Comparative Characterization of the Sindbis Virus Proteome from Mammalian and Invertebrate Hosts Identifies nsP2 as a Component of the Virus Nucleocapsid and Sorting Nexin 5 as a Significant Host Factor for Alphavirus Replication

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Running head: Host proteins involved in Sindbis virus replication
Abstract

Recent advances in mass spectrometry methods and instrumentation now allow for more accurate identification of proteins in low abundance. This technology was applied to Sindbis virus, the prototypical alphavirus to investigate the viral proteome. To determine if host proteins are specifically packaged into alphavirus virions, Sindbis virus (SINV) was grown in multiple host cells representing vertebrate and mosquito hosts and total protein content of purified virions was determined. This analysis identified host factors not previously associated with alphavirus entry, replication, or egress. One host protein, sorting nexin 5 (SNX5), was shown to be critical for the replication of three different alphaviruses, Sindbis, Mayaro and Chikungunya virus. The most significant finding was that in addition to the host proteins, SINV non-structural protein 2 (nsP2) was detected within virions grown in all host cells examined. The protein and RNA-interacting capabilities of nsP2 coupled with its presence in the virion support a role for nsP2 during packaging and/or entry of progeny virus. This function has not been identified for this protein. Taken together, this strategy identified at least one host factor integrally involved in alphavirus replication. Identification of other host proteins provides insight into alphavirus-host interactions during viral replication in both vertebrate and invertebrate hosts. This method of virus proteome analysis may also be useful for the identification of protein candidates for host-based therapeutics.
Importance

Pathogenic Alphaviruses, such as Chikungunya and Mayaro virus, continue to plague public health in developing and developed countries alike. Alphaviruses belong to a group of viruses vectored in nature by hematophagous (blood-feeding) insects and are termed Arboviruses (arthropod-borne viruses). This group of viruses contains many human pathogens such as dengue fever, West Nile and Yellow fever viruses. With few exceptions there are no vaccines or prophylactics for these agents leaving one third of the world population at risk of infection.

Identifying effective antivirals has been a long term goal for combating these diseases not only because of the lack of vaccines but also because they are effective during an ongoing epidemic.

Mass spectrometry-based analysis of the Sindbis virus proteome can be effective in identifying host genes involved in virus replication and novel functions for virus proteins. Identification of these factors is invaluable for the prophylaxis of this group of viruses.

Keywords:

Alphavirus; Sindbis virus; Viral Proteomics; Proteomics; Mass Spectrometry
Introduction

Epizootics of re-emerging and novel pathogenic viruses have increased in recent years, causing infections of susceptible human populations in some of the poorest countries in the world and imposing constant risks on public health. With increasing weather events, travel, and sexual transmission, an even larger population may be at risk. The Alphavirus genus (Family *Togaviridae*) includes emerging and well characterized vector-borne diseases including Eastern, Western, and Venezuelan Equine Encephalitis Viruses (EEEV, WEEV, VEEV), Chikungunya virus (CHIKV) Ross River (RRV), and Mayaro virus (MAYV). Sindbis virus (SINV), the prototype of this group is nonpathogenic (1). These pathogens are vectored in nature from zoonotic reservoirs, through mosquitoes or ticks to humans, and cycle from vertebrate to invertebrate hosts (arboviruses) (2). Upon emergence, these viruses spill over into the human population and cause severe disease. Viruses are obligate parasites of their hosts and require the host’s biosynthetic machinery to propagate all virus infections, necessitating prophylactic treatments that do not harm the host. With few exceptions all arbovirus diseases lack effective vaccines and antivirals (3). Host-based therapeutics are thought to be alternative avenues, but identifying targets for this approach requires a detailed understanding of how these viruses interact with their host throughout the replication cycle (4, 5). Identification of host cofactors to virus enzymes or specific host factors involved in viral replication and packaging is challenging. The research presented herein focused on the identification of host proteins within SINV virus particles. This was done in an effort to identify host factors associated with virus replication and address any functional relevance these specific proteins might have in viral propagation. In an effort to determine if mass spectrometry could 1) identify host proteins in the virus particle that could participate in subsequent infections or 2) identify host proteins trapped in the particle...
The formation of this complex is proposed to induce conformational changes within the virus that produce a pore within the membrane and facilitates the release of the positive sense, single-stranded viral RNA directly into the cell (13-16). In the cytoplasm, the RNA is then translated into four non-structural proteins, nsP1, nsP2, nsP3, and nsP4 (17). Together, these proteins, in association with unknown host proteins, comprise the replication complex which is found associated with the plasma membrane in vertebrate cells (18). Transcription of viral RNA produces polycistronic full length and a subgenomic 26S RNAs. In both vertebrate and invertebrate cells the non-structural proteins are translated from full length RNA while the structural proteins are translated from the subgenomic 26S RNA. The 26S RNA encodes the proteins in the order Capsid (C) PE2 (processed to E3 and E2), 6K/TF (transframe), and E1, assembled in the endoplasmic reticulum (ER) and processed by host signalase and furin proteins.
(19). The matured virus from all host species contains the structural proteins C, E1 and E2. 6K is not found associated with Sindbis virions but LC-MS/MS analysis of the 6K chimera-frameshift TF protein have identified the latter in Sindbis particles (20). In vertebrate hosts, the glycoproteins mature via the exocytic pathway and are delivered to the plasma membrane. Concurrently, the capsid protomers are pre-assembled into viral nucleocapsids with full length RNA found within the cytoplasm and are transported to the plasma membrane, where they acquire their glycoprotein-modified outer shell (12). The virus fully matures as E1 and E2 modified plasma membrane surrounds the nucleocapsid (NC) core and is extruded into the surrounding environment (21, 22) in a process that excludes the preponderance of host plasma membrane proteins (23). In invertebrate cells the virus takes an alternate path to maturation. Translation and replication complexes are housed in cytoplasmic endosomes termed “virus factories” where all assembly mechanics occur. Fully formed virions are then secreted into the media by fusion of the endosomes with the cell plasma membrane (24, 25). In both hosts, the final virion is composed of each of the structural proteins, C, E1 and E2, in a 1:1:1 stoichiometric ratio producing a highly stable and symmetrical virus (26). The mature structure contains an outer protein shell composed of the glycoproteins E1 and E2 separated by a host-derived membrane from the inner NC core. The lipid composition of the membrane will vary between vertebrate or invertebrate hosts especially with respect to cholesterol, which is not synthesized by the class Insecta (27, 28). In nature, arboviruses are maintained in a complex cycle involving infections between the virus vertebrate reservoir, the insect vector and the human host and can replicate in many host backgrounds (29). For this reason, we used cell lines of various tissue types and host backgrounds to characterize the compliment of host cell-derived proteins. To determine the host protein content of the model Alphavirus particle, SINV, a mass spectrometry-
based approach was utilized to identify host proteins within virions that might not be resolvable using other methods (30). Additionally, mass spectrometry can allow for the identification of proteins that are specifically associated with the virus structure or alternatively have become trapped or assembled into the structure during the process of maturation.

In this study, highly purified SINV preparations derived mosquito and mammalian cells were analyzed using LC-MS/MS to determine if previously undetected host proteins were packaged into virus particles. More than 124 unique host proteins were discovered in virus replicated in the human cell backgrounds of HEK293 (human embryonic kidney cells) or HepG2 (human hepatic cancer) cells and in hamster BHK21 (baby hamster kidney) cells more than 38 host proteins were identified. When the host protein profiles between mammalian species were cross compared, eight proteins were found in common. SINV was also prepared in a mosquito C7-10 Aedes albopictus cell line, an Aedine cell line which exhibits both lytic and persistent infections (31). Although more than 50 host proteins were identified in the mosquito background, phospholipid scramblase was the most abundant and interestingly was the only protein found in both the invertebrate and vertebrate backgrounds. A detailed mapping of the viral proteome revealed a nearly 75% coverage of SINV’s structural polyprotein, which contains peptides detected for capsid, E3, E2, and E1, and TF but not 6K. An unexpected and intriguing finding was that peptides from SINV’s non-structural polyprotein were also detected, but specifically only for nsP2, the viral protease, which was not previously detected (32-34).

In order to determine a related function of these co-identified proteins during SINV replication we attempted to generate stable homozygous knockouts of each of the eight identified host proteins, in HEK293 cells. We were able to obtain homozygous knockouts for sorting nexin 5 (SNX5) and RNA binding protein 3 (RBM3), others were lethal. Using these knockout cell
lines, we observed a significant suppression in Sindbis virus production when compared to wild-type HEK293 cells. To confirm this observation, we also performed the same analysis using Mayaro and Chikungunya virus. Viral replication was also inhibited for both these viruses. To the best of our knowledge, this is the first report of a sorting nexin, SNX5, playing a critical role in alphavirus replication. Collectively, this LC-MS/MS approach has expanded the tools that can be used for understanding the mechanics of alphavirus replication while also identifying novel host targets with therapeutic potential.

Results

Determination of Viral Purity

In order to effectively analyze the virus associated host protein content, ultra-pure preparations of virions were prepared. Host background contamination from cellular vesicles or debris not specifically incorporated needs to be eliminated by the virus purification procedure prior to mass spectrometry analysis. The high particle/PFU ratio of SVHR, its structural stability and known exclusion of host proteins from the virus modified membrane make this an ideal experimental model. First, virus was grown and supernatant collected from infected and non-infected cells. The supernatant was harvested from a single cycle infection prior to cell lysis; crude viral preparations were clarified by low speed centrifugation to remove large complexes and particulates. Negative control fractions were run through the same process as virus-containing fractions. The clarified material was then run through two serial potassium tartrate density gradients using isopycnic ultracentrifugation. Virus from the second purification was then pelleted through PBS and the pellet was washed as the final purification step. To determine if this purification scheme (Fig. 1A) resulted in visually pure preparations of virus particles free of
contaminating organelles or membrane fragments, fractions were visualized using transmission electron microscopy by negative stain (Fig. 1B). The electron micrographs of the three-step purified SINV preparations visually show high purity virus preparations, with little evidence of cell debris or other cell-associated membrane-bound vesicles. To determine if there was any other protein contamination present in the preparations, each purified prep was resolved on an SDS-PAGE gel and stained with silver or coomassie blue as described above. Similar to the transmission electron micrographs, the SDS-PAGE analysis also confirmed that there was no major protein impurities in the preparations (Fig. 1C). The primary bands in the viral preparations were from the virus structural proteins E1, E2, and capsid.

Host and Virus Protein Identification and Functional Analysis of Purified SINV Virions

Following the confirmation of the purity of the virus preparations, SINV and negative control preparations were analyzed, in parallel, using a FASP LC-MS/MS method. Viral preparations and negative controls from BHK21 (hamster, *Cricetulus griseus*), C7-10 (mosquito, *Aedes albopictus*), HEK293 (human), and HepG2 (human) cell host backgrounds were analyzed to determine if any host proteins could be detected. Each sample was analyzed in technical triplicate on a virgin C18 HPLC column to ensure that carry over from sample to sample would not result in false host protein identifications. LC-MS/MS spectral data was searched using the SEQUEST algorithm in Proteome Discoverer 1.4 (Thermo Fisher) to identify peptides and align them to known host proteins from each background. Each purified virus preparation yielded unique host protein identifications with only a limited number of host proteins being identified in the negative controls. Keratin was often identified in the negative control and was not considered for functional interpretation because it is shed by cells in culture or introduced during sample processing. In the human backgrounds 124 host proteins were identified; 91 in HepG2 cells and
58 in HEK293 cells (Table S1). Using the PANTHER classification to characterize these protein identifications, a significant portion of the proteins fell into three major functional classes; binding, catalytic activity, and receptor activity.

Compared to the human proteins found associated with SINV, an additional 38 host proteins were identified from the BHK21 hamster background and 51 proteins from the C7-10 mosquito background (Table S1). It is of note that mammalian and insect cells do not always express mammalian protein orthologs. Both hamster and human genomes and proteomes are well characterized; however, the same level of characterization does not exist in the mosquito host *Aedes albopictus* as the genome sequence was very recently published (35). To analyze the raw data obtained from the C7-10 cell background, a curated draft proteome from *Aedes albopictus* was utilized (see methods). Many of the proteins identified in the C7-10 background classify in similar functional categories and classes as the proteins identified in the mammalian backgrounds. Interestingly, for the mosquito background, phospholipid scramblase was overwhelmingly represented when compared to the other mosquito host proteins identified. Scramblase was detected as 8 unique peptide sequences a total of 57 times (PSMs) for total sequence coverage of 30% (Table S1).

**Coinciding Host Protein Signatures between Human, Hamster, and Mosquito Cell Backgrounds**

When comparing human virus preparations (HEK293 vs. HepG2) only to each other, we observed a 34% overlap or identified 20 proteins, respectively (Fig. 2). Given that the limiting factor which dictates detection by LC-MS/MS is dynamic range and instrument sensitivity, the host proteins that were commonly detected between each preparation were also in the highest abundance (indicated by the total number of PSMs, ∑#PSMs) within these virus particles. In
addition to comparing preparations within the same species, we also compared the host proteins identified across species. Using this strategy we identified 8 protein signatures that were conserved between hamster and human (Fig. 2). Cellular nucleic acid binding protein 6 (CNBP6), RNA binding protein 3 (RBM3), myosin (MYO), and claudin (CLDN) were present in virus particles grown in all backgrounds. Interestingly, the exact peptide sequence was identified for CNBP6, RBM3, and sorting nexin 5 (SNX5) from all vertebrate derived samples. When we compared the mammalian backgrounds to the mosquito background we found that only phospholipid scramblase was conserved. Also of note, we detected that the scramblase peptides overlapped even though only 51% of the amino acid sequence is conserved between mosquito and human proteins (Fig. 3).

**Viral Structural and Non-structural Proteins Identified in Purified SINV Virions**

In addition to the detailed analysis of the host proteome contained within the SINV virion, the viral proteome within SINV virions was also examined. Raw data gathered from each SINV preparation regardless of host background was pooled together to generate a comprehensive sequence map of SINV structural and non-structural polyprotein coverage (Table S2). For SINV’s structural polyprotein, 91 unique peptide sequences were detected a total of 10,833 times, resulting in a sequence coverage total of 74%. Peptides from every processed structural protein were detected except 6K. However, we did detect a single peptide (RLPGGEGR) corresponding to TF protein, which is reported to co-purify with NC (36). In this study, we also searched our raw data against SINV non-structural polyprotein. From this analysis, we only detected peptides from nsP2, the virus protease. No peptides from any other non-structural protein were detected. For nsP2, we detected 20 unique peptide sequences a total of 121 times.

When compared to the structural polyprotein, peptides corresponding to nsP2 were detected with
a frequency of roughly 1%. To confirm that nsP2 is not co-purifying with other structural proteins via protein-protein interactions, we performed an additional high salt wash in 1M KCl on viral pellets prepared in the BHK21 background. LC-MS/MS analysis of these preparations still resulted in high sequence coverage of nsP2 and no other non-structural protein (Table S1).

To identify if the nsP2 protein was associated with the SINV outer shell or inner core, we attempted to fractionate the inner core from the outer shell containing the glycoproteins. NC cores were fractionated by treating purified virus particles with 4% or 5% Triton X-100 for 1 hr. at 37°C to remove the glycoprotein shell and the fractions were separated by ultracentrifugation. Silver stain of 4-12% Bis-Tris gradient gels of the purified fractions (Fig. 4) demonstrate that the NC fractions shown in lanes 2 and 3 are not completely stripped of glycoproteins showing visible amounts of E1 and E2 on the gel after treatment with 5% Triton. From these silver stained fractions it could be concluded that the Triton X-100 treatment was not sufficiently stringent to eliminate all of the outer glycoprotein shell from the cores. Additionally, it was clear that treatment of the fractions with Triton appeared to shift the mobility of C, E1 and E2 proteins as seen by the appearance of slower migrating proteins in the 4% Triton X-100 fraction (Fig. 4 lane 2) compared to the 5% Triton X-100 fraction (Fig. 4 lane 3) and the observed E1 and E2 proteins in the glycoprotein fraction seen in figure 4 lane 4. Additionally the doublet C band in figure 4 has unlabeled C protein bands similar to those seen in Fig. 5B-lane 1 described below.

Thus, none of the extractions utilizing treatment of the purified virus with 4% Triton X-100 produced fractions pure enough to unambiguously assign the location of nsP2 to a particular sub fraction of the virus. However, to definitively identify the virus protein species which were seen in the extracted fractions, western blots were done using polyclonal antibodies against expressed capsid protein only (36) (raised in rabbits, a gift from S. Mukhopadhyay) or a
combination of anti-C Ab and anti-SVHR whole virus (raised in rabbits) and is shown in figure 273B. This analysis showed that the anti-capsid Ab only recognized the bottom band of the ~30kDa. doublet while both C bands were recognized by the anti-whole SVHR Ab. The anti-SVHR C/E2 antibody recognized only E2 at roughly 50kDa. These observations indicate that after Triton treatment, the doublet band running at ~30kDa. contains 2 forms of C protein. The lower band only binds to the polyclonal anti-C Ab while the whole SINV Ab virus reacted with both bands of C. The specific lot of anti-whole virus Ab used does not recognize E2, while E1 and C are recognized (data not shown). To positively identify nsP2 protein association with purified SINV, a second blot was made and probed with anti-nsP2 antibody (a gift of R. Hardy) shown in figure 5A. Thus we verified that nsP2 was associated with purified virus but could not definitively identify the location in the particle.

Virus-associated Mammalian Host Protein Requirement for Virus Replication

Stable knockouts of each of the conserved host proteins were attempted in human HEK293 cells. We were able to obtain homozygous knockouts for sorting nexin 5 (SNX5) and RNA binding protein 3 (RBM3). To establish that this observation is not specific just to Sindbis virus, but rather to alphaviruses, we repeated these studies using the ‘new-world’ Mayaro, a pathogenic virus (27) and CHIKV which produces acute febrile polyarthritis (37). Using these knockout cell lines, we observed a significant suppression in Sindbis, Mayaro and Chikungunya virus production by standard plaque assay when the cells were grown in the SNX5, or RBM3 knockout cell lines when compared to wild-type HEK293 (Fig. 6). Because the virus produced from these knockout cell lines compared to virus produced from wild type HEK293 cells was not as infectious as determined by plaque assay on BHK cells, we conclude that these proteins are not absolutely required for infectivity. While SNX5 is important in the efficient maturation of
infectious alphavirus intracellular trafficking of virus was not affected because there was not a significant amount of intracellular virus found in the SNX5 negative cells (data not shown). All three viruses assayed, SINV, MAYV and CHIKV replicated to high titers in wild-type HEK293, with titers of $5 \times 10^9$, $1 \times 10^8$, and $4.2 \times 10^7$ PFU/mL, respectively. By contrast, the titers of SINV were significantly reduced >100 fold ($p=0.03$), ~10 fold for MAYV ($p=0.003$) and 40 fold for CHIKV ($p=0.01$) in the HEK293 ΔSNX5 (p value determined by student’s T-test). For MAYV reduced viral replication was also observed in HEK293 ΔRBM3, however the effect was modest with a ~5-fold reduction for both SINV and MAYV. CHIKV was more reduced in virus production than MAYV from HEK293 ΔSNX5 and HEK293 ΔRBM3 showing a relative inhibition of 40 and 36 fold, respectively. Of the two proteins identified, the data demonstrates that the deletion of SNX5 is much more deleterious to alphavirus replication than RBM3, thus RBM functions are not further discussed.

**Discussion and Conclusions**

The lack of a significant advancement in the development of effective antivirals, vaccines, and investigative tools for the study of pathogenic viruses remains a major gap in the ability to protect susceptible populations from diseases caused by emerging and re-emerging pathogens. Basic understanding of virus-host biology remains central to the development of new approaches, therefore; novel techniques must be harnessed to increase this basic scientific knowledge. Here, LC-MS/MS was utilized to determine if the prototypical alphavirus, Sindbis virus, specifically packaged host proteins into its virus particles. A two-stage purification process for SINV virions, followed by FASP digestion procedure and mass spectrometry analysis, revealed that a variety of host proteins were incorporated into SINV particles from each host.
background. Central to this approach was ensuring that the viral preparations were free of virion-associated impurities and cellular debris. Transmission electron microscopy and a silver-stained SDS-PAGE gel revealed that our approach yielded high purity viral preparations that were not contaminated with other cellular debris, membrane fragments, or any significant protein contamination (Fig. 1B&C). The observation that 6K was not found associated with the SINV particles is expected since 6K is not virion-associated in SVHR (38). The *Aedes albopictus* mosquito proteome is not well annotated so processing of mass spectrometry data from the C7-10 background required our group to generate a manually curated mosquito proteome. Despite this limitation, the functional role of these proteins being associated with SINV replication will aid in experimental validation of their proteomic annotation. In the mammalian backgrounds, the identification of a core group of coinciding host proteins creates strong leads for further antiviral potential (Fig. 3). These proteins should serve as the first set of host proteins interrogated for their functional relevance during the SINV replication cycle and will be discussed first.

In this study, sorting nexin 5 (SNX5) was found to be associated with virus particles when SINV was grown in two mammalian cell lines HEK293 and BHK21. This host protein has cellular functions which are germane to the replication pathway of SINV because virus replication and maturation is integrally associated with the endo and exocytic host pathways. The sorting nexins (SNX) are a family of cytoplasmic proteins associated with a larger protein complex known as the retromer complex (39). This complex plays a vital role in endosomal protein sorting and this machinery is conserved across all eukaryotes (40). This complex is involved in endosome to Golgi trafficking and also in endosome to plasma membrane sorting and signaling. These membrane movement events are required to recycle receptors and other plasma membrane components during normal cellular processes. Members of this family contain
a phox (PX) domain (41), which is a phosphoinositide binding domain (PIP) (42). Specific SNX5 functions include endosomal sorting via the phosphoinositide-signaling pathway and micropinocytosis (43). However, while SNX5 was found only in two of the three mammalian cell lines tested, SNX5 is known to be expressed in HepG2 cells, the third mammalian line (44). Although no SNX5 was found associated with virions produced from HepG2 cells, this may be a result of the level of expression of SNX5 by the HepG2 cells as well as the dynamic range of the LC-MS/MS analysis. This may also explain why SNX5 was not found associated to virions from C7-10 cells because SNX orthologs are also expressed in the Class Insecta (45). An alternative explanation for the lack of detection of SNX5 from insect cells is that alphaviruses mature via secretion from internal vesicles and not through the budding at the plasma membrane.

To investigate the requirement of this protein by SINV during replication, stable HEK293 SNX5 knockout cell lines were constructed. By replicating SINV in these cell lines we were able to show that SNX5 is essential for the growth of Sindbis, Chikungunya, and Mayaro viruses. In the absence of SNX5, replication of virus was found to be significantly inhibited by infectious virus titration by plaque assay of progeny virus. SNX5 has been shown to be critical in the replication of other intracellular pathogens as well, which is relevant to this analysis. Currently, a scenario is beginning to emerge in which the retromer complex may be involved in the restriction of pathogens to the host cell by its’ functional exploitation or repression (46). The best documented pathogen is Chlamydia, a common human bacterial disease. Recently, a pair of studies have elucidated a role for SNX5 as a restriction factor for pathogenesis. The intracellular bacteria Chlamydia require membrane structures to replicate and produce infectious progeny within host cells. Both studies determined that SNX5 can negatively impact the membrane.
rearrangements required by Chlamydia to replicate efficiently (47-49). To counter this restrictive effect, Chlamydia sequesters SNX5 in replicative bodies and prevents its action on membrane rearrangements, allowing replication. It is known that membrane spherules are involved in plus strand RNA replication of alphaviruses (50, 51). It has also been shown that membrane rearrangements and composition are required for assembly of the alphaviruses during virus maturation or budding (52). Additionally, SNX5 was recently been found to co-localize with Ebolavirus during the entry phase in an internalization process via micropinocytosis (53).

Collectively these observations raise the question of whether membrane reorganization occurs during alphavirus penetration of the host membrane or budding at the plasma membrane by an SNX mediated process. Although we have shown that SNX5 is essential for alphavirus replication, the exact effect on replication or the mechanism of inhibition is unknown. Other viruses, such as human papilloma virus (HPV) and human immunodeficiency virus (HIV) are known to commandeer the retromer complex for efficient infection via specific mechanisms involving virus entry into the cell (6, 11). Evidence also implicates the retromer complex in a role during hepatitis C virus (HCV) infection as a method to recruit host proteins to the replication complex on a phophoinositol-4-phosphate rich membrane (54, 55). It is possible that SNX5 might be hijacked by SINV proteins directly or indirectly during particle penetration, early steps of the replication cycle or conversely during particle assembly to induce events necessary for infectious particles to be formed efficiently. Research to uncover the exact role of SNX5 during SINV replication is warranted and could further uncover potential host candidates for anti-viral therapeutics.

While SNX5 has been implicated in the replication of infectious alphaviruses, the other proteins identified which were found in common (Fig. 2) in the viral proteome by the reported
method have less-clear potential roles in SINV replication. We did not detect phospholipid scramblase in two (both mammalian) of the four cell lines investigated and was the only host protein which vertebrate and invertebrate derived SINV particles displayed in common.

For the co-identified proteins to provide meaningful information on the SINV replication pathway a putative functional role for the virion associated proteins must first be identified. Some hypothetical functions of these proteins during SINV replication are suggested below and are amenable to testing. Phospholipid scramblase functions to passively reorganize phosphatidyl inositols (PIPs) interchangeably from the outer and inner leaflet of the cell membranes. During lytic death induced by infection of vertebrates by alphaviruses, membranes are key targets for destruction upon the initiation of cell death by apoptosis and necrosis (56, 57). It has become increasingly documented that PIPs are involved in these processes (58). Thus, PIPs may accumulate during the latter part of the infection as cell lysis approaches. The mosquito C7-10 cells line is the invertebrate cell line which expresses the most SINV induced lysis of the Aedes derived linages (59). Because this process is passive, there is no clear signaling network between scramblase and SNX5. Additionally, phospholipid scramblase 1 has been found to interact with influenza A suppressing nuclear import and thereby inhibiting virus replication (60).

It is of special interest that junctional adhesion molecule C (JAMC), claudin (CLDN), and myosin are all components of the epithelial cells tight junctions (TJ) that provide a physical barrier between cells (61). These cell structures have been shown to be important for hepatitis C (HEP C) viral entry, replication, dissemination, and exit from the cell (54, 61). Desmoplakin is a component of the desmosome, a cell structure that is also involved in cell to cell adhesion. How this protein relates to SINV replication is also not clear. Cellular nucleic acid binding protein 6 (CNBP6) and RNA binding protein 3 (RBM3) both have the ability to bind single stranded RNA,
the species of RNA encoding the SINV genome (62). In fact, RBM3 has already been shown to be associated with the SINV particle (63). Possible functions for these host proteins are to participate in virus translation and/or also participate as components of the RNA replication complex (64, 65). These RNA-binding proteins could protect viral RNA from host cell antiviral sensors while also promoting translation of viral polyproteins upon progeny virus entry into the host cell cytoplasm (65, 66). The heat-shock proteins HSP70/71 are important throughout viral replication cycles, although it remains unclear if these molecules play a role by incorporation into alphavirus and SINV virions (67, 68). HSP70 is known to be involved in the assembly of other RNA-containing viruses such as cucumber necrosis virus (69), and hepatitis C virus (55) and are physically associated with lentivirus particles (70). Another protein chaperone, BIP (HSPA5), belonging to the HSP70 superfamily has been shown to be required for the assembly of SINV (71, 72). The identification of multiple host factors within SINV particles using this method opens avenues for continued exploration into how these sequestered proteins influence the production of infectious virions or some part of the replication cycle. To definitively identify a role of these proteins further analysis is required.

Of major significance is the identification of a SINV non-structural protein, nsP2 associated with the virus particles, an unpredicted and fascinating finding. Alphavirus non-structural proteins are important for virus replication, but have not been found encapsidated into the virus particle. In this study, LC-MS/MS data lead to the discovery that nsP2 is incorporated into SINV virus particles when grown in each of the different host cells tested. It was considered that nsP2 could be co-purifying with SINV as a non-specific contaminant but its’ association with the virion was confirmed by performing additional high salt washes of the virus preparations to eliminate non-specific binding. To determine whether the nsP2 protein was
associated in the outer glycoprotein shell or was held in the nucleocapsid core, the inner and outer shells of purified virus were prepared as described above, dissociated from one another with Triton X-100 and the fractions were run on a 4-12% Bis-Tris SDS PAGE gradient gel. To definitively identify the proteins, western blots were performed on the nucleocapsid enriched fraction using anti-whole SINV Ab and anti-capsid Ab. Capsid proteins and E2 were identified in the NC fraction. nsP2 was found incorporated into whole purified virus although a specific association with the outer shell or inner core could not be established.

This finding is supported by evidence that nsP2 plays a direct or indirect role in RNA encapsidation in Venezuelan Equine Encephalitis virus (VEEV) another alphavirus (73). This discovery is significant because nsP2 is a multifunctional enzyme previously thought only to function in RNA replication as the viral protease and triphosphatase (NTPase) (74). As the viral protease, its’ functions range from cis and trans cleavage of viral non-structural polyproteins to innate immune evasion early in viral replication (75-80). While all proposed enzymatic roles for nsP2 have not been verified nor all the identified roles fully understood, one role of this protein is as a transcription factor i.e. a structural role. The presence of nsP2 in the SINV suggests that this protein may have an additional structural role within the SINV virion or may be required upon virus entry because it was identified in virions produced in all host backgrounds. The incorporation of an enzyme in a structural role is not novel among viruses. Thymidylate synthase and dihydrofolate reductase are DNA synthesis and replication enzymes encoded by the phage T4. However, they are also essential structural components of the phage baseplate and thus essential elements for phage infectivity (81). Conversely, by incorporating nsP2 into progeny virions, SINV may use its enzymatic and immune antagonist roles to facilitate the infection at the earliest stages of viral entry, before translation of the plus-stranded viral RNA occurs. It may be
involved in recruitment of capsid protein to the RNA nucleation site thereby participating in NC assembly. Alternatively nsP2 associated with the genomic RNA could establish the translation complex which initiates production of the non-structural proteins and then switches to replicate viral RNA. Any or all of these functions are possible and will be investigated. It is feasible that prophylactic targeting of viral nsP2, which has no other known substrates (17), would serve to disrupt the infection. Functional and structural roles for nsP2 both in cis and trans imply that the protein can assume multiple conformations and may not be amenable to rational design of functional inhibitors for non-enzymatic structures. However, pharmacological targeting of nsP2 is an excellent candidate for an anti-viral therapeutic and would fulfill the first tenant of a good therapeutic, virus specificity.

Overall, this study has produced significant results that contribute to the understanding of alphavirus-host biology while identifying SNX5 as a potential target for antiviral therapy. LC-MS/MS analysis of highly pure viral particles determined that SINV specifically incorporates host proteins into progeny virus particles. These proteins have a variety of functions that can be investigated for a role in SINV replication, with a core group of proteins conserved in multiple host backgrounds serving as a logical starting point. Finally, the novel discovery of SINV nsP2 incorporation into virus particles suggests a specific interaction and a possible role for nsP2, during replication or assembly. By interrogating SINV particles for members of the host background proteome, a better understanding of the virus-host interactions necessary for alphavirus entry, replication, protein processing, and particle assembly prior to budding can be established.

Material and Methods
Cells, Virus Growth, Preparation, and Purification

The heat resistant SINV (SVHR) strain used in this study was provided to Dr. Dennis Brown (NC State University). This strain was isolated by Burge and Pfefferkorn in 1966 by collecting virus that was resistant to heating to 54°C (82). The choice of strain is important because this strain produces high titers ($10^{10}$ pfu/mL) of highly infectious and physically stable virus.

Chikungunya virus West African strain 37997 was grown and harvested as described in (83).

Mayaro virus strain CH was utilized for the study and was kindly provided by Dr. Scott Weaver at UTMB. Propagation of HepG2 cells (human), and C7-10 cells (mosquito) was as described previously (84). Influenza A/PR/8/34 and the MDCK cells used to propagate and titer the virus were a kind gift of S. Laster and propagated as detailed in (85). All cells were obtained from American Tissue Culture Collection (Vero: CCL-81, HEK293: #CRL-1573, HepG2: #HB-8065, BHK21: #C-13) except for the C7-10 (*Aedes albopictus*) cells which were obtained from internal collections (86). Culture medium from each uninfected cell culture was harvested and served as a negative control. Virus and negative controls were harvested from 10 T-75 flasks (Corning) which produces enough virus to form a visible band in a 30 mL potassium tartrate gradient. Cells were infected at an MOI of 10 PFU/mL, allowed to replicate for a single cycle and harvested at 18 hours post infection to ensure that no cell lysis took place. The supernatants were clarified by low speed centrifugation. Twenty mL of the resulting virus was loaded onto a 15-35% linear potassium tartrate gradient and twice purified by isopycnic ultracentrifugation (Beckman SW-28 rotor, 18 hours at 24,000 RPM). The resulting band of purified virus was collected and washed twice by pelleting the virus in 1X PBS. A sample of the purified population was then visualized by transmission electron microscopy to check for contaminating cellular organelles or membrane fragments as previously described (87). Additionally, to check for co-purified protein
contaminants, each preparation was resolved on a 4-12% Bis-Tris SDS-PAGE gradient gel (Invitrogen) as described previously (88) and stained with silver in the method of Wray (89) or with coomassie blue. Visualized bands (silver stain only) were excised and an in-gel digestion was performed as described previously (90) prior to LC-MS/MS analysis for protein identification.

**Sindbis virus particle analysis for nsP2**

Gradient purified SINV was washed with 1M KCl to remove non-specifically bound proteins. Purified SINV particles were then separated into glycoprotein and nucleocapsid fractions by treatment with 4% or 5% Triton X-100 for 1 hour at 37°C and pelleted for 45 min through PBS at 45,000 RPM in a Beckman XL 90 centrifuge (91). The supernatant (glycoprotein fraction) was saved and the resulting pellet (nucleocapsid fraction) was washed twice by pelleting through PBS in the same manner. The nucleocapsid fraction was then subjected to LC-MS/MS analysis, SDS-PAGE, or fluorography. Location of the nsP2 protein within the outer or inner shells of the particle was done using staining of 4-12% Bis-Tris gradient gels and western blot of the glycoprotein and NC fractions of the particle. Virus particles (~5µg per lane) were prepared in SDS-PAGE sample buffer supplemented with 5% beta-mercaptoethanol and boiled for approximately 10 minutes. The samples were run on an SDS-PAGE Bis-Tris 12% gradient gel and transferred to a PVDF membrane. The membrane was blocked with 5% Carnation nonfat dry milk in PBS for 1 hour at ambient temperature. The membrane was then probed for one hour with a rabbit antibody raised against nsP2 (a gift from R. Hardy) or anti C protein (a gift from S. Mukhopadhyay) diluted in PBS supplemented with 5% dry milk and 0.1% TWEEN 20 at ambient temperature. The blot was washed 3 times for 5 minutes each with PBS supplemented with 0.1% TWEEN 20 at ambient temperature. Finally, the membrane was probed with an anti-
rabbit secondary antibody conjugated with IRDye® 800CW near infrared dye diluted in PBS supplemented with 5% dry milk and 0.1% TWEEN 20, and 0.02% SDS at ambient temperature. The blot was subsequently washed 3 times for 5 minutes each with PBS supplemented with 0.1% TWEEN 20 at ambient temperature, washed once with water, dried, and imaged according to LI-COR Odyssey CLx instructions.

Protein Extraction and Digestion

Viral preparations and their respective negative controls were processed for LC-MS/MS analysis using the filter-aided sample preparation (FASP) method as previously described (92). Briefly, following purification the total protein concentration was determined and all preparations were normalized using sterile PBS to 0.5 µg/µL, aliquoted in 10µg fractions, and stored at -80°C. A total of 10µg of total protein (20µL) was mixed 1:1 with MPER (Thermo Fisher) supplemented with 50 mM dithiothreitol (DTT) and heated to 95°C for 10 minutes. Once cooled to room temperature, the samples were mixed with 200µL of UA (8M urea, 100mM Tris-HCl pH 8.5), placed over a 30kDa filter spin column (Millipore Ultracell YM-30), and centrifuged at 14,000G for 30 minutes at room temperature to collect all proteins on the filter membrane.

Denatured and reduced proteins were then alkylated by adding 100µL of IAA solution (0.05M iodoacetamide in UA) to each filter and incubating at room temperature in the dark for 20 minutes. Following alkylation, the samples were centrifuged at 14,000G for 20 minutes to remove the alkylation solution. Next, each sample was washed three times with 100µL of UA and then three times with 100µL of 100mM triethylammonium bicarbonate (TEAB).

Centrifugation at 14,000G for 30 minutes was used to remove each wash including the final wash. To digest the captured protein, 100µl of a trypsin digestion solution (10 µg/mL in 100mM TEAB) was placed on each membrane and incubated in a sealed tube overnight at 37°C with...
shaking. After incubation, the peptides were collected for LC-MS/MS analysis in a clean tube by centrifuging each tube for 30 minutes at 14,000G. The membrane was washed by centrifugation one time with 50µL 100mM TEAB and once with 50µL of 0.5M NaCl. All washes were collected and pooled with the final peptide eluate. Each sample was then acidified using 10% trifluoroacetic acid until the final pH was roughly 2-3. Prior to mass spectrometric analysis, each sample was desalted using C18 desalting columns (Thermo Fisher) according to the manufacturer’s directions.

**Liquid Chromatography Mass Spectrometry Analysis**

Tryptic peptides were analyzed (technical triplicates) on an Orbitrap ELITE mass spectrometer coupled with the Easy-nLC II liquid chromatography pump system. Dried peptides were reconstituted in 3% acetonitrile/0.1% formic acid and resolved on virgin Picofrit 15cm X 75µm ID HPLC column packed with 5µm BioBasic C18 particles 300Å (New Objective) using a 130 minute multistep gradient [0-5 min: 5-10%B, 6-110 min: 10-35%B, and 111-130 min: 35-95%B]. For the gradient, the A buffer is 3% acetonitrile/0.1% formic acid and the B buffer is 95% acetonitrile/0.1% formic acid. Orbitrap MS1 scans were performed at a resolution of 120,000 at 400 m/z, with a scan range of 110-2000 m/z. The top 20 precursors were selected for MS2 data-dependent fragmentation. An MS2 spectrum was acquired using the iontrap scanning in normal mode (Top 20 Method). The minimum signal required to trigger a data-dependent scan was 5000. Collision induced dissociation (CID) was used to generate MS2 spectra with the following settings: normalized collision energy 35%, default charge state 2, isolation width 2 m/z, and activation time 10 ms. AGC target was set to 1 x 10⁶ for MS and 5 x 10⁴ for MS/MS with a maximum accumulation time of 200 ms. Dynamic exclusion was set for 60 seconds for up
to 500 targets with a 5 ppm mass window. A lock mass of 445.120025 was used for internal calibration to improve mass accuracy.

**Mass Spectrometry Data Processing**

Spectra data were processed using Proteome Discoverer 1.4 with the SEQUEST search algorithm against a Sindbis virus polyprotein database (Uniprot ID: P03317) merged with either *Homo sapiens* (RefSeq Tax ID: 9606) or *Cricetulus griseus* (RefSeq Tax ID: 10029). The *Aedes albopictus*-deducted proteome (35) data was downloaded from VectorBase (93). Additional peptides were downloaded from the NCBI TSA database. The non-redundant proteomes were organized on excel spreadsheets (94) and annotated as described previously (95). The resulting FASTA file was merged with the SINV polyprotein and used to search against the SINV mosquito preparations. Dynamic modifications were set for carbamidomethylation of cysteine [+57.02 Da], oxidation of methionine [+15.99 Da], and N-terminal acetylation [+42.011]. MS/MS spectra were searched with a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.6 Da. Trypsin was specified as the protease with a maximum number of missed cleavages set to 2. A false discovery rate was calculated using PERCOLATOR and was set at <1% to score high confidence peptide identifications. Grouping and functional analysis was performed using the PANTHER classification system for the human background only using each protein id’s accession number (96).

**Phenotypic Studies with Stable Knockout HEK293 Cells**

HEK293 knockout (KO) cell lines were purchased from Applies Biological Materials Inc. (Richmond, BC, Canada). SNX5 and RBM3 KO was confirmed by Sanger sequencing. Cell lines were propagated at 37°C with 5% CO2 in Dulbecco’s Minimal Essential Medium (DMEM).
containing 10% (v/v) fetal bovine serum (FBS), sodium pyruvate (1 mM), 1% (v/v) non-essential amino acids, and 50 μg/mL gentamicin. Briefly, HEK293 monolayers were infected at equal MOIs (approximately 10 PFU/cell) of SVHR, CHIKV or MAYV and allowed to incubate for 20 hours. Supernatants were titrated on BHK cells by the standard plaque assay to determine the virus titer. As a measure of the knockout cells’ ability to replicate an unrelated enveloped virus, Influenza A/PRF/8/34 virus was used to infect the wild type and ∆SNX5 HEK293 cells. Infectious virus titer was determined by plaque assay. Both ∆SNX5 HEK293 cells and wild-type cells produced an equivalent amount of infectious virus after 24 hrs.

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Figure Legends

**Fig. 1.** Sindbis virus (SINV) virion purification strategy and production flowchart.

(A) Clarified viral preparations were purified over two potassium tartrate density gradients done in series using isopycnic ultracentrifugation and a final wash and pellet step. Negative controls (uninfected cell culture supernatant) were processed in parallel with the virus preparations. (B) Transmission electron micrographs were taken of the purified virions to confirm efficiency of this enrichment procedure. (C) An SDS-PAGE gel was stained with silver or with coomassie blue for each of the viral preparations. HepG2 preparations are presented here, and are representative of all viral preparations from all host cellular backgrounds.

**Fig. 2.** Comparison of the Mammalian Viral Host Proteomes

**Fig. 3.** Mosquito Phospholipid Scramblase Detection.
(A) The NCBI BLAST alignment tool was used to align the protein sequence of phospholipid scramblase for *Aedes albopictus* and *Homo sapiens*. The “Query” is the *Aedes albopictus* sequence (Accession#: 604777565) and the “Sbjct” is the *Homo sapiens* sequence (Accession#: 10863877). Peptide sequences presented below the alignment are highlighted, red (mosquito) and yellow (human). (B) Is the MS2 spectra used to identify phospholipid scramblase in HEK293 and C7-10 cells.

**Fig. 4. Characterization of SINV nucleocapsid (NC) and glycoprotein fractions.**

Silver stain of whole virus and NC fractions after increasing treatments of Triton X-100. M, protein marker size (kDa.), lane 1: stained whole SINV, lane 2: whole virus treated with 4% Triton X-100 [NC fraction], lane 3: whole virus treated with 5% Triton X-100 [NC fraction], lane 4: Triton X-100 fractions containing glycoproteins. Note that capsid, E1 and E2 glycoproteins run slower (denoted by *) after treatment with Triton.

**Fig. 5. Western Blot Analysis of NC fractions of purified SVHR.**

(A) Western blot of whole purified SINV bound to anti-nsP2 Ab using the LI-CORE image-capturing system. M is the same marker as in Fig. 4; the 260, 70, and 38 kDa. markers do not fluoresce at the 700 and 800 nm. NIR wavelength used. (B) Western blot of same fraction as in panel A. Probed first with anti-C Ab (the yellow band) and second, with anti-whole SINV Ab, green bands. The arrows point to a lower C band which is the only band that fluoresces with the anti-C Ab and the upper arrow which indicates the upper green C band which only fluoresces with the anti-whole virus Ab. Thus, two capsid bands are resolved in this fraction. E2 is above C in green. *Note that E1 does not label with this Ab, while the capsid protein does.*
Fig. 6. Sindbis virus (SINV), Mayaro virus and CHIKV production from HEK293 cells.

Cells lacking RBM3 or SNX5, or wild type cells were infected with wild type virus and infectious virus titers were determined by plaque assay. All three viruses assayed, SINV, MAYV and CHIKV replicated to high titers in wild-type HEK293. By contrast, the titers of SINV were significantly reduced >100 fold (p=0.03), ~10 fold for MAYV (p=0.003) and 40 fold for CHIKV (p=0.01) in the HEK293 ΔSNX5 with p values as determined by student’s t-test. For MAYV reduced viral replication was also observed in HEK293 ΔRBM3, however the effect was modest with a ~5-fold reduction for both SINV and MAYV. CHIKV was more reduced in virus production than MAYV from HEK293 ΔSNX5 and HEK293 ΔRBM3 showing a relative inhibition of 40 and 36 fold, respectively.
Fig. 1

Highly purified viral preparation confirmed by transmission EM.

Repeat this procedure one additional time with only the viral band.

A

B

C

KDa
96
62
49
38
28
17
14

Coomassie Stain

Silver Stain

E1/E2

Capsid

Downloaded from http://jvi.asm.org on July 22, 2018 by guest
Fig. 2
Fig. 3

A

B

Phospholipid Scramblase

FTIQNEK

DQSGNTVLR

Human

[HEK293]

Mosquito

[C7-30]
Fig. 4
Fig. 5

- A: nsP2
- B: α - E2/C whole virus, α-capsid protein

[Image of gel electrophoresis with bands labeled nsP2, α - E2/C whole virus, and α-capsid protein]
Fig. 6

Virus titer pfu/mL

Cell lines

HEK 293 Wt HEK 293 ΔSNX5 HEK 293 ΔRBM3

SINV MAYV CHIKV

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