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A Novel System for Visualizing Alphavirus Assembly

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Abstract

Alphaviruses are small, enveloped RNA viruses that form infectious particles by budding through the cellular plasma membrane. To help visualize and understand the intracellular assembly of alphavirus virions we have developed a bimolecular fluorescence complementation-based system (BiFC) that allows visualization of capsid and E2 subcellular localization and association in live cells. In this system, N- or C-terminal Venus fluorescent protein fragments (VN- and VC-) are fused to the N-terminus of the capsid protein on the Sindbis virus structural polyprotein, which results in the formation of fluorescent capsid-like structures in the absence of viral genomes that associate with the plasma membrane of cells. Mutation of the capsid autoprotease active site blocks structural polyprotein processing and alters the subcellular distribution of capsid fluorescence. Incorporating mCherry into the extracellular domain of the E2 glycoprotein allows the visualization of E2 glycoprotein localization and showed a close association of the E2 and capsid proteins at the plasma membrane as expected. These results suggest that this system is a useful new tool to study alphavirus assembly in live cells and may be useful in identifying molecules that inhibit alphavirus virion formation.

Keywords

Sindbis; Alphavirus; virus budding; fluorescence microscopy

Introduction

Alphaviruses (Family *Togaviridae*) are small, enveloped RNA viruses that cause significant human disease worldwide. Alphaviruses have a single strand positive strand genome that possesses a 5' guanine cap structure and a polyadenylated 3' terminus (E. G. Strauss et al., 1984). The 42S genomic RNA is translated by host ribosomes to produce the nonstructural proteins (nsP1-nsP4) that catalyze viral RNA replication (Rice and J. H. Strauss, 1981; E. G.

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Strauss et al., 1984). During RNA replication, a 26S subgenomic RNA is generated that is responsible for producing the viral structural polyprotein. The viral structural proteins (capsid / E3 / E2 / 6K / E1) are necessary for the formation of infectious virion particles. Alphavirus virions contain a T=4 icosahedral symmetry nucleocapsid composed of the capsid protein that binds and encapsidates the genomic 42S RNA via specific and non-specific RNA:capsid interactions (Linger et al., 2004; Perera et al., 2003; 2001). The highly ordered nucleocapsid is assembled from the self-association of 240 units of the capsid protein (30kDa) with the 42S genomic RNA in the cytoplasm of infected cells. The nucleocapsid interacts with the short cytoplasmic domain of E2 (in the glycosylated E2/E1 heterodimer) at the cytoplasmic surface of the plasma membrane, resulting in the budding and release of 70 nM diameter spherical virion particles from infected cells (Paredes et al., 1993; Tang et al., 2011).

The alphavirus capsid is a multi-functional protein, which contains domains for specific and non-specific viral RNA binding (Linger et al., 2004; Warrier et al., 2008), self-association (Jones et al., 2003; Perera et al., 2003; 2001), glycoprotein interaction (Zhao et al., 1994), and proteolytic activity (Hahn and J. H. Strauss, 1990; Melancon and Garoff, 1987). Each of these domains is critical for the efficient assembly of virions. For example, the C-terminal protease domain releases the capsid protein from the structural polyprotein on the cytoplasmic surface of the endoplasmic reticulum, allowing it to be trafficked to the cytoplasmic surface of the plasma membrane. Additionally, capsid associates with the E2 glycoprotein via a conserved YxL motif on the cytoplasmic tail of E2 (Zhao et al., 1994). Assembly of the nucleocapsid takes place within the cytoplasm, although the mechanism of assembly is unclear. In mature virions, the nucleocapsid is surrounded by a viral E2/E1 studded lipid bilayer derived from the cellular plasma membrane to form infectious viral particles. There are currently two models of how the nucleocapsid is formed. The first model proposes that the nucleocapsid encapsidates viral RNA in the cytoplasm and subsequently directs budding through the plasma membrane by concentrating E2 and E1 glycoprotein. The second model suggests that the high local concentrations of the E2 and E1 glycoproteins on the plasma membrane drive the dimerization and RNA association of the capsid protein at the plasma membrane surface. Commonly used techniques to study alphavirus assembly, such as cryo-electron microscopy and immunofluorescence microscopy, have proven to be insufficient to distinguish between these two models, requiring new approaches to understand alphavirus virion assembly.

To aid in the study of alphavirus assembly, we have developed a novel system for directly visualizing the assembly of alphavirus virus-like particles in live cells. This system is based on the principle of bimolecular fluorescence complementation, in which two halves of a fluorescent protein (Venus N-terminus (VN) and C-terminus (VC)) are individually linked to the prototypic Sindbis virus structural proteins, specifically the N-terminal capsid protein. Expression of the individual VN- or VC-linked constructs alone is insufficient to generate a fluorescent signal, whereas co-expression of the VN- and VC-linked structural protein constructs results in a stable fluorescent signal due to the capsid:capsid interaction reconstituting the Venus fluorophore (Kerppola, 2008). Mutation of the capsid protein alters its subcellular localization and processing, indicating that this system can be used to probe the molecular functions of the capsid proteins. Additionally, we have found that fusion of

mCherry with the E2 glycoprotein allows for the direct visualization of E2 localization and association of the glycoproteins with the BiFC labeled capsids, suggesting that this technique can be used to study virion assembly. This novel method provides an enhanced visual method to monitor the formation of alphavirus virions in live cells.

Results

Co-expression of Sindbis capsid-BiFC pairs results in membrane associated nucleocapsid-like structures

We first wanted to test if the BiFC system could be used to detect the interaction of alphavirus capsid proteins in live cells. In normal virion formation, the capsid monomers are held together by interaction with a viral RNA packaging signal to form dimers and higher order oligomers (Geigenmüller-Gnirke et al., 1993; Linger et al., 2004), resulting in spherical nucleocapsid structures that interact with E1/E2 glycoproteins located in the plasma membrane during the budding process. Interestingly, it has been previously shown that nucleocapsid cores and virus-like particles can be formed in the absence of viral RNA or a viral RNA packaging signal by introducing a coiled-coil into the capsid protein (Perera et al., 2003; 2001), indicating that the requirement for viral RNA or a packaging signal in nucleocapsid assembly can be circumvented. We hypothesized that the VN-capsid and VC-capsid interaction may be sufficient to drive capsid association and allow visual confirmation of the nucleocapsid assembly process in live cells in the absence of viral RNA.

To develop this BiFC-capsid system, we fused either the Venus N-terminal or C-terminal (VN or VC) protein fragments to the N-terminus of the Sindbis virus structural polyprotein, specifically to the N-terminus of the capsid protein. The VN and VC fusion proteins were cloned into a cytomegalovirus IE promoter expression plasmid to generate plasmids pBG262 and pBG265 (Figure 1A). Transfection of pBG262 or pBG265 into BHK cells alone produced no fluorescence as expected (data not shown), whereas transfection of pBG262 and pBG265 into BHK cells produced BiFC Venus fluorescence 48 hr post-transfection. We observed distinct punctate Venus fluorescence in what appeared to be the periphery of the cells (Figure 1B, left panel). Overlay of Venus fluorescence with a differential interference contrast (DIC) image demonstrated that the Venus signal is localized primarily to the plasma membrane of cells, with a minor population of diffuse fluorescence and fluorescent puncta appearing to be cytoplasmically localized (Figure 1B, right panel), indicating that the capsids are interacting primarily with the plasma membrane.

Mutation of capsid protease activity alters capsid BiFC localization

Alphavirus structural proteins are translated as a polyprotein, and capsid protein is released from the structural polyprotein via the action of a C-terminal capsid protease domain whereas the E3/E2/6K/E1 proteins are processed by furin protease (Hahn and J. H. Strauss, 1990). To determine what effect disrupting proteolytic processing of the capsid protein would have on Venus puncta formation, we abolished capsid protease activity in our BiFC expression constructs. The normal capsid protease domain contains a catalytic triad of 3 residues (His141, Asp163, and Ser215) (Hahn and J. H. Strauss, 1990). In plasmids pBG262 and pBG265, we introduced a proline mutation at His141 in the capsid protease active site to

block capsid protease cleavage activity and impair polyprotein maturation, resulting in plasmids pBG291 and pBG292, respectively (Figure 2A)(Aggarwal et al., 2014; Hahn and J. H. Strauss, 1990; Morillas et al., 2008; Tong et al., 1993). Transfection of pBG291 and pBG292 into BHK cells resulted in a very different cellular distribution of Venus signal as compared to wild-type. Fluorescence was observed only in the cytoplasm, in a dispersed perinuclear pattern reminiscent of interaction with the endoplasmic reticulum (Figure 2B). No plasma membrane-associated puncta were observed in cells transfected with the protease deficient clones, suggesting that protease activity is essential for the formation of the puncta observed with wild-type structural protein expression (Figure 1B). The overall morphology of the Venus positive pBG291/pBG292 cells was abnormal as compared to the pBG262/pBG265 transfected cells (Figure 1B), indicating that expression of protease-deficient capsid and structural proteins disrupts normal cellular physiology. It is currently unclear why uncleaved capsid and structural proteins would cause this affect, although it may be possible that uncleaved Capsid/E3 protein is causing a disruption of the endoplasmic reticulum homeostasis or inducing apoptosis. We plan on examining this in future experiments.

To determine if viral capsid proteins can be released into the media of transfected cells, we collected cells and media from pBG262/pBG265 and pBG291/pBG292 transfected cells, pelleted the cells at low speed (10K RPM), and pelleted virus-like particles in 10K clarified media with high-speed centrifugation (100K RPM). Capsid protein was detected by western blot analysis using a hemagglutinin (HA) epitope present between the capsid and Venus fragments in each construct (Figure 2C). Cells transfected with pBG262/pBG265 (wildtype) showed two dominant HA containing proteins at approximately 42 kDa and 55 kDa, which are the sizes predicted for correctly processed VN-capsid and VC-capsid proteins, respectively. A similar pattern of VN-capsid and VC-capsid reactivity was observed in clarified media that had been pelleted at $100,000 \times G$, suggesting that the VN-capsid and VC-capsid proteins had been released into the media. The slightly higher apparent molecular weight is likely due to less total protein and large molecular weight molecules present in the $100,000 \times G$ samples (media) as compared to the $10,000 \times G$ samples (lysed cells), although we cannot rule out post-translational modification of the proteins (such as phosphorylation or palmitoylation) in either sample that could alter their apparent molecular weights. Correctly processed VN-capsid and VC-capsid were not observed either in cells transfected with pBG291/pBG292 (H141P), whereas a high molecular weight band correlates with unprocessed structural proteins. VN-capsid or VC-capsid bands were not observed in clarified media, indicating that the capsid protease activity was necessary for release of capsid into the media.

Truncated capsid proteins associate with nucleoli

The N-terminal region of the capsid protein is involved in binding viral RNA. To test if removing the RNA binding regions of the capsid protein alters its ability to self-associate, we first deleted 81 amino acids from the N-terminus of the capsid protein on plasmids pBG262 and pBG265. This mutation removed helix I and the non-specific RNA binding domain (Hong et al., 2006; Perera et al., 2001) and resulted in plasmids pBG263 and pBG266 respectively. Co-transfection of pBG263 and pBG266 resulted in a mixed phenotype. A small portion of the Venus signal segregated to the plasma membrane in a similar fashion as

the full length BiFC pairs, whereas a large portion of the Venus signal was found within the nucleus in structures appearing to be nucleoli (Figure 3A). Semliki Forest virus capsid protein has been previously shown to be able to interact with nucleoli structures (Michel et al., 1990), and the findings that deletion of the first 81 amino acids of the capsid protein suggest that the N-terminus of the capsid protein is responsible for excluding the capsid protein from the nucleus. This also demonstrated that residues 1-81 were not required for capsid self-association. We then tested if removing all RNA binding regions from the capsid protein (Linger et al., 2004; Owen and Kuhn, 1996) altered capsid association and the nucleoli interaction phenotype. Deletion of the specific RNA binding region plasmids (pBG264 and pBG267, AA 1-114) resulted in a different phenotype, with diffuse weak fluorescence in cells (Figure 3B, oversaturated). No nucleolar fluorescence was observed in cells transfected with the AA 1-114 deletion mutants. The observation of fluorescence even in the absence of the known capsid dimerization and RNA binding domains suggest that the VN and VC fragments can stabilize transient interactions between the capsid monomers in the absence of first 81 residues in the capsid protein.

mCherry-labeled E2 glycoprotein interacts with the Venus-BiFC capsid structures

Nucleocapsids interact with E2 at the plasma membrane to form mature virions that are subsequently released from the cell. We next wanted to determine if we could use this system to visualize viral glycoprotein association with capsid proteins in live cells. The E2 glycoprotein has been previously modified to include a Staphylococcus protein A ZZ domain to facilitate binding to the antibody Fc chain (Bergman et al., 2003), indicating that the E2 glycoprotein can tolerate insertions in its extracellular domain without destroying its interaction with the capsid protein. To test if a fluorescent protein could be inserted into the E2 protein without compromising E2 function, we fused the coding sequence for the mCherry fluorophore into E2 in the plasmid pBG265, resulting in the plasmid pBG337. Co-transfection of BHK cells with pBG262 and pBG337 (Figure 4A) resulted in the same Venus puncta observed previously (Figure 4B top panel), and a large number of E2-mCherry puncta as well (Figure 4B bottom panel). Almost all capsid puncta are associated with an E2-mCherry puncta, indicating a specific association of the nucleocapsid with the E2 glycoprotein. However, a large number of E2-mCherry puncta had no visible association with capsid puncta, suggesting that glycoprotein clustering on the plasma membrane is not a function of association with oligomerized capsid proteins.

Analysis of the diameters of the capsid-Venus puncta and E2:mCherry puncta resulted in an average size of 238 ± 18 nm for capsid-Venus puncta and 308 ± 55 nm ($n=8$) for E2-mCherry puncta. The optical resolution of traditional fluorescence microscopy (Rayleigh limit) is 200 nm for most microscope systems and alphaviruses are of an average diameter of 70 nm based on electron microscopy (Paredes et al., 1993; Sherman and Weaver, 2010; Tang et al., 2011), so the true diameter of the fluorescent particles cannot be determined by our analysis and would require super-resolution microscopy to determine the accurate sizes. However, the increased size of the E2-mCherry puncta compared to the capsid-Venus puncta suggested that the E2 puncta were as expected bigger than the capsid puncta. Distances between capsid-Venus and E2-mCherry puncta were then determined. The centerpoints of the Venus puncta were an average distance of 150 ± 30 nm from

centerpoints of E2 puncta (n=6), indicating a close association between capsid structures and E2 patches. Several of the E2 puncta associated capsid puncta showed a convex shape (Figure 4B zoomed view, white arrows), suggesting that capsid structures were interacting with and causing curvature of E2 patches. E2 patches not associated with capsid puncta did not show this curvature. This suggests that the capsid structures were initiating the budding process through the E2 patches, forming structures similar to the diagram shown in Figure 4C.

Discussion

In this report we describe the construction and characterization of a new system to monitor the formation of alphavirus particles in live cells. We demonstrate that bimolecular fluorescence complementation can be used to form and detect alphavirus nucleocapsid-like structures and assess the effects of mutations within the capsid protein on subcellular localization and function. This provides an interesting method to further dissect the function and location of the capsid protein in viral replication. For example, the capsid protein from New World alphaviruses has been shown to reduce mammalian nuclear import and host transcription (Atasheva et al., 2008). This system could be useful in further elucidating the role of capsid in virus-mediated manipulation of the host cell, including host cell metabolism, transcription, translation, and pathogenesis. We also demonstrate that this system can be used to probe the interactions between capsids and mCherry labeled E2 proteins on membranes. The interaction between E2 and capsid structures is critical for the formation of viable virus particles and has been an active area of research. It has been previously demonstrated that conserved E2 residues Y400 and L402 are involved in the interaction between E2 and capsid protein (West et al., 2006), and one potential application of this system is to directly visualize if mutating these residues alters capsid/E2 association.

In addition to being useful as a tool to probe basic virion assembly mechanisms, this system may be used to screen chemical libraries for inhibitors of alphavirus virion assembly. There are currently no clinically available alphavirus inhibitors, and identifying potent and specific inhibitors of alphavirus assembly would be of great interest for the development of antiviral drugs. For example, establishing a stable human cell line where inducible expression of viral structural proteins (such as those presented in Figure 4 (pBG262 and pBG337)) would allow for simultaneous screening of inhibitors of capsid protease activity, capsid/capsid interactions, E2 puncta formation, E2/capsid subcellular localization, and E2/capsid association. Using this type of system in a high-content microscopic screen could identify novel antiviral molecules that inhibit specific parts of the alphavirus replication cycle that would be difficult to screen using other technologies. Many screening technologies either target individual processes in pathogen replication or take a blind approach towards antiviral identification, but this system could overcome those limitations and provide specific data on the viral processes that inhibitors target. In addition, expression of the viral structural proteins in the absence of viral RNA replication results in minimal biohazard risk, allowing for screening of libraries in non-biohazard environments.

Conclusions

This report describes a new BiFC-based system that allows for the study of alphavirus virion assembly in live cells. This system will be valuable for dissecting how alphavirus virion particles are formed during infection, providing new information about the viral lifecycle. In addition, this system can be adapted to a high-content screening format to identify novel inhibitors of alphavirus virion assembly, providing potential new therapeutics for the treatment of alphavirus infection.

Materials and Methods

Cell Culture and Transfection

Baby hamster kidney (BHK) cells were cultured in Dulbeccos's modified Eagle medium supplemented with 10% fetal bovine serum (FBS) as previously described (Steel et al., 2011). BHK cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

Construction of BiFC Plasmids

Mammalian BiFC expression plasmids containing actin genes were generously provided by Dr. Chaoping Chen (Chen et al., 2007). Wild-type expression plasmids (pBG262 and pBG265) were generated by PCR amplification of the Sindbis structural protein open reading frame from the TE/3'2J/TR339 Sindbis virus infectious clone (Hahn et al., 1992) using primers BG363 (5'-catggaattctcatgaatagaggattcttaacatgc) and BG366 (5'-cagaggtacatctctgtgtgtagtcagc), restriction digestion of the PCR product with EcoRI and KpnI enzymes, and ligated in EcoRI/KpnI digested the pCMV-VN-actin or pCMV-VC-actin vectors digested with EcoRI/KpnI.

To generate capsid 1-81 deletions, we PCR amplified the truncated structural protein open reading frame from pBG262 with the primer pair BG364 (5'-catggaattctcatgaagccgaagaacaaaaacgcagg) / BG366. The PCR products were digested with EcoRI/KpnI and ligated into pCMV-VN or pCMV-VC to generate the final plasmids pBG263 and pBG266, respectively. Capsid 1-114 deletion plasmids were generated using the same approach using the primer pair BG365 (5'-catggaattctcatgtgttcgacgtcaagaacg) / BG366 to produce plasmids pBG264 and pBG267. To generate pBG337, a HindIII restriction site was introduced into pBG265 using QuickChange Mutagenesis (Stratagene, Inc) using primers BG415 (5'-cgctacatgtcgcttaag cagaagctaccgttaaagaaggcacc) and BG416 (5'-ggtgccttcttaacggtaagcttctgcttaagcagatgtagcg) to generate plasmid pBG336. The mCherry open reading frame was then PCR amplified from pmCherry (Clontech) using primers BG417 (5'-caccaagcttatggtgagcaaggcgagg) and BG418 (5'-atacaagcttctgtacagctcgtcc), the PCR product and pBG336 were digested with HindIII, and the mCherry gene was ligated into pBG336 to generate pBG337. All plasmid constructs were verified by sequencing.

Fluorescent Microscopy

For live-cell imaging, transfected BHK cells were grown on acid-washed coverslips for 48 hrs, then coverslip were transferred to a optically transparent imaging chamber filled with phenol-red free L-15 media supplemented with 10% FBS. Live cells were imaged on a Deltavision Personal DV (Applied Precision) live cell imaging system with environmental chamber. Images were collected with either 60X or 100X oil immersion lenses (Olympus) and a CoolSNAP HQ2 camera (Photometrics/Roper Scientific). Image deconvolution was performed using the SoftWoRx analysis package (Applied Precision). Particle diameter and central distance analysis was performed using ImageJ (NIH). The pixelation seen in the expanded view within Figure 4B is due to the resolution limit of the CoolSNAP HQ2 camera at 100X magnification.

Western Blot Analysis

To prepare cell samples, BHK cells were trypsinized from transfected wells, pelleted, and 100,000 cells were resuspended in 100 µl of 1x SDS loading buffer. Cells were boiled for 5 minutes and clarified by centrifugation at 10,000 × G prior to loading onto 12% SDS-PAGE gels. For experiments examining released VLPs, transfected cells were incubated as above, and at 48 hrs the cells and supernatant were harvested. The 100K pellet was derived from media collected from the cell culture that was initially centrifuged at 10K at 4°C for 20 min to clarify large debris, followed by centrifugation of the clarified media at 100,00 × G at 4°C for 1 hr. Pelleted VLPs were resuspended in 20ul of 1x Laemmli SDS sample buffer and protein was resolved on SDS-PAGE gels. Resolved proteins were transferred to nitrocellulose membranes. Membranes were blocked with 2% non-fat dry milk in phosphate buffered saline supplemented with 0.1% Tween 20. Membranes were incubated with polyclonal anti-hemagglutinin antibody (Abcam), followed by incubation with horse-peroxidase-conjugated anti-rabbit secondary antibody. Proteins were detected with the ECL Plus Western blot detection kit (Amersham) on a Storm 860 phosphorimager.

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Highlights

- Developed a BiFC-based assay for alphavirus assembly for live cell analysis
- Mutation of capsid protease disrupted protein processing and virion assembly
- Incorporation of mCherry into E2 allows visualization of capsids and E2 on plasma membrane
- This system provides a novel technique for studying alphavirus assembly
- This system provides a novel technique to screen for alphavirus assembly inhibitors

Figure 1. Expression of BiFC tagged capsid proteins results in membrane associated fluorescent puncta

A) Diagram of open reading frames expressed from plasmids pBG262 (Top) and pBG265 (Bottom). Venus fluorophore N- and C-terminal fragments (VN and VC) and hemagglutinin (HA) epitopes are shown. B) Fluorescence microscopy images of BHK cells cotransfected with pBG262 and pBG265. Left Panel: Deconvoluted Venus fluorescence image of transfected cell. Scale bar represents 15 μ M. Right Panel: Overlay of differential interference contrast (DIC) with a representative BiFC fluorescence image from a 0.5 μ M Z-stack. Note Venus puncta and fluorescence localize primarily on the cell periphery, with a minor population present within the cytoplasm.

Figure 2. Mutation of alphavirus capsid protease results in altered capsid subcellular distribution and structural protein processing

A) Diagram of the location of H141P mutations in plasmids pBG291 and pBG292. **B)** Fluorescence microscopy images of BHK cells cotransfected with pBG291 and pBG292. **C)** Western blot analysis of HA-tagged capsid proteins in cells (10K) and in clarified media (100K).

Figure 3. Deletion of capsid RNA binding regions alters capsid subcellular localization
A) Top: Diagram of pBG263/pBG266 deletion mutants. Bottom: Fluorescence microscopy images of BHK cells co-transfected with pBG263 and pBG266. **B)** Top: Diagram of pBG264/pBG267 deletion mutants. Bottom: Fluorescence microscopy images of BHK cells co-transfected with pBG264 and pBG267. Scale bar represents 15 μm in both images.

Figure 4. BiFC labeled capsids interact with mCherry labeled E2 glycoproteins in live cells
A) Diagram of plasmids pBG337 with approximate location of mCherry insertion. **B)** Fluorescence microscopy images of BHK cells co-transfected with pBG262 and pBG337. Venus BiFC fluorescence is shown in the top panel (Capsid), and mCherry fluorescence is shown in the bottom panel (E2). An overlay of the two images is shown in the middle panel, and an expanded view of overlay showing interactions of BiFC capsids and mCherry E2 puncta (noted by white arrows) is shown on the right. Scale bar in each image represents 5 μ M and was used to calculate particle diameters and distances between puncta centers using ImageJ. **C)** Diagram of proposed organization capsid/E2 interactions in this system.