Generating enveloped virus-like particles with *in vitro* assembled cores

Fan Cheng and Suchetana Mukhopadhyay*

Department of Biology, Indiana University, 212 S. Hawthorne Drive, Bloomington, Indiana, 47405

*Corresponding author. Phone: (812) 856-3686. Fax: (812) 856-5710.

Email addresses: fancheng@umail.iu.edu (F. Cheng), sumukhop@indiana.edu (S. Mukhopadhyay)

Abstract

Alphaviruses are comprised of a nucleocapsid core surrounded by a lipid membrane containing glycoprotein spikes. Previous work demonstrated that *in vitro* assembled core-like particles are similar in structure to the nucleocapsid core in the native virus. Here we demonstrate that *in vitro* assembled core-like particles can be inserted into viral glycoprotein-expressing cells to generate enveloped virus-like particles. These virus-like particles bud from cells like native virus, are similar in size to the native virus, and can enter cells to release the contents of the core-like particle into the cytoplasm of the cell. Virus-like particles can be used to infect cells with biological and non-biological cargoes. The generation of enveloped virus-like particles containing an *in vitro* core and *in vivo* synthesized glycoproteins has applications for gene and drug delivery, medical imaging, and also basic mechanistic studies of virus assembly.

Keywords: virus assembly, enveloped virus, nanoparticle

Introduction

Alphaviruses are simple, enveloped viruses with a positive-sense RNA genome (Strauss and Strauss, 1994). The particle is ~70 nm in diameter and has three concentric layers: a central nucleocapsid core that consists of the viral genome surrounded by the capsid protein, a host-derived lipid membrane that covers the core, and, on the surface of the particle, 80 trimeric glycoprotein spikes that are embedded in the lipid membrane (Cheng et al., 1995; Mukhopadhyay et al., 2006).

Alphavirus particle assembly consists of two parallel and independent pathways, nucleocapsid core and glycoprotein spike formation. The two pathways merge during budding, the final stage of virus assembly. The five structural proteins, capsid-E3-E2-6K-E1, are synthesized from a 26s subgenomic mRNA. The capsid protein is autoproteolytically cleaved from the polyprotein during translation (Melancon and Garoff, 1987). In the cytoplasm, 240 capsid proteins and one progeny genome self-assemble to form a nucleocapsid core. The remainder of the polyprotein, E3-E2-6K-E1, is co-translationally translocated into the ER where it is cleaved into individual proteins. Spikes are assembled in the ER and Golgi, and transported to the plasma membrane via the host cell secretory machinery (Wahlberg, Boere, and Garoff, 1989; Ziemiecki, Garoff, and Simons, 1980). During budding, the nucleocapsid core protrudes through the cell membrane embedded with glycoprotein spikes and a virus particle is released from the cell. It is hypothesized that the interaction between the cytoplasmic tail of the E2 protein and the hydrophobic pocket in the capsid protein aids in assembly and stabilizes the virus particle (Lee et al., 1996; Skoging et al., 1996; Wilkinson et al., 2005; Zhao et al., 1994). Mutations in the E2 cytoplasmic tail inhibited virus budding and

reduced particle formation (Owen and Kuhn, 1997; West and Brown, 2006). While the amino acid sequence of E2 cytoplasmic tails are similar (Lee et al., 1996; Skoging et al., 1996), chimeric viruses where the glycoproteins are from one alphavirus species and the capsid is from another species, form very low levels of particles compared to the parental viruses (Lopez et al., 1994). Non-enveloped nucleocapsid cores are not released from cells. The distinct and separable assembly pathways make alphaviruses an attractive system for synthesizing enveloped virus-like particles (VLPs) using *in vitro* assembled components.

Alphavirus core-like particles (CLPs) have been assembled *in vitro* by mixing recombinant alphavirus capsid protein and single-stranded nucleic acid (Tellinghuisen et al., 1999). CLPs can also be assembled using artificial scaffolds such as nanoparticles (Goicochea et al., 2007) and polyanions such as heparin (F. Cheng and S. Mukhopadhyay, unpublished data). Cryo-electron microscopy and 3D image reconstructions of CLPs containing a DNA oligomer demonstrated that the CLPs are approximately 40 nm in diameter and that the capsid protein subunits within the CLPs are arranged with T=4 icosahedral symmetry (Mukhopadhyay et al., 2002): both are characteristic of wild-type nucleocapsid cores. However, previous studies of CLPs did not demonstrate whether or not they could interact with glycoprotein spikes to form VLPs.

In this study, we show that *in vitro* assembled alphavirus CLPs can associate with alphavirus glycoproteins to form enveloped VLPs. Thus, *in vitro* assembled CLPs retain the functionality of nucleocapsid core in the absence of any host chaperones. As with native alphaviruses, these *in vitro* assembled VLPs enter cells via the endocytotic

pathway. Furthermore, the VLPs can disassemble and release the contents within the encapsidated the CLP. This is the first demonstration that a functional enveloped virus-like particle containing an *in vitro* assembled core can be synthesized. The ability to make VLPs containing different cargos in their CLP has significance for basic mechanistic studies of virus assembly as well as drug and nucleic acid delivery and medical imaging applications.

Results and Discussion

In vitro core-like particles are assembled using different cargoes

Capsid proteins from Sindbis, Western equine encephalitis, and Ross River virus expressed in *E. coli* have been shown to assemble into CLPs under similar assembly conditions (Mukhopadhyay et al., 2002; Tellinghuisen et al., 1999). We used the Ross River virus system because full-length capsid protein can be obtained in high yields from a recombinant bacterial expression system, resulting in the reproducible synthesis of higher concentrations of CLPs (Mukhopadhyay et al., 2002). Furthermore, CLPs can be packaged with a variety of polyanions and both RNA- and DNA-containing CLPs were used in this work. Purified CLPs were homogeneous and approximately 40 nm in diameter like cytoplasmic cores from virus-infected cells and the nucleocapsid core from virus particles (Figure 1a-c). Yields of purified CLPs were 30-50% of the starting capsid protein in numerous independent preparations.

Functional alphavirus glycoproteins are synthesized in BHK cells

To create a cell line that transiently expressed surface glycoproteins for enveloping *in vitro* assembled CLPs, we cloned the gene fragment encoding the wild-type Ross River virus E3-E2-6K-E1 proteins into the pCAGGS DNA vector (pRRV-GP) for transient expression, guided by expression systems previously described (Kondor-Koch, Burke, and Garoff, 1983; Liljestrom and Garoff, 1991). As a negative control, pRRV-GP was modified (pRRV-mtGP) so amino acid residues 399-401 of the E2 cytoplasmic tail were mutated from YAL to DDD. The glycoproteins synthesized from pRRV-mtGP should not interact with CLPs because the putative hydrophobic interaction between the E2 cytoplasmic tail and the capsid protein has been disrupted (Owen and Kuhn, 1997; West et al., 2006; Zhao et al., 1994). As a second negative control, we cloned the glycoproteins from wild-type Sindbis virus (pSV-GP) into the pCAGGS vector to investigate the specificity of RRV CLPs towards other alphavirus glycoproteins.

BHK cells transfected with either pRRV-GP, pRRV-mtGP, or pSV-GP produced comparable amounts of the E2 protein and its precursor, pE2 (also called p62), as determined by western blot analysis (data not shown). The cellular distribution of glycoproteins from pRRV-GP, pRRV-mtGP, and pSV-GP were similar to each other and to virus-infected cells (Figure 1d). Cells transfected with pRRV-GP, pRRV-mtGP, and pSV-GP could undergo cell-to-cell fusion under low pH conditions (Chanel-Vos and Kielian, 2004; Hernandez et al., 2003) suggesting the expressed glycoproteins can mediate membrane fusion, whereas mock transfected cells did not form syncytia at any pH tested. These results are consistent with those reported for other alphavirus systems.

Cytoplasmic cores form virus particles with viral glycoproteins produced from pRRV-GP

In virus-infected cells, cytoplasmic nucleocapsid cores contain the viral genome. Initially we investigated whether or not we could form infectious particles by transfecting purified cytoplasmic cores into cells expressing viral glycoproteins from pRRV-GP. This approach used assembly components most similar to those found in a virus infection.

We transfected 18 μg, or 1.6×10^{12} purified cytoplasmic nucleocapsid cores from Ross River virus-infected cells into 1.4×10^{7} BHK cells previously transfected with pRRV-GP or into non-transfected 1.4×10^{7} BHK control cells. After one hour cells were washed and after an additional hour incubation in fresh medium, the latter was collected and assayed for infectious particles. The short incubation time minimized the chance of genome replication and virus assembly from a disassembled cytoplasmic core. There were 300-600 pfu (~10^{2} pfu/ml) from pRRV-GP-transfected cells. In contrast, no infectious particles were isolated from BHK control cells. This indicates that cytoplasmic cores by themselves did not disassemble and could not propagate infections in our time frame. The low titer of the infective virus likely reflects a combination of low transfection efficiency of cytoplasmic cores, the limited transport of cytoplasmic cores to the appropriate membrane for packaging, and the short time period over which we collected particles. Nevertheless, these results show that cells transfected with cytoplasmic cores can generate infectious virus particles.

Optimizing the strategy for generating VLPs

We sought to determine whether *in vitro* assembled Ross River CLPs could associate with Ross River glycoproteins expressed *in trans* to form VLPs. We first needed to determine the timing of the transfection of CLPs into BHK cells and timing of particle budding. Transfection is highly dependent on the size of the entering particle and its surface charge. Although transfection has been successfully used with cores of other viral systems (Bichko, Netter, and Taylor, 1994; Jiang and Coombs, 2005), it has not been previously reported for alphavirus nucleocapsid cores. We determined CLP transfection by monitoring the presence of capsid protein in newly transfected cells. We transfected 3 μg CLPs containing 48mer DNA (~$2.6x10^{11}$ CLPs) into $2.4x10^{6}$ non-transfected BHK control cells using Lipofectamine as used with other virus systems. At various times post-transfection, the cells were washed and lysed, and the lysates were then probed for capsid protein by western blotting (Figure 2a). The capsid protein in these cells would be from the CLPs that were transfected into them. The presence of capsid protein was detected within 15 minutes post-transfection and reached a maximum at 30-60 minutes post-transfection. Levels of capsid protein started to decrease only after 4 hours. The stability of the capsid protein suggests that CLPs are not immediately disassembling because during a native alphavirus infection, the nucleocapsid core disassembles under acidic conditions within 2 minutes of entry into the cytoplasm (Wengler, 2009; Wengler and Wengler, 2002). However, in these transfection assays, CLPs enter the cytoplasm directly and bypasses the endosome (Jiang and Coombs, 2005).

We hypothesized that the timing of virus-like particle budding would be different than during a native alphavirus infection because during production of VLPs, the core was

already assembled and ready to bud. To determine the timing of potential VLPs being released into the media, we assayed medium for the presence of capsid protein as an indicator of VLPs. We transfected 19 μg CLPs containing 48mer DNA (~$1.6x10^{12}$ CLPs) into $1.4x10^{7}$ BHK cells expressing pRRV-GP. One hour post-CLP transfection, cells were washed and fresh medium was added; this is defined as zero time. Medium from the cells were collected at 30, 60, and 120 minutes and centrifuged. Thus, medium collected at 30 min contains particles released between 0-30 minutes, medium collected at 60 min contain particles released between 30 and 60 min, and medium collected at 120 min contains particles released between 60 and 120 min. After centrifugation, each sample was analyzed for the presence of capsid protein (Figure 2b). At t=0 no capsid protein was detected indicating that non-transfected CLPs were completely removed during the wash step: all capsid protein detected subsequently is therefore from released VLPs. We could detect capsid protein in the pelleted particles at 30 min but between 30 and 60 minutes (time 60 min), much less capsid protein was detected. These results indicate that virus-like particle assembly and release was complete within 30 minutes of addition of the fresh medium. One explanation for this narrow time range for VLP production is that there is a finite number of CLPs inside the viral glycoprotein-expressing cell. Once all the CLPs form into VLPs, no more particles can be formed. In contrast, during a viral infection cytoplasmic cores are continuously being synthesized so virus particles are continuously being released from the over a long period of time.

In a viral infection, the genome replication and structural protein synthesis occurs before particle release. Particles are first detected 4 to 6 hours post-infection and continue to be released until 12 to 15 hours post-infection. In our assays, VLPs are

produced much earlier that virus particles are produced during an infection. When we transfect CLPs, we are presumably initiating assembly at the last steps of budding when the nucleocapsid cores interact with the glycoproteins and were able to form virus particles more quickly. The amount of VLPs released depends upon the amount of CLPs that have been transfected since new CLPs are not being synthesized within the cell.

Taking into account the above observations, we standardized our protocol for making VLPs. Transfect BHK cells with pRRV-GP and, 15 hours later when glycoprotein synthesis was occurring, transfect purified CLPs into the same cells. After one hour, the medium was removed, cells were washed with PBS to remove non-transfected CLPs, and fresh medium added. After an additional one hour incubation the medium was collected and analyzed for VLPs. This timing was used for two reasons. First, optimal yield of VLPs were obtained one hour post-CLP transfection (as determined by western blot). Second, if we collected all the medium post-CLP transfection and did not wash the cells, non-transfected CLPs remaining in the medium would interfere with quantitation and characterization of the VLPs. As a consequence of the timing of our assays, we may be underestimating the amount of VLPs that are formed. Hypothetically, if CLPs enter the cell within 30 minutes and can bud and be released within 15 minutes, these VLPs would not be collected in our assays.

VLPs contain viral glycoproteins and capsid protein

Our small scale optimization results, obtained by transfecting CLPs into BHK cells expressing pRRV-GP, demonstrated that VLPs were released into the media (Figure

2b). We wanted to determine if the viral glycoproteins were also present in the VLPs. We transfected 90 μg CLPs containing 48mer DNA (~$7.8x10^{12}$ CLPs) into $7.2x10^7$ BHK cells expressing pRRV-GP and into non-transfected $7.2x10^7$ BHK control cells. The medium from each sample (after the appropriate washes) were applied to a sucrose cushion and centrifuged. The pellets from pRRV-GP plus CLPs transfection contained both E2 and capsid protein as determined by western blot (Figure 3a, lane 4). In contrast, resuspended pellets from CLPs transfected into control BHK cells contained neither E2 nor capsid protein (Figure 3a, lane 3). This result is consistent with our hypothesis that VLPs only form in the presence of CLPs and viral glycoproteins. In addition, it also demonstrated that non-transfected CLPs were removed during the wash step. As a positive control, Ross River virus was pelleted and $1.5x10^8$ particles were loaded on the gel (Figure 3a, lane 2).

Virus-like particle formation requires E2-capsid interaction

The interaction between the cytoplasmic tail of the E2 protein and the hydrophobic pocket of the capsid protein promotes virus budding. We abolished this interaction when expressing an E2 mutant (pRRV-mtGP) in which residues in the cytoplasmic tail were mutated from YAL to DDD. Glycoprotein expression and syncytia formation for pRRV-mGP was comparable to cells expressing pRRV-GP and virus-infected cells. However when 108 μg CLPs containing 48mer DNA oligomer (~$9.3x10^{12}$ CLPs) into $7.2x10^7$ BHK cells expressing pRRV-mtGP, neither E2 nor capsid protein was detected (Figure 3b) in the resuspended pellet. If any VLPs formed with the mutant glycoprotein pRRV-mtGP, their amounts were severely reduced in comparison to those formed using

pRRV-GP. When the YAL->DDD mutation was made in Ross River virus, no infectious particles were detected in plaque assays. Thus, VLPs contain E2, capsid protein and show an E2-capsid protein-dependent interaction.

VLPs are morphologically similar to native virus particles

We wanted to determine if the morphology and size of the VLPs were similar to native alphavirus particles. When viewed by transmission electron microscope (TEM), the resuspended pellet from the pRRV-GP plus CLPs transfected cells show spherical particles with a diameter of approximately 70 nm by electron microscopy (Figure 4a), consistent with native alphavirus particles. The number of VLP observed was fewer than from a native infection. However, the distribution of particle size and variation in particle shape was similar to what is observed when we pelleted native virus. Some particles look less spherical in both the VLP and native virus population and we speculate this is due to pelleting virus particles. No unenveloped CLPs (40 nm diameter, spherical particles) were observed. No spherical particles with either 70 or 40 nm diameter were found in the resuspended pellet from the control experiment where CLPs transfected into non-viral glycoprotein expressing cells. The absence of both virus-like and core-like particles in this control was consistent with a western blot of the media (Figure 3a).

Cryo EM structures of both *in vitro* assembled CLPs (Mukhopadhyay et al., 2002) and isolated cytoplasmic nucleocapsid cores (Lamb et al., 2010) indicate that these complexes are flexible and undergo glycoprotein-mediated conformation changes. From negative stain TEM images, it was difficult to if the glycoprotein spikes were fully

covering the CLP. To determine if there were areas on the VLP that were not surrounded by the spikes, thus the capsid proteins were exposed, we assayed resuspended VLP pellets with anti-capsid protein antibody. The rationale was if the glycoprotein spikes were not covering the CLP then the VLP would be immunoprecipitated using an anti-capsid antibody. However, if the CLP was covered by glycoprotein spikes, as is observed in native virus particles, then the VLPs would not bind to the capsid antibody. Here, 20 μg of CLPs containing 48mer DNA (~$1.7x10^{12}$ CLPs) were transfected into $1.4x10^7$ BHK cells, both those expressing RRV-GP and control cells not expressing viral glycoproteins. Concentrated VLPs were immunoprecipitated with anti-capsid antibody and then analyzed by western blotting. No VLPs could be detected in the medium from RRV-GP plus CLPs transfected cells indicating large areas of capsid protein in the CLP was not surface exposed (Figure 4b, lane 3). A strong capsid protein band was detected when we directly added 15 μg (~$1.2x10^{12}$ particles) CLPs to the medium, pelleted the CLPs, and immunoprecipitated the resuspended pellet with anti-capsid antibody (Figure 4b, lane 6). The absence of detectable capsid protein in medium from CLPs transfected into control BHK cells (Figure 4b, lane 2) demonstrated that secretion of VLPs requires glycoproteins, consistent with native alphavirus particles.

VLPs can enter and disassemble in cells

We tested the entry and disassembly activity of our VLPs by creating particles that contain luciferase RNA reporter. We synthesized VLPs from CLPs containing luciferase RNA (18 μg CLPs transfected into $1.4x10^7$ BHK cells transfected with RRV-GP or non-

transfected BHK control cells. Medium containing the VLPs (total volume=3 ml) was incubated with $2.4x10^6$ BHK cells. After 12 hours, cells were lysed and assayed for luciferase activity. This incubation is intended to mimic an infection; no transfection reagent was included. Luciferase would only be translated if the VLPs could enter and disassemble within the new cell. Within each set of experiments, the luciferase activity detected from BHK cells that were incubated with medium from pRRV-GP plus CLP-transfected cells was set to 1 (Figure 5a). Cells that were incubated with medium from CLPs transfected into BHK control cells had background levels of luminescence (Figure 5a).

Alphaviruses have been reported to enter the host cells either via receptor-mediated endocytosis (Bron et al., 1993; Marsh, Kielian, and Helenius, 1984). Entry at neutral pH at the plasma membrane has also been reported (Paredes et al., 2004). We specifically tested the hypothesis that VLPs entered BHK cells using the same route as Ross River virus. We blocked entry using a neutralizing antibody and inhibiting pH change in the endosome.

The epitope of the Ross River neutralizing antibody T10C9 is the receptor-binding domain of the E2 protein (Smith et al., 1995; Vrati et al., 1988). When 3 ml of medium containing VLPs made with luciferase CLPs were incubated with 500 μl of T10C9 antibody in serum, the luciferase activity in the incubated BHK cells was reduced by 50% compared to VLPs not treated with the neutralizing antibody. These results demonstrate that an E2-specific neutralizing antibody is able to interfere with virus-like particle entry into host cells, consistent with what is observed with native Ross River virus (Vrati et al., 1988) (Figure 5b).

When BHK cells were pre-treated with NH_4Cl and then infected with Ross River virus, we observed a decrease in titer from 10^8 to 10^5 pfu/ml (Figure 5b) as previously reported for other alphaviruses (Glomb-Reinmund and Kielian, 1998; Helenius, Marsh, and White, 1982). Similarly, VLPs showed reduced luminescence in BHK cells that had been pre-treated with NH_4Cl; the luciferase activity was reduced by 70% suggesting that VLP entry occurs via the endosomal pathway, where either fusion and/or core disassembly may be affected by the change in pH of the NH_4Cl-incubated cell.

When blocking entry of the VLPs, the luciferase activity of the cells is decreased compared to untreated VLPs but still remains higher than the background CLP luminescence value (Figure 5a, CLP). Possible explanations could include that some VLPs do not enter cells via the receptor-mediated endosomal pathway or the arrangement of the spikes around the particle does not allow for complete neturalization by the T10C9 antibody. In Ross River virus treated with antibody and pre-treatment of cells with NH_4Cl, there was only a partial decrease in viral titer which correlates with our luciferase assay results (Figure 5b). An alternative explanation for the luciferase activities not return to background levels is the CLP may be enveloped by any viral glycoprotein-containing membrane and, as a result, particles may show increased luminescence in cells. To test this hypothesis, we transfected 3.6 μg (~$3.1x10^{11}$ CLPs) made with Ross River capsid protein into $2.4x10^6$ BHK cells expressing Sindbis virus glycoproteins, pSV-GP. After the appropriate washes, medium was collected and added to $2.4x10^6$ BHK cells. Here, the luminescence of cells treated with medium from the RRV CLP + SV-GP VLPs was equal to background levels. Previous reports have demonstrated that in chimera viruses where the Ross River capsid protein was

exchanged with Sindbis capsid protein in a Ross River background, or Sindbis capsid protein was exchanged with a Ross River capsid protein in a Sindbis background (Lopez et al., 1994), grew very poorly or ~10^3 pfu/ml (Figure 5b). Our low levels of luciferase activity are consistent with these results and suggest that VLPs are not being formed when Ross River CLPs are transfected into pSV-GP cells.

Concluding remarks

Animal viruses have natural and efficient entry mechanisms making them ideal vector systems for real-time imaging and drug delivery. However, potential dangerous effects due to the presence of the parental virus genome are concerns. In the alphavirus based VLPs, the cell entry properties associated with the glycoprotein spikes are retained but the genome and its pathogenic effects are eliminated because the cargo within the CLP is not specific to the viral genome. We have demonstrated that VLPs can enter and disassemble within a new host cell and there is a strong correlation between the biological activity of VLPs as determined by luciferase activity and virus infectivity as determined by plaque assay.

We can expand our work with Ross River VLPs to generate other alphavirus VLPs, extending specificity and cell targeting. Many alphavirus capsid proteins form CLPs using nucleic acid (Hong, Perera, and Kuhn, 2006; Mukhopadhyay et al., 2002; Tellinghuisen et al., 1999; Tellinghuisen and Kuhn, 2000; Tellinghuisen, Perera, and Kuhn, 2001). In addition, CLP formation using inorganic cargoes towards medical imaging has also been demonstrated (Goicochea et al., 2007). It has been

demonstrated in mice that Sindbis viral vectors specifically infect tumors because the virus particles bind to over-expressed laminin receptor synthesized in many tumors (Scheiman et al., 2010; Tseng et al., 2004), suggesting oncolytic potential for this system (Scheiman et al., 2010; Tseng et al., 2004).

The enveloped VLP system provides a means to deliver toxic and labile cargoes. Toxic cargoes will only be released once the VLPs enter the target cell. Similarly, labile cargoes are protected until they are inside the target cell. Applications using VLPs include intracellular delivery of small molecule drugs, siRNAs of modulation of gene expression, and nanoparticles for imaging. The VLP system will require further optimization for maximum production of VLPs and that may be specific to the cargo of the CLP. Our results demonstrate the first steps in the use of alphavirus nanoparticles in basic mechanistic virology studies as well as in application-driven uses of the VLPs.

Materials and Methods

Viruses and cells

The T48 strain of Ross River virus (Kuhn et al., 1991) was used in this work. BHK-21 cells (American Type Tissue Culture, Rockville, MD) were grown in minimal essential medium (MEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), non-essential amino acids, glutamine, and penicillin/ streptomycin. Cells were grown at 37°C in the presence of 5% CO_2. The neutralizing Ross River antibody, T10C9, was generously provided by Richard Kuhn, Purdue University.

Transient expression of alphavirus glycoproteins

The viral glycoproteins E3-E2-6K-E1 from Ross River virus were cloned into a pCAGGS vector; pRRV-GP. A mutant of RRV-GP where amino acid residues E2-399-401 are mutated from YAL to DDD was created by Quick Change mutagenesis (Stratagene, La Jolla, CA); pRRV-mtGP. The viral glycoproteins E3-E2-6K-E1 from Sindbis virus were cloned into a pCAGGS vector; pSV-GP. BHK cells were transfected with pRRV-GP/pRRV-mtGP/pSV-GP using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Viral glycoprotein expression was determined by western blotting of cell lysates and immunofluorescence assays using a polyclonal Ross River E2 or Sindbis E2 antibody (Cocalico, Reamstown, PA), and FITC- or TRITC-conjugated goat anti-rabbit secondary antibody.

Syncytia assays

Syncytia assays were performed to determine if cells expressing pRRV-GP, pRRV-mtGP, and pSV-GP were able to mediate fusion. BHK cells were transfected with pRRV-GP, pRRV-mtGP, or pSV-GP using Lipofectamine 2000 (Invitrogen). After fifteen hours, cells were treated with 200 µl MEM with 20 mM HEPES or 20 mM MES at the specified pH (ranging from pH 7.5 to 5) for 3 minutes at 37°C. The medium was removed, MEM plus 10% FBS was added, and the cells were incubated for an additional 1 hour at 37°C. Syncytia were clearly visible under bright field.

Isolation of cytoplasmic cores from virus-infected cells

Cytoplasmic cores were isolated as described previously (Lopez et al., 1994). Briefly, BHK cells were infected at MOI=5 with Ross River virus and 18 hours post-infection cytoplasmic cores were harvested in 10 mM Tris (pH 7.5), 10 mM NaCl, and 20 mM EDTA. Lysed cells were incubated on ice for 20 minutes and then Triton X-100 was added to a final concentration of 3%. Cells were vortexed gently and then centrifuged at 4,000 *x g* for 10 minutes at 4°C. Lysates were loaded on a 0-50% sucrose gradient prepared in 20 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, and 0.1% TX-100 and centrifuged in a SW41 rotor at 178,000 *x g* for 90 minutes at 12°C (Lopez et al., 1994). The fraction containing the nucleocapsid cores was concentrated and buffer-exchanged into PBS. Bradford assays were used to determine the concentration of the cytoplasmic cores.

In vitro assembly of core-like particles

Ross River capsid protein was cloned into a pET 29b vector (Novagen, EMD Chemical Inc., Gibbstown, NJ) and expressed in Rosetta2 cells (Novagen, EMD Chemical Inc.). Capsid protein purification was performed as described previously (Mukhopadhyay et al., 2002; Tellinghuisen et al., 1999). Ross River capsid protein was concentrated and buffer exchanged into 20 mM HEPES, 150 mM NaCl, and 0.1 mM EDTA for use in CLP reactions. For composition and morphology studies, the nucleic acid cargo was a 48mer DNA oligomer (5'-CCGTTAATGCATGTCGAGATATAAAGCATAAGGGACATGCATTAACGG-3') (IDT, Coralville, IA). For the cell entry and disassembly studies firefly luciferase mRNA was the cargo (Nickens and Hardy, 2008). Capped and polyadenylated luciferase mRNA

was generated by *in vitro* transcription using SP6 polymerase and standard reaction components (Nickens and Hardy, 2008), and was purified using an RNeasy Mini Kit (Qiagen, Valencia, CA). Concentrations of DNA and RNA were determined using a NanoDrop 1000 (Thermo Scientific, Wilmington, DE).

Ross River CLPs were assembled *in vitro* by incubating 100 μg capsid protein and 60 μg of nucleic acid (either DNA or RNA) at room temperature for 10 minutes. CLPs were purified to remove residual capsid protein and nucleic acid by applying the reaction mixture onto a 27% sucrose cushion (10 ml) and centrifuging at 130,000 x *g* for 2.5 hours at 4°C. The pellet was resuspended in PBS to give a protein concentration between 0.30-0.45 mg/ml as determined by a Bradford assay. CLP purification yield was 35-50% of starting capsid protein. CLP formation, both before and after concentration, was verified by an agarose gel shift assay and by TEM analysis (Mukhopadhyay et al., 2002; Tellinghuisen et al., 1999).

Assembly of VLPs with CLPs

The general procedure to assemble VLPs is described below. The total amount of CLPs and the number of cells used were adjusted based on the experiment being conducted and the amount of VLPs needed for analysis. However, the CLP:BHK cell ratio remained similar. In most experiments, the amount of CLPs and cells used are indicated in the text. A 35 mm dish containing $2x10^6$ BHK cells was transfected with pRRV-GP. Fifteen hours later, 3 μg of purified CLPs (approximately $2.6x10^{11}$ particles) were transfected into the pRRV-GP-transfected cells using 10 μl of Lipofectamine 2000. After one hour, cells were washed three times with PBS to remove residual CLPs and

Lipofectamine reagent and 0.5 ml of MEM + 10% FBS were added to the cells. After one hour additional hour, medium which contain VLPs was collected.

We tested several transfection agents for transfecting CLPs including Lipofectamine 2000 (Invitrogen), Fugene 6 (Roche), and PEI (Sigma). In addition we tested reagents specific for protein and peptide transfection, including Chariot (Motif Active), and microinjection and syringe loading (Clarke and McNeil, 1992) of CLPs. Lipofectamine 2000 yielded the highest efficiency of transfection.

Concentrating VLPs

VLPs were concentrated for western blot and TEM studies. Medium containing VLPs was filtered through a 0.45 μm syringe filter to remove cells and cell debris. The medium was applied onto a 27% sucrose cushion (10 ml) and centrifuged at 130,000 x *g* for 2.5 hours at 4°C. The pellet, which contains the VLPs, was resuspended in PBS to yield ~0.15 mg/ml of VLPs based on protein concentration.

Western blot and immunoprecipitation of VLPs

To determine the composition of VLPs, 10 μl of the resuspended VLPs was analyzed by SDS-PAGE and probed for RRV capsid and E2 protein using polyclonal antibodies. For immunoprecipitation experiments, anti-capsid protein antibodies were coupled to the agarose coupling resin columns of a ProFound Co-Immunoprecipitation Kit (Pierce, Rockford, IL) according to the manufacturer's protocol. As a control for non-specific binding of CLPs/protein to the agarose beads, a sample not including antibody was also performed. Ten microliters of the resuspended VLPs were incubated with the

agarose beads (+/-antibody) at room temperature for two hours. Samples were eluted from the column by SDS and analyzed by western blotting.

Transmission electron microscopy

Samples of concentrated VLPs (3-5 μl) were applied to 400-mesh carbon-coated formvar copper grids and stained with 1% uranyl acetate. The grids were examined on a JEOL 1010 transmission electron microscope (Tokyo, Japan) at 80 kV. Images were recorded using a Gatan UltraScan 4000 CCD camera (Pleasanton, CA).

Luciferase assay

VLPs containing luciferase RNA were added to cells to determine if they were capable of entering and disassembling within a cell. Medium containing VLPs from 6-35 mm dishes were collected (total volume=3 ml) and incubated with $2.4x10^6$ BHK cells. After 12 hours, luciferase assays were performed using a Luciferase Assay Kit (Sigma, St. Louis, MO) according to the manufacturer's directions. Cells were lysed in 150 μl lysis buffer. Samples were measured using a BIO-TEK synergy HT multimode microplate reader (BIO-TEK, Winooski, VT). Samples were measured in triplicate and at least six experiments were averaged for each sample.

The range of luminescence minimum and maximum between experiments varied even though the relative values within an experiment were consistent. The absolute luminescence values were normalized for each experiment. To do so, background values determined from BHK cell lysates was subtracted from all samples. Within each set of experiments, luminescence from particles isolated from cells expressing RRV-GP

that had been transfected with CLPs (medium containing VLPs) was set to 1. Standard error was determined between sets of experiments.

Treatment of VLPs and virus with neutralizing antibody

Three milliliters of medium from containing VLPs were incubated with 500 μl T10C9 antibody in serum for 30 minutes at room temperature. The mixture was applied to BHK cells and after 12 hours luciferase activity was determined as described above. One milliliter of wild-type Ross River virus was incubated with 500 ml of T10C9 antibody for 30 minutes at room temperature. Infectivity was determined by plaque asay.

Treatment of cells with ammonium chloride

BHK cells were pre-treated with 20mM freshly prepared ammonium chloride for 20 minutes before being incubated with 3 ml of VLPs. After one hour of incubation, the medium containing the particles were removed and fresh medium containing 20mM ammonium chloride was added. After 12 hours, cell lysates were assayed for luciferase activity. Infectivity of Ross River virus was determined in a similar manner. Cells were pre-treated with 20 mM NH_4Cl for 20 minutes before being infected with Ross River virus. After 24 hours, medium from virus-incubated cells were removed after and titered.

Acknowledgements

We thank Adam Zlotnick and members of our laboratory for helpful discussions and critical comments on the manuscript. This work was supported through a Lilly Endowment METACyte Award.

References

Bichko, V., Netter, H. J., and Taylor, J. (1994). Introduction of hepatitis delta virus into animal cell lines via cationic liposomes. *J Virol* **68**(8)**,** 5247-52.

Bron, R., Wahlberg, J. M., Garoff, H., and Wilschut, J. (1993). Membrane fusion of Semliki Forest virus in a model system: correlation between fusion kinetics and structural changes in the envelope glycoprotein. *Embo J* **12**(2)**,** 693-701.

Chanel-Vos, C., and Kielian, M. (2004). A conserved histidine in the ij loop of the Semliki Forest virus E1 protein plays an important role in membrane fusion. *J Virol* **78**(24)**,** 13543-52.

Cheng, R. H., Kuhn, R. J., Olson, N. H., Rossmann, M. G., Choi, H. K., Smith, T. J., and Baker, T. S. (1995). Nucleocapsid and glycoprotein organization in an enveloped virus. *Cell* **80**(4)**,** 621-30.

Clarke, M. S., and McNeil, P. L. (1992). Syringe loading introduces macromolecules into living mammalian cell cytosol. *J Cell Sci* **102 (Pt 3),** 533-41.

Glomb-Reinmund, S., and Kielian, M. (1998). The role of low pH and disulfide shuffling in the entry and fusion of Semliki Forest virus and Sindbis virus. *Virology* **248**(2)**,** 372-81.

Goicochea, N. L., De, M., Rotello, V. M., Mukhopadhyay, S., and Dragnea, B. (2007). Core-like particles of an enveloped animal virus can self-assemble efficiently on artificial templates. *Nano Lett* **7**(8)**,** 2281-90.

Helenius, A., Marsh, M., and White, J. (1982). Inhibition of Semliki forest virus penetration by lysosomotropic weak bases. *J Gen Virol* **58 Pt 1,** 47-61.

Hernandez, R., Sinodis, C., Horton, M., Ferreira, D., Yang, C., and Brown, D. T. (2003). Deletions in the transmembrane domain of a sindbis virus glycoprotein alter virus infectivity, stability, and host range. *J Virol* **77**(23)**,** 12710-9.

Hong, E. M., Perera, R., and Kuhn, R. J. (2006). Alphavirus capsid protein helix I controls a checkpoint in nucleocapsid core assembly. *J Virol* **80**(18)**,** 8848-55.

Jiang, J., and Coombs, K. M. (2005). Infectious entry of reovirus cores into mammalian cells enhanced by transfection. *J Virol Methods* **128**(1-2)**,** 88-92.

Kondor-Koch, C., Burke, B., and Garoff, H. (1983). Expression of Semliki Forest virus proteins from cloned complementary DNA. I. The fusion activity of the spike glycoprotein. *J Cell Biol* **97**(3)**,** 644-51.

Kuhn, R. J., Niesters, H. G., Hong, Z., and Strauss, J. H. (1991). Infectious RNA transcripts from Ross River virus cDNA clones and the construction and characterization of defined chimeras with Sindbis virus. *Virology* **182**(2)**,** 430-41.

Lamb, K., Lokesh, G. L., Sherman, M., and Watowich, S. (2010). Structure of a Venezuelan equine encephalitis virus assembly intermediate isolated from infected cells. *Virology*.

Lee, S., Owen, K. E., Choi, H. K., Lee, H., Lu, G., Wengler, G., Brown, D. T., Rossmann, M. G., and Kuhn, R. J. (1996). Identification of a protein binding site on the surface of the alphavirus nucleocapsid and its implication in virus assembly. *Structure* **4**(5)**,** 531-41.

Liljestrom, P., and Garoff, H. (1991). A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Biotechnology (N Y)* **9**(12)**,** 1356-61.

Lopez, S., Yao, J. S., Kuhn, R. J., Strauss, E. G., and Strauss, J. H. (1994). Nucleocapsid-glycoprotein interactions required for assembly of alphaviruses. *J Virol* **68**(3)**,** 1316-23.

Marsh, M., Kielian, M. C., and Helenius, A. (1984). Semliki forest virus entry and the endocytic pathway. *Biochem Soc Trans* **12**(6)**,** 981-3.

Melancon, P., and Garoff, H. (1987). Processing of the Semliki Forest virus structural polyprotein: role of the capsid protease. *J Virol* **61**(5)**,** 1301-9.

Mukhopadhyay, S., Chipman, P. R., Hong, E. M., Kuhn, R. J., and Rossmann, M. G. (2002). In vitro-assembled alphavirus core-like particles maintain a structure similar to that of nucleocapsid cores in mature virus. *J Virol* **76**(21)**,** 11128-32.

Mukhopadhyay, S., Zhang, W., Gabler, S., Chipman, P. R., Strauss, E. G., Strauss, J. H., Baker, T. S., Kuhn, R. J., and Rossmann, M. G. (2006). Mapping the Structure and Function of the E1 and E2 Glycoproteins in Alphaviruses. *Structure* **14**(1)**,** 63-73.

Nickens, D. G., and Hardy, R. W. (2008). Structural and functional analyses of stem-loop 1 of the Sindbis virus genome. *Virology* **370**(1)**,** 158-72.

Owen, K. E., and Kuhn, R. J. (1997). Alphavirus budding is dependent on the interaction between the nucleocapsid and hydrophobic amino acids on the cytoplasmic domain of the E2 envelope glycoprotein. *Virology* **230**(2)**,** 187-96.

Paredes, A. M., Ferreira, D., Horton, M., Saad, A., Tsuruta, H., Johnston, R., Klimstra, W., Ryman, K., Hernandez, R., Chiu, W., and Brown, D. T. (2004). Conformational changes in Sindbis virions resulting from exposure to low pH and interactions with cells suggest that cell penetration may occur at the cell surface in the absence of membrane fusion. *Virology* **324**(2)**,** 373-86.

Scheiman, J., Tseng, J. C., Zheng, Y., and Meruelo, D. (2010). Multiple functions of the 37/67-kd laminin receptor make it a suitable target for novel cancer gene therapy. *Mol Ther* **18**(1)**,** 63-74.

Skoging, U., Vihinen, M., Nilsson, L., and Liljestrom, P. (1996). Aromatic interactions define the binding of the alphavirus spike to its nucleocapsid. *Structure* **4**(5)**,** 519-29.

Smith, T. J., Cheng, R. H., Olson, N. H., Peterson, P., Chase, E., Kuhn, R. J., and Baker, T. S. (1995). Putative receptor binding sites on alphaviruses as visualized by cryoelectron microscopy. *Proc Natl Acad Sci USA* **92**(23)**,** 10648-52.

Strauss, J. H., and Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* **58**(3)**,** 491-562.

Tellinghuisen, T. L., Hamburger, A. E., Fisher, B. R., Ostendorp, R., and Kuhn, R. J. (1999). In vitro assembly of alphavirus cores by using nucleocapsid protein expressed in Escherichia coli. *J Virol* **73**(7)**,** 5309-19.

Tellinghuisen, T. L., and Kuhn, R. J. (2000). Nucleic acid-dependent cross-linking of the nucleocapsid protein of Sindbis virus. *J Virol* **74**(9)**,** 4302-9.

Tellinghuisen, T. L., Perera, R., and Kuhn, R. J. (2001). In vitro assembly of Sindbis virus core-like particles from cross-linked dimers of truncated and mutant capsid proteins. *J Virol* **75**(6)**,** 2810-7.

Tseng, J. C., Levin, B., Hurtado, A., Yee, H., Perez de Castro, I., Jimenez, M., Shamamian, P., Jin, R., Novick, R. P., Pellicer, A., and Meruelo, D. (2004).

Systemic tumor targeting and killing by Sindbis viral vectors. *Nat Biotechnol* **22**(1)**,** 70-7.
Vrati, S., Fernon, C. A., Dalgarno, L., and Weir, R. C. (1988). Location of a major antigenic site involved in Ross River virus neutralization. *Virology* **162**(2)**,** 346-53.
Wahlberg, J. M., Boere, W. A., and Garoff, H. (1989). The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to low pH during virus maturation. *J Virol* **63**(12)**,** 4991-7.
Wengler, G. (2009). The regulation of disassembly of alphavirus cores. *Arch Virol* **154**(3)**,** 381-90.
Wengler, G., and Wengler, G. (2002). In vitro analysis of factors involved in the disassembly of Sindbis virus cores by 60S ribosomal subunits identifies a possible role of low pH. *J Gen Virol* **83**(Pt 10)**,** 2417-26.
West, J., and Brown, D. T. (2006). Role of a conserved tripeptide in the endodomain of Sindbis virus glycoprotein E2 in virus assembly and function. *J Gen Virol* **87**(Pt 3)**,** 657-64.
West, J., Hernandez, R., Ferreira, D., and Brown, D. T. (2006). Mutations in the endodomain of Sindbis virus glycoprotein E2 define sequences critical for virus assembly. *J Virol* **80**(9)**,** 4458-68.
Wilkinson, T. A., Tellinghuisen, T. L., Kuhn, R. J., and Post, C. B. (2005). Association of sindbis virus capsid protein with phospholipid membranes and the E2 glycoprotein: implications for alphavirus assembly. *Biochemistry* **44**(8)**,** 2800-10.
Zhao, H., Lindqvist, B., Garoff, H., von Bonsdorff, C. H., and Liljestrom, P. (1994). A tyrosine-based motif in the cytoplasmic domain of the alphavirus envelope protein is essential for budding. *Embo J* **13**(18)**,** 4204-11.
Ziemiecki, A., Garoff, H., and Simons, K. (1980). Formation of the Semliki Forest virus membrane glycoprotein complexes in the infected cell. *J Gen Virol* **50**(1)**,** 111-23.

Figure legends

Figure 1. Purified core-like particles and cytoplasmic cores. Purified core-like particles containing recombinant Ross River capsid protein and (a) 48mer DNA oligomer and (b) luciferase RNA are shown in negative stain TEM images. Purified cytoplasmic cores isolated from BHK infected with Ross River virus are shown in (c). Core-like particles (a and b) and cytoplasmic nucleocapsid cores (c) are 40 nm in diameter and are homogeneous, spherical particles. (d) Expression of viral glycoproteins from pRRV-GP-, pRRV-mtGP-, and pSV-GP-transfected cells. Cells were transfected with pRRV-GP, pRRV-mtGP, or pSV-GP for 15 hours and then fixed with 1.5% paraformaldehyde in PBS before being treated with RRV E2 (pRRV-GP and pRRV-mtGP) or Sindbis E2 (pSV-GP) antibody. Untreated cells (Mock) and virus-infected cells (RRV infection and Sindbis infection) were used as negative and positive controls, respectively. All panels were stained with TRITC-conjugated Goat anti-rabbit secondary except RRV infection which was treated with FITC-conjugated secondary antibody. Cells were imaged at 20x magnification on an Olympus 1X71 fluorescence microscope.

Figure 2. Timing of virus-like particle production. (a) Time course for core-like particle transfection. BHK cells (2.4×10^6) were transfected with CLPs containing 48mer DNA oligomer (3 μg, 2.6×10^{11} particles) and after the indicated time, cells were washed to remove non-transfected CLPs, and then lysed. Cell lysates were run on a SDS-PAGE and probed for capsid protein. Each data point represents a separate transfection. (b) Time course for release of VLPs. BHK cells transfected with pRRV-

GP ($1.4x10^7$ cells) were transfected with CLPs containing 48mer DNA oligomer (19 μg, $1.6x10^{12}$ particles). After one hour, cells were washed and new medium was added. After one additional hour, medium was collected (9 ml) at different times, centrifuged, and the pellet probed for capsid protein. Zero time is defined as immediately after washing cells and adding fresh media. At 30 min medium contains VLPs released between 0 and 30 min. Similarly 60 min and 120 min correspond to collecting VLPs released between 30 and 60 minutes and 60 and 120 minutes after adding medium, respectively. The absence of capsid protein at zero time indicates that the washing step removed all non-transfected CLPs. Far left lane is a positive control of purified capsid protein.

Figure 3. Viral protein components of VLPs. (a) Western blot analysis of pelleted VLPs. Medium containing VLPs (made from 90 μg CLPs containing 48mer DNA oligomer transfected into pRRV-GP-tranfected cells) was centrifuged. Pellets were resuspended in ~50 μl PBS to yield 0.15 mg/ml of VLPs. Ten μl of pelleted VLPs were probed for E2 and capsid protein. Native Ross River virus was used as a control (lane 2). (b) VLPs production reduced with pRRV-mtGP. BHK cells were transfected with pRRV-mtGP and 108 μg CLPs containing 48mer DNA oligomer. Medium was collected, centrifuged, and the pellet probed for E2 and capsid protein. Native Ross River virus was pelleted as a control. Lane 1 contains $1x10^8$ Ross River virus particles.

Figure 4. Morphology of VLPs. (a) TEM images of VLPs. Concentrated VLPs were stained with uranyl acetate and visualized by TEM. A sample of Ross River virus

prepared the same way is also shown. (b) VLPs are covered by viral glycoproteins. Immunoprecipitation experiments were performed on concentrated VLPs using anti-capsid antibody. Twenty μl of resuspended pelleted VLPs (~3 μg) were added to beads alone (- antibody) or beads coupled with capsid protein antibody (+ antibody). No capsid protein was detected (lane 3, pRRV-GP + CLPs). No capsid protein was detected in pellets from medium of CLPs transfected into control BHK cells (lane 2, BHK + CLPs). The positive control of CLPs containing 48mer DNA (18 μg, ~$1.6x10^{12}$ CLPs) added to the medium of BHK cells showed a strong band corresponding to capsid protein in lane 6 (Media + CLPs), demonstrating that CLPs can be detected in growth medium. Purified capsid protein is shown in lane 1 as a control.

Figure 5. Biological activity of VLPs and Infectivity of virus. (a) Biological activity of VLPs**.** VLPs containing Ross River capsid protein and luciferase RNA were made and 3 ml of the preparation were incubated with BHK cells ($2.4x10^5$) for one hour. After 12 hours, the cells were lysed and assayed for luciferase activity. Within each set of experiments, luminescence values from cells incubated with medium from cells transfected with pRRV-GP and CLPs were normalized to 1 (VLP) since the minimum and maximum luminescence varied between experiments. Cells were incubated with medium from BHK cells transfected with CLPs only (CLP). VLPs were incubated with the Ross River neutralizing antibody, T10C9, for 30 minutes at room temperature before being added to BHK cells (VLP + antibody). BHK cell were pre-treated with NH_4Cl before addition of VLPs (VLP+ NH_4Cl). VLPs from CLPs containing luciferase RNA were transfected into BHK expressing pSV-GP were collected and used to infect BHK

cells (RRV CLP+ pSV-GP). (b) Ross River virus infectivity. Infectivity of Ross River virus alone (WT RRV), in the presence of T10C9 neutralizing antibody (RRV + antibody), and after being amplified in the presence of ammonium chloride (RRV + NH_4Cl). The titer from the chimera virus, SIN(RRc), which contains Ross River capsid protein in a Sindbis background is taken directly from Lopez et al. The virus "CP" is a replicon derivative that contains only the non-structural proteins and the capsid protein. The average of two experiments for CP, RRV+antibody, and RRV+NH_4Cl is shown. Panels (a) and (b) are only to show trends in the data and cannot be directly compared.