129-35.
321 Dojima, T., Nishina, T., Kato, T., Ueda, H., Park, E.Y.,2010. Production of scFv
322 displaying BmNPV in silkworm larvae and its efficient purification. Biotechnol
323 Appl Biochem.
324 Dojima, T., Nishina, T., Kato, T., Uno, T., Yagi, H., Kato, K., Park, E.Y., 2009.
325 Comparison of the N-linked glycosylation of human
326 beta1,3-N-acetylglucosaminyltransferase 2 expressed in insect cells and
327 silkworm larvae. J Biotechnol 143, 27-33.
328 Fanning, T.G., Taubenberger, J.K., 1999. Phylogenetically important regions of the
329 influenza A H1 hemagglutinin protein. Virus Res 65, 33-42.

330 Johnson, M.C., Spidel, J.L., Ako-Adjei, D., Wills, J.W., Vogt, V.M., 2005. The
331 C-terminal half of TSG101 blocks Rous sarcoma virus budding and sequesters
332 Gag into unique nonendosomal structures. J Virol 79, 3775-86.
333 Joshi, S.M., Vogt, V.M., 2000. Role of the Rous sarcoma virus p10 domain in shape
334 determination of gag virus-like particles assembled in vitro and within
335 Escherichia coli. J Virol 74, 10260-8.
336 Kampani, K., Quann, K., Ahuja, J., Wigdahl, B., Khan, Z.K., Jain, P., 2007. A novel
337 high throughput quantum dot-based fluorescence assay for quantitation of virus
338 binding and attachment. J Virol Methods 141, 125-32.
339 Kato, T., Kajikawa, M., Maenaka, K., Park, E.Y., 2010. Silkworm expression system as
340 a platform technology in life science. Appl Microbiol Biotechnol 85, 459-70.
341 Keller, P.W., Johnson, M.C., Vogt, V.M., 2008. Mutations in the spacer peptide and
342 adjoining sequences in Rous sarcoma virus Gag lead to tubular budding. J Virol
343 82, 6788-97.
344 Kenney, S.P., Lochmann, T.L., Schmid, C.L., Parent, L.J., 2008. Intermolecular
345 interactions between retroviral Gag proteins in the nucleus. J Virol 82, 683-91.

346 Kondo, Y., Fushikida, K., Fujieda, T., Sakai, K., Miyata, K., Kato, F., Kato, M., 2008.
347 Efficient delivery of antibody into living cells using a novel HVJ envelope
348 vector system. J Immunol Methods 332, 10-7.
349 Krishna, N.K., Campbell, S., Vogt, V.M., Wills, J.W., 1998. Genetic determinants of
350 Rous sarcoma virus particle size. J Virol 72, 564-77.
351 Ma, Y.M., Vogt, V.M., 2002. Rous sarcoma virus Gag protein-oligonucleotide
352 interaction suggests a critical role for protein dimer formation in assembly. J
353 Virol 76, 5452-62.
354 Nandhagopal, N., Simpson, A.A., Johnson, M.C., Francisco, A.B., Schatz, G.W.,
355 Rossmann, M.G., Vogt, V.M., 2004. Dimeric rous sarcoma virus capsid protein
356 structure relevant to immature Gag assembly. J Mol Biol 335, 275-82.
357 Noad, R., Roy, P., 2003. Virus-like particles as immunogens. Trends Microbiol 11,
358 438-44.
359 Roy, P., Noad, R., 2008. Virus-like particles as a vaccine delivery system: myths and
360 facts. Hum Vaccin 4, 5-12.
361 Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating
362 inhibitors. Proc Natl Acad Sci U S A 74, 5463-7.

363 Scheifele, L.Z., Kenney, S.P., Cairns, T.M., Craven, R.C., Parent, L.J., 2007.
364 Overlapping roles of the Rous sarcoma virus Gag p10 domain in nuclear export
365 and virion core morphology. J Virol 81, 10718-28.
366 Silverman, L., Glick, D., 1969. The reactivity and staining of tissue proteins with
367 phosphotungstic acid. J Cell Biol 40, 761-7.
368 Vogt, V.M., Simon, M.N., 1999. Mass determination of rous sarcoma virus virions by
369 scanning transmission electron microscopy. J Virol 73, 7050-5.
370 Waelti, E.R., Gluck, R., 1998. Delivery to cancer cells of antisense L-myc
371 oligonucleotides incorporated in fusogenic, cationic-lipid-reconstituted
372 influenza-virus envelopes (cationic virosomes). Int J Cancer 77, 728-33.
373 Wills, J.W., Craven, R.C., Achacoso, J.A., 1989. Creation and expression of
374 myristylated forms of Rous sarcoma virus gag protein in mammalian cells. J
375 Virol 63, 4331-43.
376 Yu, F., Joshi, S.M., Ma, Y.M., Kingston, R.L., Simon, M.N., Vogt, V.M., 2001.
377 Characterization of Rous sarcoma virus Gag particles assembled in vitro. J
378 Virol 75, 2753-64.
379

380 **Table 1**

381 Purification of VLPs using ultracentrifugation.

	Protein amount after purification (mg)	Total Protein before ultracentrifugation (mg)	Yield of VLPs (%)
5th fraction of D6 cell line	0.78	17*	4.50
Hemolymph (Upper layer)	2.60	384**	0.67
Hemolymph (Lower layer)	6.40	384**	1.67

382 *Total protein was collected from 100 ml of culture supernatant

383 **Total protein was collected from 10 silkworms

384

385 **Figure Legends**

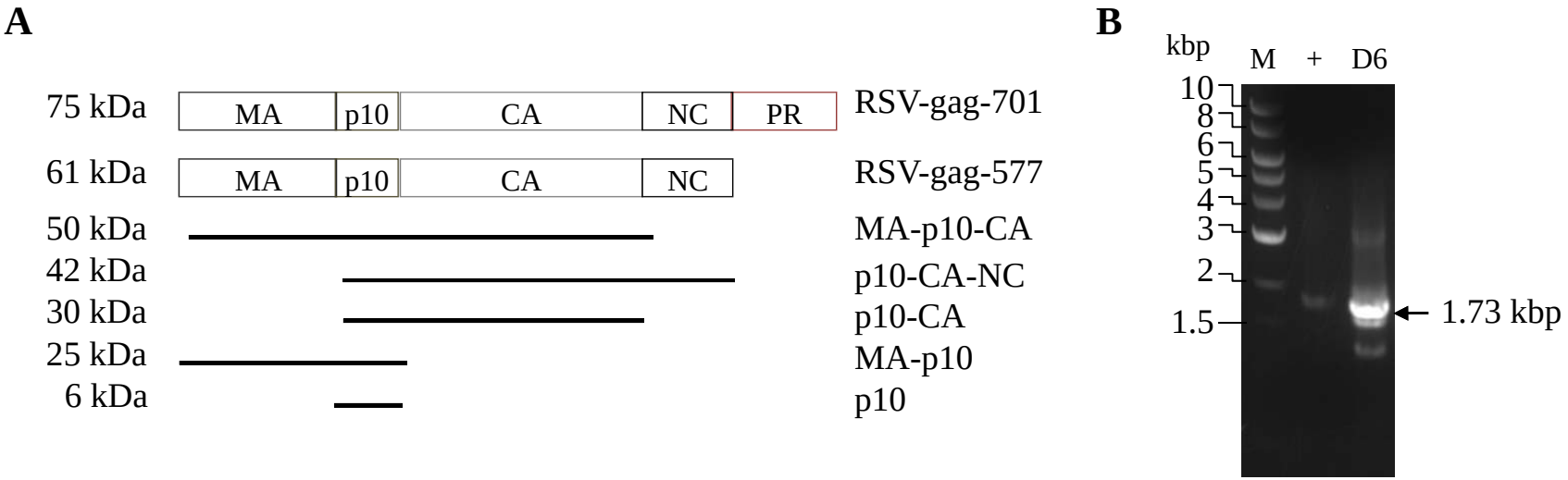
386 **Fig. 1. A.** Schematic representation of the RSV-gag-701 and RSV-gag-577 and various
387 protein components in un-assembled form expressed in insect cell line and silkworm
388 larvae when RSV-gag is expressed. MA, CA, NC, and PR denote matrix associated
389 protein, capsid associated protein, nucleocapsid associated protein, and protease region,
390 respectively. **B.** PCR product using genomic DNA from clones D6 expressing RSV-gag
391 where runned on an agaorse gel 0.75% (w/v). M, 1 kbp DNA ladder (New England
392 Biolabs); +, PCR product using pENTR/RSV-gag as template; D6 PCR products using
393 genomic DNA as template. The primers used were specific for the cDNA as mentioned
394 in the materials and method. An Arrow indicates the expected size of RSV-gag-577.

395 **Fig. 2. A.** Western blot from 100 ml of suspension culture collected on 2nd day after
396 passage. The membranes were probed using primary antibody anti-RSV-gag as
397 mentioned in materials and method. M: MagicMark^{TM} XP western marker (Invitrogen).
398 **B.** Western blots of fractions 1-6 collected from sucrose gradient after removing the
399 sucrose using MWCO 10K (Pall Corporation) for D6 clone. M, MagicMark^{TM} XP
400 western marker (Invitrogen); lanes 1-6, fractions 1-6.

401 **Fig. 3. A.** Picture taken after the sucrose step gradient purification step from silkworm
402 larval hemolymphs. Two distinctive layers as highlighted by the white arrows were

observed. **B.** Expression of RSV-gag-577 in silkworm was confirmed by western blotting using anti-RSV-gag. 30 μg protein per well of each sample was loaded as follows; lane 1, larval hemolymph sample; lane 2, higher density layer; lane 3, lower density layer; lane 4, purified baculovirus (Negative control); lane M, MagicMark^{TM} XP Molecular marker. **C.** 12% SDS-PAGE was loaded with 45 μg protein per well was loaded and stained with Coomassie Blue as followed; 1ane 1, larval hemolymph sample; lane 2, higher density layer; lane 3, lower density layer; lane 4, purified baculovirus (Negative control); lane M, dual precision molecular marker (Bio-Rad).

Fig. 4. A. D6 (from a to d) clone cells stained for ER (a), Anti-RSV-gag conjugated with Qdot525 (b), light (c), and a,b,c merged (d). Bar denotes 20 μm. **B.** Reconstructed image of D6 clone cell utilizing the fluorescence data. Zen LE software available on Carl Zeiss website simulated image using the time scanned images from Carl Zeiss LSM 700. Bar denotes 15 μm. **C.** TEM images of VLPs collected from D6 at 50,000× magnification. Bar denotes 100 nm. **D.** TEM images of VLPs collected from silkworm larval hemolymph and purified using sucrose step gradient, and were observed under TEM at 50,000× magnification. Bar denotes 100 nm.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75 kDa
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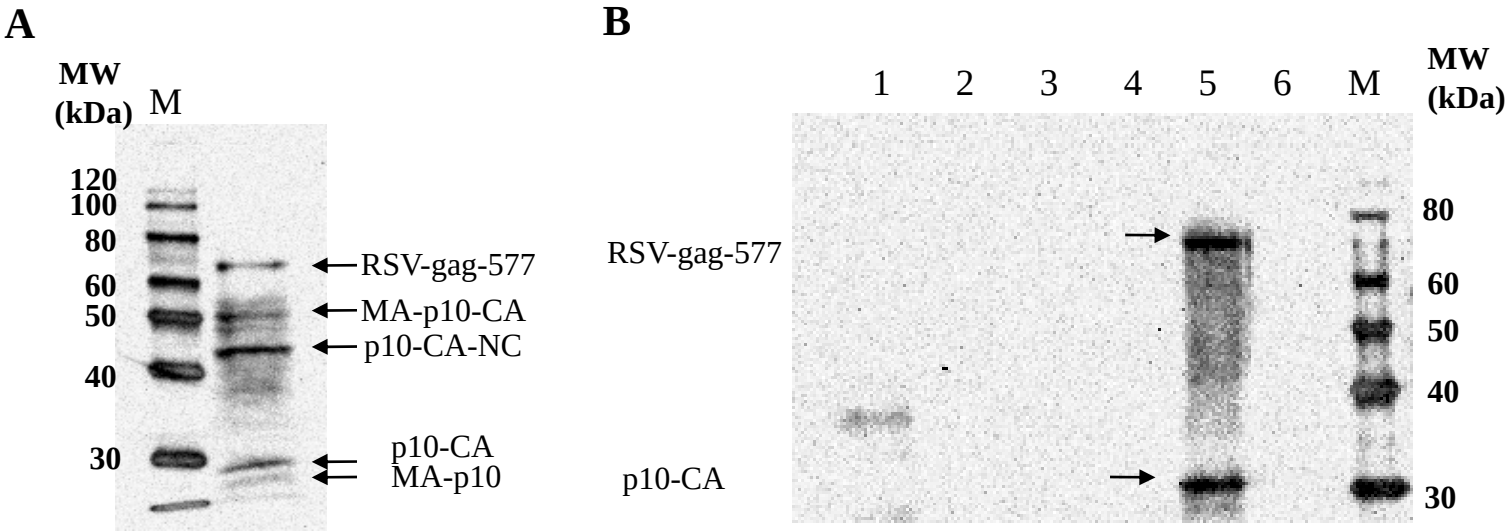
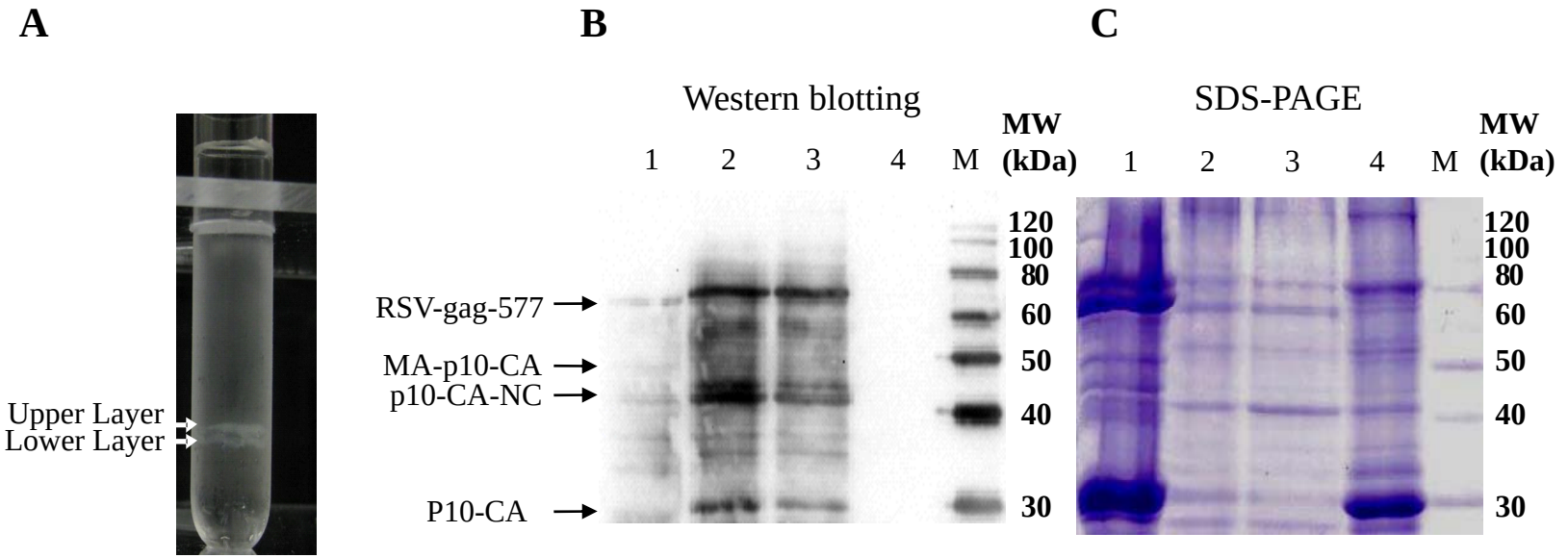
p10
CA
NC
PR
RSV-gag-701
61 kDa
MA
p10
CA
NC
RSV-gag-577
50 kDa
MA-p10-CA
42 kDa
p10-CA-NC
30 kDa
p10-CA
25 kDa
MA-p10
6 kDa
p10
B
kbp
M
+
D6
10
8
6
5
4
3
2
1.5
1.73 kbp


A
MW
(kDa)
M
120
100
80
60
50
40
30
RSV-gag-577
MA-p10-CA
p10-CA-NC
p10-CA
MA-p10
B
1
2
3
4
5
6
M
MW
(kDa)
RSV-gag-577
p10-CA
80
60
50
40
30


A
B
C
Upper Layer
Lower Layer
Western blotting
SDS-PAGE
1
2
3
4
M
MW
(kDa)
120
100
80
60
50
40
30
RSV-gag-577
MA-p10-CA
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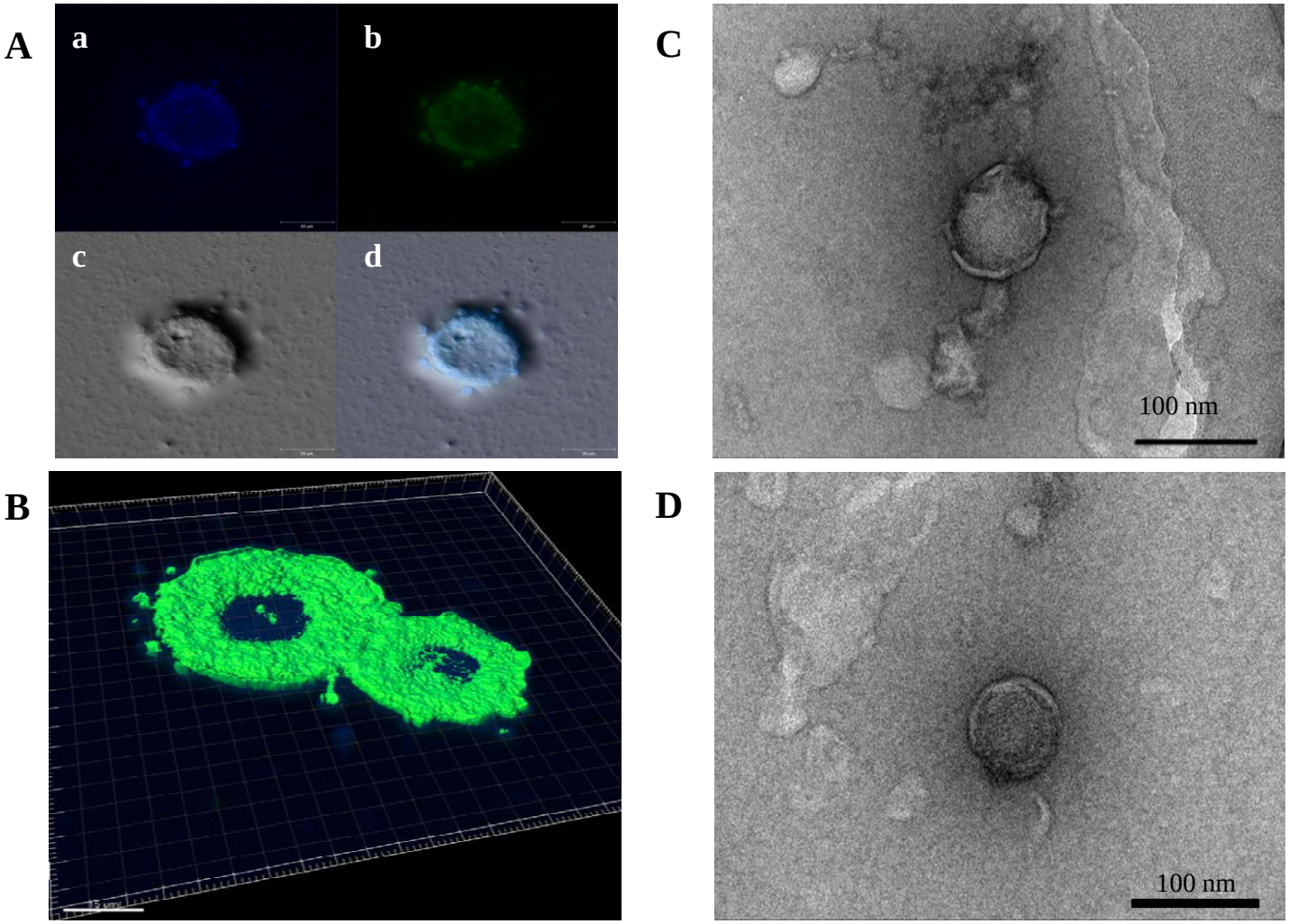
P10-CA


A
a
b
c
d
B
C
100 nm
D
100 nm