A PROTEOMICS ANALYSIS OF IN VITRO HOST-PROTEIN BINDING PARTNERS OF WESTERN EQUINE ENCEPHALITIS VIRUS (WEEV) CAPSID PROTEIN

by

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A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

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Ampicillin resistance gene ..... Amp-R
Antibiotic Free ..... AB-free
Bacteriophage RNA polymerase promoter ..... T7 promoter
Cytomegalovirus ..... CMV
Dulbecco's Essential Modified Medium ..... DMEM
Eastern equine encephalitis virus ..... EEEV
Green fluorescent protein ..... GFP
Heterogenous Ribonucleoproteins ..... HnRNP
Immunoprecipitation ..... IP
Immunoprecipitation/Mass spectrometry ..... IP/MS
Mass spectrometry ..... MS
Nuclear export signal ..... NES
Nuclear localization signal ..... NLS
Nuclear pore complex ..... NPC
Nucleoporin ..... NUP
Peptide spectral match ..... PSM
Polyvinylidene difluoride ..... PVDF
Sindbis virus ..... SINV
Venezuelan equine encephalitis virus ..... VEEV
Western blot ..... WB
Western equine encephalitis virus ..... WEEV

ABSTRACT

A PROTEOMICS ANALYSIS OF IN VITRO HOST-PROTEIN BINDING PARTNERS OF WESTERN EQUINE ENCEPHALITIS VIRUS (WEEV) CAPSID PROTEIN

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Western equine encephalitis virus (WEEV) is a New World (NW) encephalitic alphavirus. WEEV has been documented in North and South America with most human cases occurring in the Western U.S. and epizootic episodes occurring in S. America. WEEV is transmitted to humans via mosquito and clinical manifestations may include mild flu-like symptoms or more extreme manifestations including encephalitis, seizures or neurological complications that contribute to 3-5% fatality rates. NW alphaviruses including WEEV have 11 kB, positive-sense single-stranded RNA genomes that encode non-structural proteins and produce structural proteins from a subgenomic promoter. A critical structural protein, capsid, is the first protein transcribed from the subgenomic promoter and is composed of 240 repeating capsid monomers that contribute to the structure of the viral nucleocapsid. Other NW alphavirus capsid proteins such as VEEV capsid, have been previously elucidated as critical modulators in inhibition of host-cell mRNA transcription. In addition, VEEV capsid interacts with host-cell importin-alpha and -beta along with exportin-1 (CRM1) to traverse and block the nuclear pore, which

contributes to its transcriptional inhibition activity. Specific information regarding pathogenesis and the role of WEEV capsid has not been identified. The current study investigates the specific role of WEEV capsid in pathogenesis through proteomic-interaction studies. A WEEV capsid expression construct was designed, transfected and expressed in Vero and BSR-T7 cells. Following validation of protein expression in both cell types, WEEV capsid was immunoprecipitated with V5 antibody and utilized for mass spectrometry (MS) analysis of protein interactions. Several proteins were identified as potential interactors by MS including those involved in cellular roles involving nuclear import and export, composition of the nuclear pore complex, mRNA splicing, and proteins involved in cellular RNA metabolism. Exportin-1 (CRM1) and importin-alpha, host nuclear export and import proteins were identified and validated by IP/WB as interacting with WEEV capsid. Future studies will include validation of other MS-identified protein interactors and observation of knockdown of CRM1 and importin-alpha and subsequent effect on capsid cellular localization. Studies involving CRM1 and importin-alpha could lead to further insight regarding the pathogenesis of WEEV and provide foundation for host-based therapeutics that target capsid-host protein interactions.

INTRODUCTION

Alphaviruses

Alphaviruses belong to the family *Togaviridae* and are characterized as zoonotic pathogens that are transmitted by mosquito (14). Alphaviruses remain to be some of the most clinically relevant zoonotic disease-causing viruses due to categorization as potential weapons of bioterrorism or re-emergence (18). There are 29 members of the alphavirus genus and members cause infection in humans, livestock, domesticated animals, wild mammals and fish (14, 18). Alphaviruses are widely distributed and have been documented on all seven continents.

The alphavirus genus consists of arboviruses that are divided into New World (NW) and Old World (OW) viral groups according to the geographic region in which these viruses originated. OW alphaviruses are characteristically arthralgic and consist of viruses originating in Asia, Africa, Europe and Australia. Some representative OW alphavirus members include chikungunya virus (CHIKV), Ross River virus (RRV), Sindbis virus (SINV) and Semliki Forest virus (SFV). NW alphaviruses are encephalitic and endemic to North, Central and South America. NW alphaviruses include Venezuelan (VEEV), eastern (EEEV) and western equine encephalitis viruses (WEEV) (5,7).

The phylogeny of the alphavirus genus has been characterized through observations of genomic conservations in genes for viral structural proteins E1, 6K and

E2 (17,18). The current phylogeny is defined by genomic and antigenic characteristics that define the alphavirus complex. The seven distinct complexes within the genus include EEE, VEE, WEE, Semliki Forest, Barmah Forest, and Middelburg complexes. As more antigenic and genomic information is obtained, additional sub-complexes have been allocated to include both Trocara and Ndumu complexes.

The WEE alphavirus complex consists of viruses such as WEEV, Sindbis (SINV) Buggy Creek virus (BCV), Fort Morgan virus (FMV), Highlands J virus (HJV), Aura Virus (AURAV) and Whataroa virus (WHAV) (19, 21). The WEE complex is considered to be monophyletic when defined by glycoprotein characteristics but consists of members that are derived from viral recombination events. WEE, HJV and FMV are members of the WEE complex that are derived from recombination events between SINV and EEEV, a member of the EEE alphavirus complex. WEEV maintains antigenic similarity in glycoproteins to SINV but shares genomic characteristics to that of EEEV (7, 19, 21).

Western Equine Encephalitis Virus (WEEV)

WEEV is a member of the NW alphavirus lineage and first encephalitic equine virus noted in North America. WEEV was first documented in the brain of an equine located in the San Joaquin Valley of California in 1930 (14, 15). The first human infection of WEEV that resulted in fatality was documented in 1938. Following initial documentation, WEEV disseminated to western parts of North America. WEEV occurs in the western halves of both North and South America. Occurrences in S. America are noted in Guyana, Ecuador, Brazil, Uruguay and Argentina. Most outbreaks of WEEV in

S. America have been restricted to smaller equine related outbreaks and human cases have not occurred.

Transmission

WEEV circulates in a primary enzootic cycle between birds and mosquito vectors such as *Culex tarsalis*. *C. tarsalis* are abundant mosquito species located in agricultural settings and act as amplifying hosts for epizootic episodes (14, 15). There is also an assumed secondary cycle that primarily contributes to epizootic episodes in which WEEV cycles between small rodents or mammals and *Aedes* mosquitos and is amplified within this cycle (14, 15). Transmission of WEEV to humans and horses is completed by bridge mosquito vectors such as *Ochlerotatus melanimon* in California, *Aedes dorsalis* in Utah and New Mexico, and *Ae. Campestris* in New Mexico. The annual WEEV transmission cycle has not been fully characterized in temperate regions and climate or migratory birds are suspected to contribute to the cycle of transmission (14, 15).

In addition to mosquito transmission, NW alphaviruses such as WEEV may be transmitted through laboratory-related, accidental aerosol exposures and result in fatality as high as 40% in humans exposed by this route of transmission. Subsequently, VEEV, EEEV and WEEV are categorized as Category B agents by the National Institute for Allergy and Infectious Diseases (NIAID) (2).

Clinical Manifestations

There were 639 confirmed human cases of WEEV in the U.S. ranging from 1964 to 2005. WEEV infection generally presents as asymptomatic but mild symptoms may manifest following a 2-7-day incubation period. Some mild symptoms may include

nausea, vomiting, malaise, fever or headache (2, 11, 14). In severe cases, usually in infants or children, symptoms may present as encephalomyelitis and contribute to neck stiffness, confusion, tonic-clinic seizures, somnolence, coma and death (4, 8, 11). The fatality rate in humans infected with WEEV ranges for 3 -7% (11, 14). The case fatality rate in infected horses is much higher and ranges from 20 - 30% but can be as high as 50% during equine epidemics (11, 14).

Widely distributed vaccines for WEEV have not been developed. Formalin-inactivated vaccines have been developed for laboratory workers at risk or for horses (11, 14). Inactivated horse vaccines are administered as a double vaccine in combination with EEEV and used in veterinary medicine for horses. The combination horse vaccine is administered twice a year due to the low immunogenicity (11, 14).

Genome and Replication Cycle

Of the NW alphaviruses WEEV is a recombinant virus that consists of SINV-like glycoproteins and EEEV-like genome (7). WEEV is subsequently subcategorized as a recombinant virus originating from EEEV (7). Alphaviruses have + ssRNA genomes that rely on replication activities occurring in the host-cell cytoplasm (5, 7). The positive sense genome is approximately 11 kB in length and contains mRNA-like features such as at 5’ cap and 3’ poly-A tail (5, 10). The first open reading frame is directly translated into proteins and the second reading frame requires production of minus strand RNA intermediate prior to translation (5, 10). At the 5’ end, the first reading frame encodes several non-structural proteins, nsP1, nsP2, nsP3 and nsP4 that are translated as the polyprotein P1234. These proteins are essential for replication and transcription of the

viral RNA (5, 10). Within the second reading frame resides a 26S subgenomic promoter for production of the minus strand RNA (5, 10). The second reading frame encodes structural proteins such as E1 and E2 surface glycoproteins and capsid protein (5, 10).

What is known of NW alphavirus viral replication is primarily derived from studies involving VEEV. Viral replication begins with binding of E1 and E2 surface glycoproteins to receptors on the host-cell surface. Following binding, the viral particle enters through clathrin-mediated endocytosis and enters the early endosome (5, 10). Following pH reduction, the viral particle is released into the cytoplasm through fusion of endosomal and viral membranes (5, 10). Following fusion, the viral RNA is released into the cytoplasm of the cell where the first reading frame is translated and the nsP1- 4 replication complex forms (5, 10). Following formation of the complex, these proteins aid in the production of a minus strand RNA, which is used for production of positive sense viral RNA. The minus strand RNA also contains the subgenomic promoter which is used for transcription of the 26S subgenomic mRNA, which encodes for capsid, E2, and E1 glycoproteins (5, 10). Following minus strand synthesis for use in production of structural proteins and virion generation, the capsid protein is directly translated, and the other structural proteins are translated at the ER membrane (5, 10). Glycoproteins are transported to the plasma membrane and capsid binds viral positive sense RNA in the formation of the nucleocapsid (5, 10). Capsid interacts with glycoproteins which aid in budding of progeny virion from the plasma membrane. In the case of VEEV, circulating cellular capsid protein will also associate with the host-cell nuclear pore to inhibit nuclear trafficking (5, 10).

NW Alphavirus Capsid Proteins

The capsid structural protein of alphaviruses has been elucidated to play critical roles in pathogenicity of the virus. The capsid structural and functional role has been defined by early work specifically involving VEEV and EEEV capsid. VEEV capsid is a 275 amino acid protein that consists of N and C terminal domains that provide unique and critical roles to the protein (5, 21). The C-terminal domain of capsid is highly conserved across alphaviruses and contributes to capsid's protease activity and autocleavage from the structural polyprotein. The C terminal domain also has glycoprotein binding activities that aid in the formation of the mature virion during assembly (5).

The N terminal domain of capsid is varied and not as conserved across alphaviruses. The N terminal domain of capsid is associated with viral roles of nucleocapsid assembly and RNA binding of the viral genome. This domain contributes to the assembly of the mature virion through what is assumed to be charge neutralization interactions with the viral RNA (5, 21). Additionally, the N terminal domain of capsid contributes to host-transcriptional shut-off as demonstrated in early studies with EEEV, which revealed mRNA accumulation and enhanced phosphorylation of eukaryotic initiation factor 2 alpha (eiF2α). Additionally, early studies with VEEV revealed a critical capsid amino acid sequence within the N terminal domain that aids in transcriptional inhibition (4, 5, 21). The capsid N terminal domain also contains nuclear localization signals (NLS) and nuclear export signals (NES). The NLS and NES of capsid contribute to the localization of capsid within host-cells and role in transcription inhibition (4, 5,

21). These NLS and NES signals are thought to contribute to the binding of VEEV capsid to nuclear import proteins, importin-alpha/beta and nuclear export protein, exportin-1 (CRM1) (4, 5, 21). It is suggested that the simultaneous binding of VEEV capsid to importins and exportins results in blockage of the nuclear pore subsequently inhibiting mRNA transcription of the host (4, 5, 21). The NLS and NES of NW alphaviruses is conserved amongst EEEV, VEEV and WEEV suggesting potential conserved proteomic interactions (5).

There has been minimal research into the replication activities and pathogenesis of WEEV. Previous studies involving WEEV, have elucidated the role of WEEV capsid in inhibiting pattern recognition receptor pathways in neuronal cells through inhibition of pathway components downstream of interferon regulatory factor 3 (IRF-3) (5, 23). The exact mechanism of this receptor pathway inhibition has not been defined (5, 23). Additionally, it is not known if WEEV capsid inhibits host-cellular processes such as transcription or translation or if potential inhibition mechanisms are like that of VEEV or EEEV.

Rationale

Current knowledge regarding NW alphavirus pathogenesis mostly pertains to VEEV. WEEV, a recombinant NW alphavirus, has not been extensively or specifically characterized with regard to replication and pathogenesis. Previous studies with VEEV have elucidated capsid proteins as providing essential roles for inhibiting host-cell transcription via blockage of the host nuclear pore. WEEV capsid protein shares sequence homology with other NW alphavirus members such as VEEV and EEEV capsid

and there is interest in characterizing the role of WEEV capsid and elucidating potential differences in pathogenesis. The current study addresses the design of NW alphavirus constructs for use in proteomics analysis with emphasis on understanding of WEEV capsid and host-protein interactions. Through immunoprecipitation and mass spectrometry (MS) studies, potential protein binding partners will be identified, and direct interaction will be confirmed by western blot (WB). Following confirmation of direct interaction, the effect of host-protein drug inhibitors on capsid localization will be assessed. Potential therapeutics for disrupting viral-host interactions will be addressed.

MATERIALS AND METHODS

Construct Design

The WEEV capsid DNA sequence was utilized for design of molecular construct for use in assessment of host-protein interaction studies. The construct was designed with capsid DNA sequence flanked by green fluorescent protein (eGFP) or viral V5 tag (V5) (Fig. 1A, 1B). The respective constructs were cloned into pcDNA 3.1. backbone at compatible restriction enzyme sites. The vector backbone contains both cytomegalovirus (CMV) and bacterial polymerase (T7) promoters. Additionally, the pcDNA 3.1. molecular backbone contains ampicillin resistance gene (Amp-R) which is utilized in transformation of One Shot™ TOP10 Chemically Competent *E.coli.* Following transformation, ampicillin containing LB agar plates were streaked for selection of transformed bacterial colonies. Representative colonies were selected, grown in ampicillin containing LB broth for colony expansion and QIAprep Spin Miniprep Kit (Qiagen) was used for plasmid DNA isolation. Following mini-prep, isolated plasmid DNA was run on 1.5% agarose gel for visualization of plasmid size. Following visualization, plasmid DNA bands corresponding to respective capsid constructs were extracted from agarose gel with QIAquick Gel Extraction Kit (Qiagen). Eluted DNA was then utilized for sequencing validation of construct identity.

Cell Culture

Vero cells (ATCC, CCL-81) were maintained in Dulbecco's modified minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine. Cells were maintained in cell culture flasks at 37˚C with 5% CO_2. Baby hamster kidney cells stably transfected with molecular constructs encoding T7 RNA Polymerase (BSR-T7) (Buchholz et al., 1999), were used for capsid transfection studies and were maintained in Minimum Essential Medium (MEM) containing 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine. BSR-T7 were cultured with 1:100 dilution of Geneticin to maintain stable selection of cells with T7 polymerase. Cells were maintained in cell culture flasks at 37˚C with 5% CO_2.

Transfection

Vero cells were plated in 6-well plates at a density of $2.0x10^5$ cells per well in antibiotic-free (AB-free) DMEM. If transfections were used for immunofluorescent confocal microcopy, cells were seeded onto neuVitro poly-D-lysine coated sterile coverslips in 6-well plates prior to transfection. Mirus TransIT® - LT1 Transfection Reagent was utilized in a ratio of 3 µL of transfection reagent per 1 µg of WEEVcapsid-V5, EEEVcapsid-V5 VEEVcapsid-V5 or pcDNA3.1.-eGFP construct DNA. Transfection protocol was followed as outlined in Mirus TransIT® - LT1 Transfection Reagent product use instructions. Varying molecular construct DNA concentrations of 1, 2, 3 and 4 µg were used to identify optimal protein expression conditions. Following preparation of transfection complexes in Opti-MEM™ reduced serum media, DNA-transfection complexes were applied dropwise to cells and transfections proceeded for 24 hours.

Western Blot

Following 24-hour transfection of Vero cells with WEEV or other capsid molecular constructs, media was removed, and cells were washed twice gently with PBS. Protein cell lysates were collected in blue lysis buffer and boiled for 10 minutes. Protein lysates were loaded and run on a NuPAGE 4-12% Bis-Tris polyacrylamide gel at 180V for approximately 45 minutes. Proteins were transferred to Polyvinylidene difluoride (PVDF) membrane, overnight at 80 mA. Membranes were then blocked with 5% bovine serum albumin (BSA) in 1x TBS with 0.1% Tween-20 (TBS-T) for one hour. Following blocking, membranes were then washed and incubated in blocking buffer containing 1:1000 diluted Bio Rad anti-V5 (MCA1360) primary antibody in 5% BSA and TBS-T overnight at 4˚C. Membranes were washed with TBS-T once for 5 minutes, followed by three, 5-minute washes with TBS. Membranes were incubated with a 1:1300 dilution of Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP (ThermoFisher, 32430). Membranes were washed and SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher, 34095) was applied to membranes and chemiluminescent imaging was conducted through use of Bio Rad Molecular Imager ChemiDoc XRS system (Bio-Rad). Following specific antibody probing, membranes were probed for loading control actin with 1:20,000 dilution of anti-beta actin HRP conjugated antibody (Abcam, ab49900).

Western blot was conducted for confirmation of protein binding partners of WEEVcapsid-V5 following immunoprecipitation experiments. Antibodies for exportin-1 (Bethyl Laboratories Inc., CRM1 A300-469A), importin subunit alpha-1 (Bethyl Laboratories Inc., RCH1/ KPNA2 A300-484A) and importin subunit beta-1 (Bethyl

Laboratories Inc., KPNB1 A300-482A) were all used at 1:1,000 dilution for membrane probing. Following incubation with primary antibodies, respective HRP conjugated secondary antibodies (ThermoFisher) were utilized for membrane probing.

Immunofluorescent microscopy

Vero cells were plated at a density of $1.5x10^5$ cells per well in 6-well cell culture plates containing glass coverslips. Cells were transfected for 24-hours with WEEVcapsid-V5, media was removed, and cells were washed twice with PBS (without Mg^{2+} or Ca^{2+}). Slides were fixed with 4% paraformaldehyde for 10 minutes and washed PBS (without Mg^{2+} or Ca^{2+}). Slides were blocked with 1% BSA/0.3 M glycine/0.025% Triton X-100 for 45 minutes. Following blocking, primary antibody incubation, Bio Rad anti-V5 (MCA1360) primary antibody was added to wells at a 1:500 dilution in 1% BSA/0.025% Triton X-100 in PBS and incubated overnight at 4°C. Following overnight incubation, slides were washed with 0.025% Triton X-100 three times for 5 minutes. For secondary antibody incubation, Donkey anti-mouse IgG Antibody Alexa Flour® 488 was added at 1:500 dilution in blocking buffer and added to wells containing slides. Slides were incubated in the dark for two hours with secondary antibody. Following three, 5-minute washes with PBS in the dark, slides were incubated with 1:1,000 dilution of DAPI for ten minutes. Following washes, slides were mounted with Flouromount-G, and dried overnight prior to imaging. Images were obtained with Nikon Eclipse TE 2000-U microscope and Nikon NIS-Elements AR Analysis 3.2 software was utilized for image processing.

Immunoprecipitation

Immunoprecipitation (IP) experiments were conducted following 24-hour transfection of WEEVcapsid-V5 and pcDNA3.1 transfection of Vero in T-225 cell culture flasks at 40-50% confluency. Following transfection, cells were trypsinized, pelleted and resuspended in clear lysis buffer. Protein extracts from both WEEVcapsid-V5 and pcDNA3.1. transfected samples were quantitated through use of Pierce™ Detergent Compatible Bradford Assay Kit and spectrophotometric quantitation of protein concentration. BSA was used for development of protein standard curve. Following quantitation, 1 mg of protein extract was utilized for IP and the remaining protein lysate was stored at -80°C for use as WB input samples. The protein lysates for IP were incubated with Bio Rad anti-V5 (MCA1360) primary antibody overnight at 4°C. The next day, Protein G Dynabeads® were added to each IP sample in order to pull down the antibody-protein complexes. The bead-antibody complexes were incubated for 45 minutes at room temperature. Following incubation, beads were washed and stored at -80°C for MS. Similar procedures were followed for IP and MS analysis of WEEVcapsid-V5 and pcDNA3.1-eGFP in BSR-T7.

Mass Spectrometry

Transfected and IP samples were prepared for MS by in-solution digest methods and use of ZipTip for desalting as outlined in *Molecular Profiling: Methods and Protocols,* Methods in Molecular Biology, Chapter 16: Mass Spectrometry-Based Biomarker Discovery (20). The MS analysis was conducted by Weidong Zhou with use of an Orbitrap Fusion™ Tribrid™ Mass Spectrometer by Thermo Scientific™. Mass

spectrometry analysis was conducted at the Center for Applied Proteomics and Molecular Medicine (CAPMM), George Mason University.

Mass Spectrometry Analysis

Following MS of immunoprecipitated protein samples, MS output of potential protein binding partners of WEEVcapsid-V5 and pcDNA3.1.-eGFP was obtained. MS protein output was processed for both Vero and BSR-T7 transfected samples similarly. Protein output lists were subjected to several filtering parameters in order to condense protein output lists for selection of proteins for validation (Fig. 9, A, B)

Following Vero transfection and immunoprecipitation with V5 antibody, a total of 1,040 proteins were identified by MS. Several groups of proteins were immediately eliminated as potential WEEV capsid interactors due to potential non-specific interactions with construct-derived protein (Fig. 9A). Proteins identified as histones or ribosomal subunits were eliminated from potential protein selection. Keratins were also excluded from selection. Additionally, proteins identified with only one single peptide were removed from selection due low abundance, coverage and likelihood of false identification. Following application of these filtering specifications, 430 proteins remained.

Following initial elimination, the remaining 430 proteins identified from WEEV IP samples were overlapped with pcDNA3.1-eGFP IP list. Proteins were eliminated as prospective WEEV interactors if the protein was identified in the control pcDNA3.1.-eGFP MS output and PSM number or peptide number remained the same or was reduced. The remaining proteins were marked with symbol indication '●' if they were identified in

the control output but had higher number of peptides identified than that of the protein identified in the control output. If proteins identified were not present in the control protein output list, a symbol indication '◊' was provided to the protein. Following overlap of MS output lists, a total number of 272 proteins were identified and considered from WEEV IP samples.

Following control overlap, the output list was condensed to one representative protein isoform. The isoform with the highest scoring peptide number was selected and remained in the final output list. Following, isoform elimination 136 proteins remained in consideration for validation. Proteins with peptide numbers lower than five were not considered for validation and actin, myosin and tubulin proteins were not considered for validation. The condensed protein output list considered for Vero cell validation consisted of 44 proteins as indicated in Table 1.

WEEVcapsid-V5 protein output from BSR-T7 transfected cells consisted list consisted of a total of 2,516 identified proteins from immunoprecipitated WEEVcapsid-V5 transfected BSR-T7 samples (Fig. 9B). Proteins were sorted by descending peptide spectral match (PSM) and peptide number to ensure high relative abundance and accurate identification of prospective proteins selected. Following sorting, several groups of proteins were immediately eliminated due to potential non-specific interactions with construct-derived protein. Proteins identified as histones or ribosomal subunits were eliminated from potential protein selection. Keratins were also excluded from selection. Additionally, proteins identified with peptide number of 1 were removed from

consideration and the total remaining proteins identified from immunoprecipitation were reduced to 1,311 proteins (Fig. 9B).

Following initial elimination, the remaining proteins identified from WEEV IP samples were overlapped with pcDNA3.1-eGFP IP list. Proteins were eliminated as prospective WEEV interactors if the protein was identified in the control pcDNA3.1.-eGFP MS output and PSM number or peptide number remained the same or was reduced. The remaining proteins were marked with symbol indication '●' if they were identified in the control output but had higher number of PSM or peptides identified. If proteins identified were not present in the control protein output list, symbol indication '◊' was provided to the protein. Following overlap of MS output lists, a total number of 734 proteins were identified and considered from WEEV IP samples.

The remaining protein list was condensed to include only one representative protein isoform and isoform with highest PSM or peptide was included. Additionally, proteins with peptide numbers of 5 or lower were excluded from consideration and the remaining number of proteins was reduced to 348 prospective proteins. This list was condensed and proteins with peptide numbers less than 10 were excluded. Additionally, a single representative protein isoform was chosen, and myosin proteins were excluded. The final list was comprised of 92 unique proteins indicated in Table 2. The protein list with identified 5 or higher peptide numbers was still referred to for selection of protein binding partners but not of primary consideration. The list of proteins identified as having peptides of 10 or higher was used for literature search for association of proteins involved in viral pathogenesis.

RESULTS

Design of molecular alphavirus capsid expression constructs and optimization of transfection and protein expression in various cell types was conducted for all three NW alphavirus capsid proteins, WEEV, EEEV and VEEV capsid. Other NW alphavirus capsid constructs, EEEV and VEEV were utilized in parallel optimization for use in subsequent studies involving proteomic comparative studies across the three NW alphaviruses. The current study focuses on optimization of transfection and protein expression conditions for all three molecular constructs, with specific focus on the proteomic interactions of host-cells and WEEV capsid protein.

Validation of Protein Expression in Vero Cells

Alphavirus capsid expression constructs were designed with reporter genes encoding viral V5 protein, a protein epitope derived from P and V proteins of the Paramyxovirus of Simian Virus 5, and green fluorescent protein (GFP) (Fig. 1). Expression constructs were transfected into Vero cells and protein expression was evaluated by WB and immunofluorescent confocal microscopy. Following 24-hour transfection of capsid constructs, protein lysates were collected, run on SDS-PAGE, and transferred to PVDF membrane. Following transfer, membranes were probed with anti-V5 antibody for the detection of alphavirus capsid protein. Control construct, pcDNA3.1. lacking both capsid and V5 tag was transfected in parallel as a negative control.

Following transfection, VEEV and WEEV capsid protein expression in Vero cells was observed by WB when transfections of 1 and 2 µg DNA concentrations were applied to cells (Fig. 2). Protein expression of WEEV and VEEV capsid was not detected following transfection at 4 µg DNA concentration (Fig. 2). Transfections of 2 µg DNA yielded the greatest protein expression of VEEV and WEEV capsid protein in Vero cells (Fig. 2). The 2 µg DNA concentration was utilized for ongoing experiments with VEEV and WEEV capsid constructs. In contrast, EEEV capsid protein expression was not detected when transfections were conducted with 1 or 2 µg DNA concentrations. EEEV capsid protein expression was detected at low levels following transfections of 4 µg of EEEVcapsid-V5 molecular construct DNA (Fig. 2, bottom). EEEVcapsid-V5 protein expression was lower than that of VEEV or WEEV capsid regardless of increased DNA transfection concentration. Subsequent transfection experiments were conducted with 4 µg of EEEVcapsid-V5 DNA.

Following validation by WB analysis, alphavirus capsid protein expression was confirmed by immunofluorescent confocal microscopy detection of reporter protein eGFP or V5 tag. Transfections of alphavirus molecular constructs for immunofluorescence microscopy were conducted in Vero cells at DNA concentrations previously validated by WB. Vero cells were transfected for 24-hours with either eGFP or V5 tagged capsid constructs, fixed, permeabilized and probed with anti-V5 antibodies (green) or left un-probed if transfected with eGFP constructs. Nuclei were stained with DAPI (blue). Immunofluorescent imaging revealed transfection of all three alphavirus capsid constructs tagged with V5 (Fig. 3). VEEVcapsid-V5 (green) was detected in Vero

and distributed diffusely throughout the cytoplasm (Fig. 3, top panel). VEEVcapsid-V5 was also detected more densely in the nucleus, with specific accumulation of green fluorescence visualized at the nuclear pore (Fig. 3, top panel). In studies conducted with TC-83 live-attenuated VEEV, capsid localization in Vero cells was observed to be distributed throughout both the cytoplasm and the nucleus (1). The distribution of VEEV capsid protein derived from VEEVcapsid-V5 molecular construct is somewhat in agreement with what is typically observed in VEEV infected cells; however, VEEV capsid is generally more cytoplasmically located with some cells showing nuclear capsid localization. In previous studies observing the localization of EEEV capsid in Vero cells, accumulation of capsid was observed at the nucleus at 8 hours post-infection while EEEV capsid was more cytoplasmically distributed at 24 hours post-infection (24). In the current study, EEEVcapsid-V5 protein expression appeared to be less than that of VEEV capsid expression as less V5 expression was detected throughout the cells (Fig. 3, middle panel). A faint accumulation of EEEVcapsid-V5 was detected in the nucleus. WEEVcapsid-V5 protein was detected in Vero cells with protein localized primarily in the nucleus, with minor distribution throughout the cytoplasm (Fig. 3, bottom panel). As compared to VEEVcapsid-V5 protein expression, WEEVcapsid-V5 protein expression appeared to be more nuclear with less cytoplasmic, punctuate protein phenotype throughout the cytoplasm (Fig. 3, bottom panel). Overall, transfections of all three alphavirus constructs with V5 tags produced detectable protein expression. Interestingly, WEEVcapsid-V5 protein appeared to be more localized to the nuclear pore. In contrast to VEEV and WEEV, EEEVcapsid-V5 protein expression was detected minimally in Vero.

Transfection experiments were conducted for eGFP tagged capsid constructs in Vero in order to observe potential localization differences between V5 tagged and eGFP tagged capsid constructs. Alphavirus capsid constructs with eGFP reporter genes were transfected and protein expression was assessed by immunofluorescent confocal microscopy. Nuclei were stained with DAPI. VEEVcapsid-eGFP was transfected and protein expression was visualized (Fig. 4, top panel). VEEV capsid was more localized throughout the cytoplasm with diffuse protein expression throughout the cytoplasm and less nuclear accumulation (Fig. 4, top panel). EEEVcapsid-eGFP expression was visualized in Vero cells with capsid localization being more nuclear than cytoplasmic (Fig. 4, middle panel). Overall, less transfection and subsequent protein expression was detected with EEEVcapsid-eGFP transfection (Fig. 4, middle panel), similar to what was observed with EEEVcapsid-V5. WEEV capsid-eGFP was more localized to the nucleus with diffuse eGFP fluorescence detected throughout the cytoplasm (Fig. 4, middle panel). These results are somewhat different than what was observed with WEEVcapsid-V5 where capsid protein was localized primarily in the nucleus. It is possible that the GFP fusion is altering the localization of capsid.

Alphavirus capsid constructs with V5 tags were utilized for further experiments due to the small size of the tag, which should minimize interference with protein partner binding and allow accurate protein localization. Transfections of capsid-V5 molecular constructs were optimized further for more enhanced expression of EEEVcapsid-V5 in other cell types.

Validation of Protein Expression in BSR-T7

Following validation of transfection of capsid constructs in Vero, capsid-V5 constructs were used for further optimization of protein expression. Protein expression of EEEVcapsid-V5 was detected in Vero but expression was minimal and less than that of VEEV or WEEV capsid expression. During transfection of Vero, molecular construct-based protein expression is derived from the CMV promoter, which is reliant on mammalian RNA Polymerase II for expression. In a previous study conducted by Aguilar et al., EEEV capsid was observed to repress transcription of molecular expression constructs reliant on CMV promoter-based expression. In contrast, the same study observed a lack of transcriptional repression of EEEV capsid when expression of constructs was derived from Bacteriophage T7 promoters with supplement T7 polymerase-based cell systems (9). The current study seeks to observe whether reduced protein expression of EEEV capsid-V5 in Vero cells is due to the transcriptional repression of EEEV capsid through a negative feedback mechanism that impacts the CMV promoter. It was hypothesized that EEEV capsid inhibits CMV promoters resulting in subsequently less EEEV capsid expression. In order to assess a potential difference in promoter expression due to promoter type, BSR-T7 cell line with stably transfected T7 polymerase was utilized for assessment of protein expression derived from the capsid construct T7 promoter. BSR-T7 were transfected with alphavirus capsid constructs at 1, 2 and 3 μg DNA concentrations in order to assess the optimal DNA concentration for expression of protein in BSR-T7. Capsid protein expression was detected in BSR-T7 by WB following transfections at all three DNA concentrations with the highest protein

expression being detected at 3 µg transfected DNA concentrations (Fig. 5). All three capsid constructs, VEEV, EEEV and WEEVcapsid-V5 were transfected and relative protein expression was directly proportional to the relative DNA concentration of construct transfected (Fig. 5). Overall expression of EEEVcapsid-V5 was more comparable to that of VEEV and WEEV capsid expression in BSR-T7 as compared to Vero.

In addition to WB confirmation of protein expression, immunofluorescent imaging was conducted for visualization of transfection of capsid-V5 constructs in BSR-T7. All three capsid constructs were transfected, cells were fixed, permeabilized, and probed with fluorescent antibodies for V5 (green). Nuclei were stained with DAPI (blue). Protein expression of all three capsid constructs was visualized by confocal microscopy (Fig. 6). VEEVcapsid-V5 protein expression was observed in BSR-T7 with characteristic nuclear phenotype (Fig. 6, top panel). VEEV capsid was observed to be more accumulated at the nucleus with some faint and speckled distribution of fluorescence throughout the cytoplasm of cells (Fig. 6, top panel). EEEVcapsid-V5 protein expression was visualized in BSR-T7, with capsid distribution being observed throughout the cytoplasm and less at the nucleus (Fig.6, middle panel). WEEVcapsid-V5 protein expression was observed in BSR-T7 with primary phenotype being an accumulation at the nucleus, along with cytoplasmic distribution (Fig. 6, bottom panel). BSR-T7 cells were utilized and optimized for continuing protein-protein interaction studies with focus on WEEVcapsid-V5 interactions in this cell type.

Immunoprecipitation of WEEVcapsid-V5 in Vero or BSR-T7

Transfection conditions for capsid constructs were optimized for Vero and BSR-T7 and protein expression was confirmed by WB and immunofluorescent imaging. WEEVcapsid-V5 molecular expression construct was confirmed to be transfected and subsequent protein expression was observed to be abundant in both Vero and BSR-T7 cell types. Transfections were conducted in 225cm^3 cell culture flasks in to increase overall protein yield for subsequent mass spectrometry analysis. Following 24-hour transfection of WEEVcapsid-V5 in Vero and BSR-T7, protein lysates were collected for immunoprecipitation and WB. A total of 1 mg of total protein was utilized for immunoprecipitation while 100 µg of protein was utilized as a WB input sample. Following immunoprecipitation, WB and IP samples were run on SDS-PAGE, transferred to PVDF membrane and membranes were probed with anti-V5 antibody. VEEV and WEEVcapsid-V5 along with pcDNA3.1-eGFP control were used for transfection, IP and WB.

Following immunoprecipitation, both VEEV and WEEVcapsid-V5 were detected in protein lysates collected from transfected Vero (Fig. 7). A band was detected at approximately 40 kDa in both western blot samples transfected with VEEV and WEEV capsid. No band was observed in pcDNA3.1.-eGFP transfected protein lysate samples. Additionally, a band was detected at 40 kDa in protein lysate samples immunoprecipitated for V5 in both VEEV and WEEVcapsid-V5 transfected protein lysates (Fig. 7). Western blotting indicated successful immunoprecipitation of capsid protein from both VEEV and WEEV transfected samples.

Immunoprecipitation of capsid constructs was conducted in BSR-T7 cell types because enhanced protein expression was detected by previous western blot experiments. Transfections of pcDNA3.1-eGFP and WEEV capsid were conducted in BSR-T7 and following transfections protein lysates were collected for later western blot (WB) or subjected to immunoprecipitation. Immunoprecipitated protein lysate samples and total protein lysate were subjected to SDS-PAGE and followed by Coomassie blue staining and western blotting. Coomassie blue staining permitted visualization of immunoprecipitated capsid protein from both pcDNA3.1-eGFP and WEEVcapsid-V5 transfected samples (Fig. 8, A). Additionally, Coomassie blue staining permitted estimation of relative protein recovered through immunoprecipitation by visualization of BSA samples subjected to western blotting. It was approximated that 250 ng of capsid protein was recovered from immunoprecipitation subjected samples. In addition to Coomassie blue staining, western blotting was conducted, and membranes were probed with anti-V5 antibody. A band was detected in WEEV capsid transfected samples that were subjected directly to western blot or immunoprecipitated (Fig. 8, B). The results of western blot confirmed a 40 kDa protein recovered from immunoprecipitation subjected samples, indicating successful immunoprecipitation of capsid (Fig. 8, B). Following immunoprecipitation, the remaining protein lysate was utilized for mass spectrometry experiments.

Mass Spectrometry Protein Output

Immunoprecipitated protein lysates from Vero or BSR-T7 were utilized for MS with the Orbitrap Fusion. Following MS, protein output was generated utilizing Proteome

Discoverer with reference FASTA protein databases for both Vero cell, *Chlorocebus sabaeus* and Syrian Golden Hamster, *Mesocricetus auratus*. WEEV capsid reference protein sequences were included in both databases for identification of capsid protein by MS. Protein output was subjected to various filtering parameters as outlined in Materials and Methods. Filtering and elimination resulted in a condensed protein output of approximately 44 proteins identified in Vero and 91 proteins identified in BSR-T7 (see Material and Methods and Fig. 9 A, B). Proteins were compiled in Table 1 and Table 2. BSR-T7 proteomic output was used for categorization of proteins and literature search for possible associations of proteins in viral pathogenesis. Additionally, proteomic output was categorized into several protein groups based on abundant members and cellular roles. Several notable protein groups include Nuclear Pore Proteins, Fragile X family proteins, Dead-box proteins and helicases, Nuclear importers and exporters and Heterogenous ribonucleoproteins. Following protein grouping and literature search, Nuclear Importers and Exporters, specifically importin-alpha, importin-beta, and CRM1, were selected for further investigation of WEEV capsid interactions because previous studies indicated the interactions of VEEV capsid with importins and exportins at the nuclear pore complex.

Confirmation of Binding Partner Interactions

Following narrowed selection of potential binding partners identified by MS, CRM1, importin-alpha and importin-beta were selected as potential binding partners for confirmation. These proteins are of interest due to their previously documented interaction with VEEV capsid and as potential regulators of WEEV capsid trafficking.

Our data show WEEV capsid in both cytoplasmic and nuclear compartments. Protein lysates collected from previous transfections were used for immunoprecipitation or directly utilized in western blotting for validation. Western blots were probed for V5 to confirm adequate transfection and immunoprecipitation. Western blots were also probed for both importins and CRM1. Following probing, CRM1 was identified as co-immunoprecipitating with capsid with protein band detected at 90-100 kDa (Fig. 10). Additionally, when blots were probed for importin-alpha a faint band was detected at 60 kDa (Fig. 10). In contrast, blots were probed with importin-beta and no band was detected (Fig. 10). These results confirm that WEEV capsid interacts with CRM1 and importin-alpha.

FIGURES

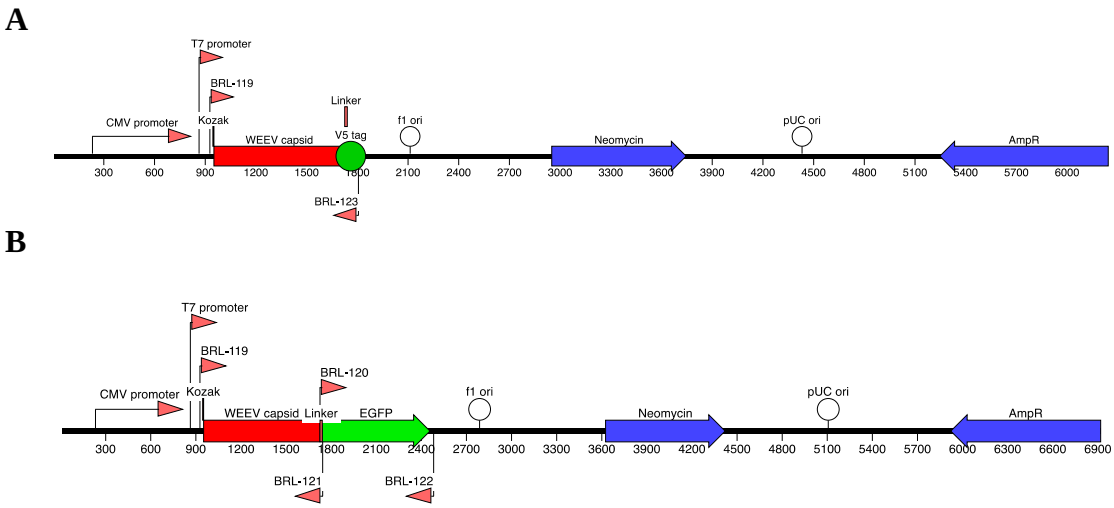

Figure 1.

Expression constructs with WEEV capsid sequence flanked by viral V5 or green fluorescent protein (eGFP) tags were used for host-protein interaction studies. Constructs include both mammalian cytomegalovirus (CMV) and bacterial polymerase T7 promoters for use of construct expression. Each construct includes Ampicillin resistance (AmpR) gene for transformant selection. Constructs of similar composition were designed for VEEV and EEEV capsid with differences occurring only in the genomic sequence of the capsid.

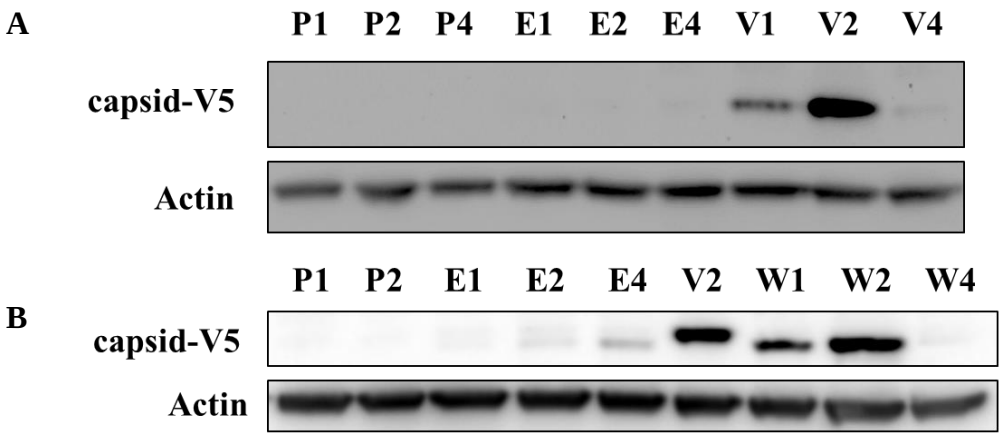

Figure 2.

Western blot of transfected viral capsid constructs and detection of capsid-V5 protein expression. Capsid constructs along with pcDNA3.1.-eGFP empty vector controls were transfected into Vero cells for 24 hours. Capsid constructs are indicated as 'V' for VEEVcapsid-V5, 'W' for WEEVcapsid-V5, 'E' for EEEVcapsid-V5 and 'P' for pcDNA3.1.-eGFP. Each respective number indication represents the transfected DNA concentration i.e. pcDNA3.1-eGFP at 2 μg as 'P2.'

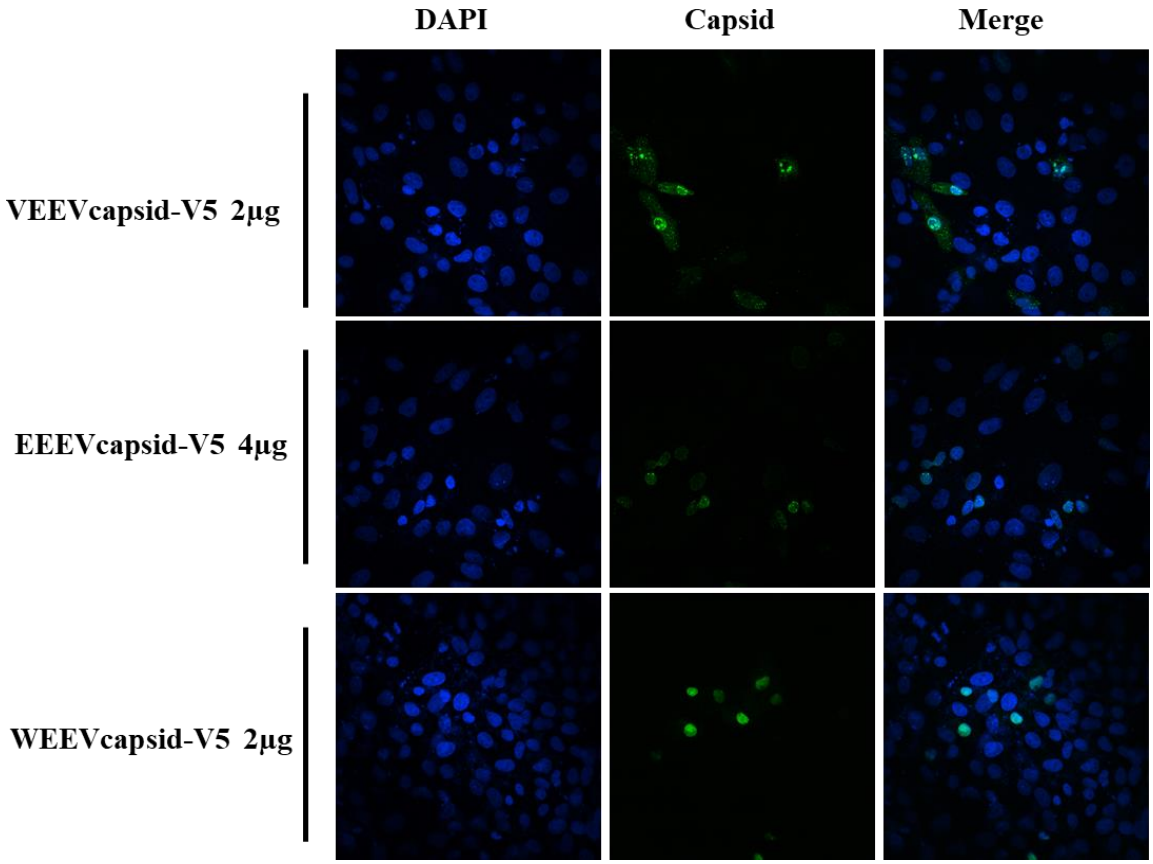

Figure 3.

Visualization of localization of alphavirus capsid-V5 protein following transfection of Vero cells. Immunofluorescent microscopy was used to visualize cells transfected with alphavirus capsid-V5 constructs. Cells were fixed 24-hours post-transfection with capsid construct and probed with anti-V5 antibody (green) and nuclei are stained with DAPI (blue).

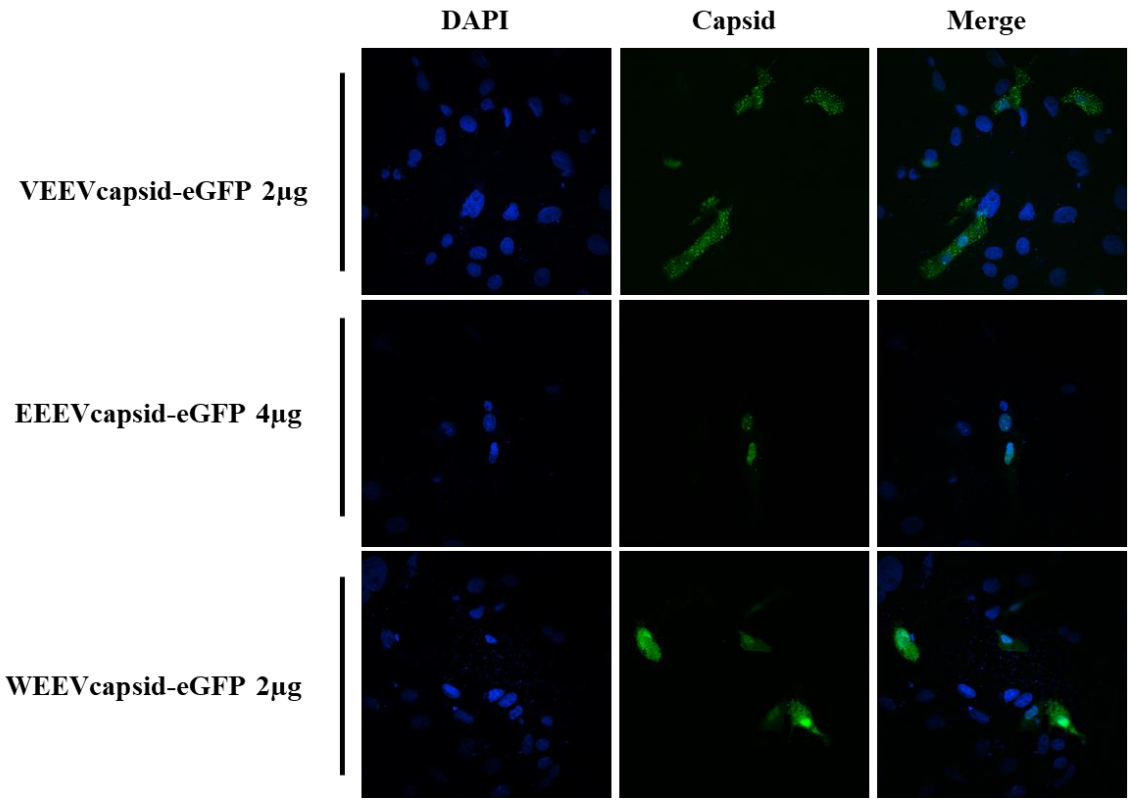

Figure 4.
Visualization of localization of alphavirus capsid-eGFP protein following transfection of Vero cells. Immunofluorescent microscopy was used to visualize Vero cells following transfection of alphavirus capsid-eGFP constructs. Cells were fixed at 24-hours post-transfection with capsid construct and probed with anti-V5 antibody (green) and nuclei were stained with DAPI (blue).

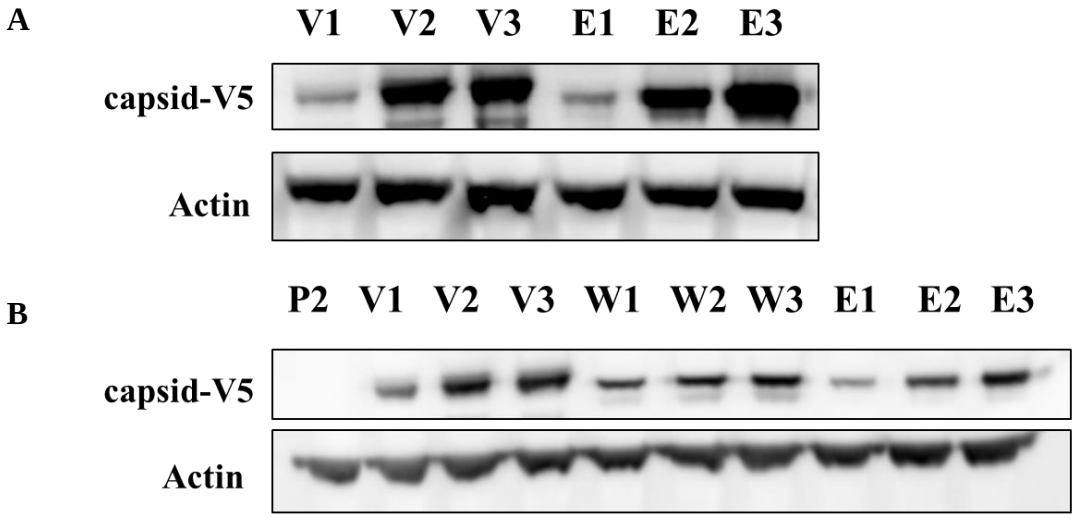

Figure 5.

Transfection of BSR-T7 with viral capsid constructs to assess efficiency of T7 promoter-derived expression. Cells expressing T7 polymerase were transfected with viral capsid constructs for 24-hours and protein expression was assessed through WB. Capsid constructs are indicated as 'V' for VEEVcapsid-V5, 'W' for WEEVcapsid-V5, 'E' for EEEVcapsid-V5 and 'P' for pcDNA3.1.-eGFP. Representative numbers indicate transfected DNA concentrations i.e. pcDNA3.1-eGFP at 2 µg as 'P2.'

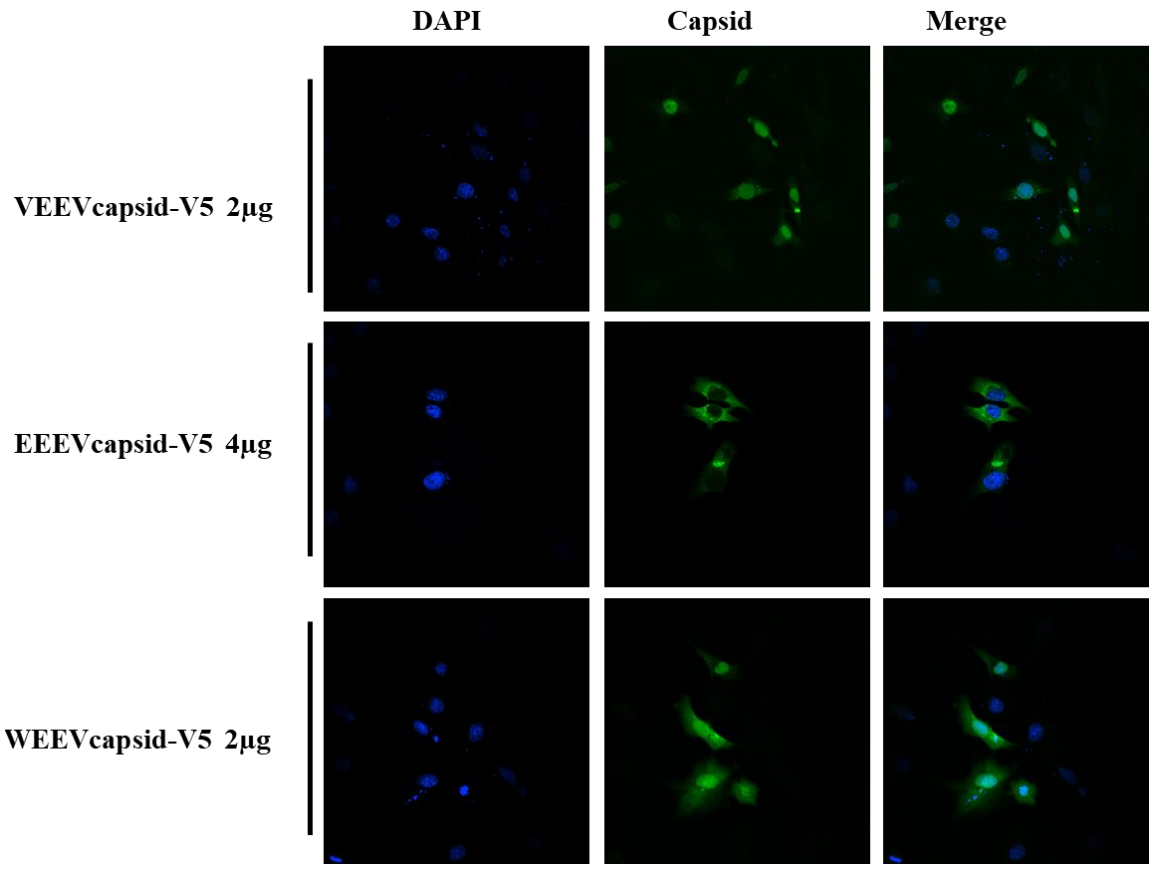

Figure 6.
Visualization of localization of transfected capsid constructs by immunofluorescent confocal microscopy in BSR-T7 cells. In order to assess the level of protein expression derived from the construct T7 promoter, BSR-T7 were transfected with capsid constructs for 24-hours. Cells were fixed and probed with anti-V5 antibody and stained with DAPI (blue) and imaged by immunofluorescent confocal microscopy.

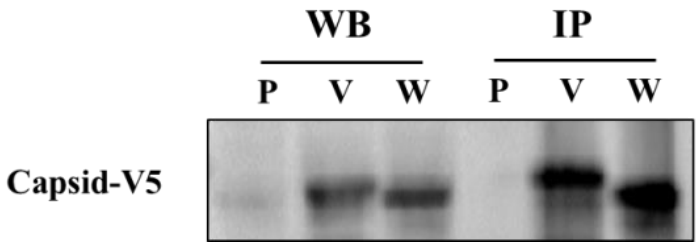

Figure 7.

Immunoprecipitation of capsid-V5 constructs and pcDNA3.1.-eGFP empty vector control in transfected Vero cells. Vero cells were transfected with capsid constructs or empty vector control for 24 hours. Protein lysate was either run directly or utilized for immunoprecipitation of capsid-V5. All samples were run on SDS-PAGE and probed for V5 in order to assess transfection and immunoprecipitation. Capsid constructs are indicated as 'V' for VEEVcapsid-V5, 'W' for WEEVcapsid-V5 and 'P' for pcDNA3.1.-eGFP.

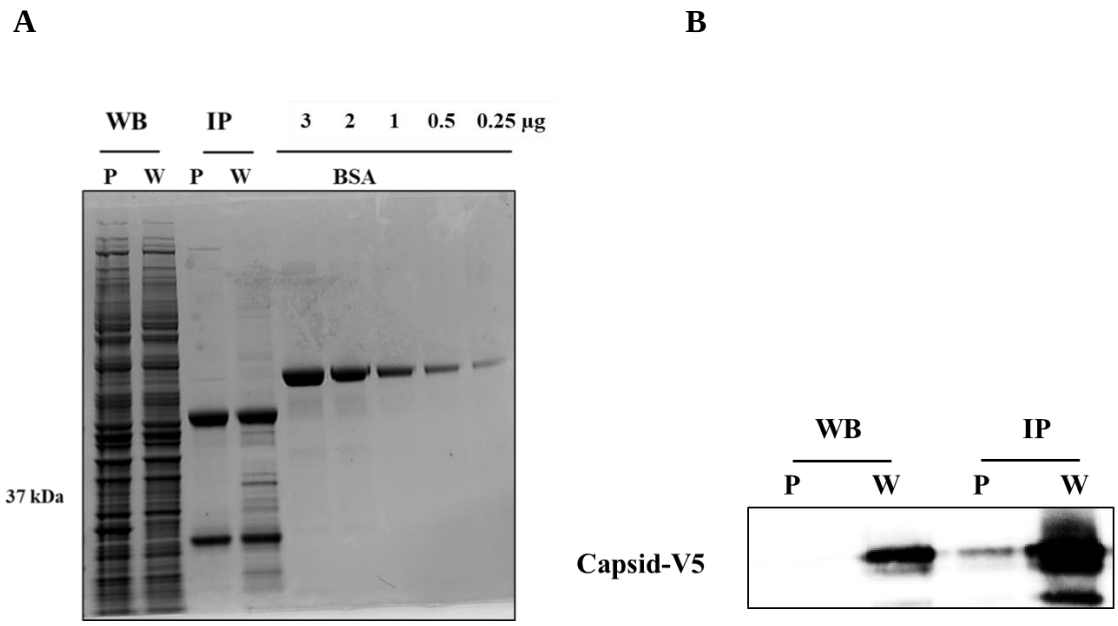

Figure 8.

Immunoprecipitation of WEEVcapsid-V5 constructs and pcDNA3.1.-eGFP empty vector control in transfected BSR-T7 cells. Protein lysates from BSR-T7 transfected cells, were subjected to immunoprecipitation or left as whole protein lysates, run on SDS-PAGE and Coomassie blue staining and western blotting was conducted. Coomassie blue staining (left) was conducted for pcDNA3.1. control and WEEVcapsid-V5 samples directly collected and run (WB) or subjected to immunoprecipitation (IP) and run on SDS-PAGE. Bovine Serum Albumin (BSA) protein samples were run on SDS-PAGE in concentrations of 3 µg, 2 µg, 1 µg, 0.5 µg and 0.25 µg. Samples were subjected to western blot and membranes were probed for V5 (right).

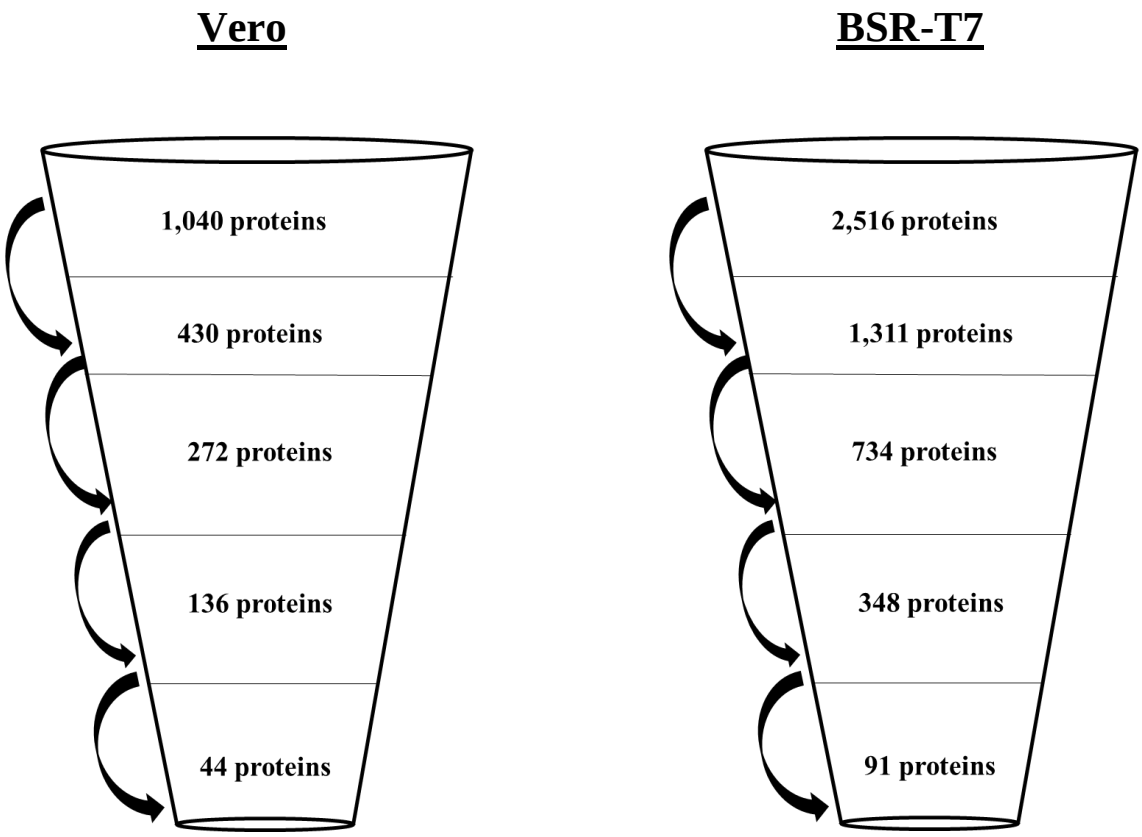

Figure 9.
Filtering parameters were applied to protein output obtained from WEEVcapsid-V5 transfected Vero and BSR-T7. Several protein types were eliminated from protein output including histones, ribosomal subunits and keratins. Proteins were compared to control output protein lists to identify novel proteins or those found more abundantly in WEEVcapsid-V5 transfected samples. Additionally, a single representative isoform was selected for each representative protein. Proteins with corresponding peptide numbers of five or ten were selected for consideration from Vero and BSR-T7 protein output lists. Specific filtering applications to protein output data-sets are referenced in Materials and Methods and final protein output for consideration is displayed in Table 1. and Table 2. respectively.

Table 1. Protein interactors identified by mass spectrometry following immunoprecipitation of WEEVcapsid-V5 in Vero cells.

Protein Description	Coverage	Peptides	PSMs	AA	Control
splicing factor 3B subunit 1 isoform X1	31.98	33	49	1304	●
splicing factor 3B subunit 3	24.65	25	36	1217	●
splicing factor 3B subunit 2 isoform X1	26.48	19	40	895	●
polyadenylate-binding protein 1	31.13	19	27	636	●
serine/arginine repetitive matrix protein 2 isoform X1	9.41	17	17	2752	●
putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	22.26	15	21	795	◊
fragile X mental retardation syndrome-related protein 1 isoform X1	18.42	13	17	825	◊
calcium homeostasis endoplasmic reticulum protein isoform X1	15.21	13	15	927	◊
WEEV capsid	37.45	12	41	259	◊
pre-mRNA-processing-splicing factor 8	6.68	12	13	2335	◊
protein SEC13 homolog isoform X1	36.14	11	24	368	●
far upstream element-binding protein 2 isoform X1	22.22	11	15	747	●
regulator of nonsense transcripts 1 isoform X1	11.70	11	13	1137	◊
splicing factor 1 isoform X5	18.99	10	16	611	●
polyadenylate-binding protein 3	13.36	10	12	636	●
cleavage and polyadenylation specificity factor subunit 7 isoform X1	26.96	9	14	471	●
transformer-2 protein homolog beta isoform X1	32.29	9	12	288	◊
heterogeneous nuclear ribonucleoprotein U isoform X1	12.73	9	11	825	◊
U5 small nuclear ribonucleoprotein 200 kDa helicase	5.52	9	11	2136	◊
U2 snRNP-associated SURP motif-containing protein isoform X1	8.75	9	10	1028	◊
polyadenylate-binding protein 4 isoform X1	14.39	8	11	660	●
fragile X mental retardation syndrome-related protein 2	15.16	8	10	673	◊
splicing factor 45	23.19	8	10	401	◊
ATP-dependent RNA helicase A isoform X1	7.76	8	9	1275	◊
probable ATP-dependent RNA helicase DDX5	14.66	8	8	614	◊
heterogeneous nuclear ribonucleoprotein H	24.42	7	19	430	●
78 kDa glucose-regulated protein	12.69	7	13	654	◊
cleavage and polyadenylation specificity factor subunit 6 isoform X1	16.13	7	11	589	●
PHD finger-like domain-containing protein 5A	67.27	7	11	110	●
splicing factor 3A subunit 1	11.73	7	11	793	●
splicing factor 3A subunit 3	18.09	7	9	492	●
cleavage and polyadenylation specificity factor subunit 5	43.17	7	9	227	◊
putative hexokinase HKDC1 isoform X1	8.66	7	8	947	●
fibronectin type III domain-containing protein 3B	7.39	7	7	1204	◊
putative helicase MOV-10 isoform X1	8.97	7	7	1003	◊
heterogeneous nuclear ribonucleoproteins C1/C2 isoform X1	26.14	6	14	306	●
heat shock-related 70 kDa protein 2	11.11	6	13	639	●
fragile X mental retardation protein 1 isoform X1	10.80	6	7	611	◊
casein kinase I isoform alpha isoform X4	23.19	6	6	345	●
heterogeneous nuclear ribonucleoprotein M	13.25	6	6	664	●
EPM2A-interacting protein 1	12.52	6	6	607	◊
peroxiredoxin-1	25.63	6	6	199	◊
heterogeneous nuclear ribonucleoprotein F	17.83	5	9	415	●

* Protein interactors unique to WEEVcapsid-V5 IP/MS output are indicated with '◊' and those noted in the control but with higher peptide numbers are indicated with '●'.

Table 2. Protein interactors with peptide numbers of 10 or greater identified by mass spectrometry following immunoprecipitation of WEEV capsid-V5 in BSR-T7 cells.

Protein Description	Coverage	Peptides	PSM	AAs	Control
myb-binding protein 1A isoform X2	55.40	61	107	1343	●
nucleolar RNA helicase 2	51.72	40	61	816	●
heterogeneous nuclear ribonucleoprotein M isoform X1	51.10	36	78	730	●
ribosome-binding protein 1	44.01	35	50	993	●
polyadenylate-binding protein 1	49.76	33	66	635	●
epiplakin-like	15.32	33	35	3095	●
polyadenylate-binding protein 4 isoform X1	43.98	32	63	664	●
heterogeneous nuclear ribonucleoprotein U	44.43	31	62	799	●
elongation factor 2	42.54	29	42	858	●
serine/arginine repetitive matrix protein 2 isoform X1	15.74	28	36	2706	●
ATP-dependent RNA helicase DDX3X	43.05	26	40	662	●
probable ATP-dependent RNA helicase DDX5	42.93	25	46	615	●
matrin-3 isoform X1	35.11	23	32	846	●
nucleolin	36.48	23	35	699	●
putative ATP-dependent RNA helicase Pl10 isoform X1	35.91	23	32	646	●
importin subunit beta-1	33.90	22	33	876	●
nuclear pore complex protein Nup214 isoform X1	16.19	22	28	2075	◊
WEEV capsid	60.46512	21	284	258	◊
heterogeneous nuclear ribonucleoprotein K	42.89	19	31	464	◊
vigilin	22.48	19	22	1268	●
cytoskeleton-associated protein 4	43.73	18	20	574	●
DNA replication licensing factor MCM5	28.88	18	21	734	●
heterogeneous nuclear ribonucleoprotein A3	43.27	18	30	379	●
pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15 iso. X1	27.42	18	27	795	●
fibronectin type III domain-containing protein 3B isoform X1	19.82	17	22	1206	●
regulator of nonsense transcripts 1 isoform X1	18.67	17	19	1130	●
rRNA 2'-O-methyltransferase fibrillarin	61.89	17	27	328	●
ATP-binding cassette sub-family F member 1 isoform X1	29.87	16	21	847	●
constitutive coactivator of PPAR-gamma-like protein 1	21.06	16	19	1116	●
nuclear fragile X mental retardation-interacting protein 2	30.06	16	25	692	●
U5 small nuclear ribonucleoprotein 200 kDa helicase	10.25	16	17	2136	●
asparagine--tRNA ligase, cytoplasmic	33.81	15	20	559	●
guanine nucleotide-binding protein-like 3	41.81	15	24	531	●
RNA cytidine acetyltransferase	21.95	15	20	1025	●
structural maintenance of chromosomes protein 2	15.45	15	16	1191	●
exportin-1	19.70	14	18	1071	●
fragile X mental retardation syndrome-related protein 1 iso.X1	28.51	14	23	677	●
nucleolar protein 56	35.70	14	15	591	◊
PDZ and LIM domain protein 5 isoform X1	37.10	14	17	593	●
polypyrimidine tract-binding protein 1	46.67	14	35	555	●
probable 28S rRNA (cytosine(4447)-C(5))-methyltransferase	23.23	14	15	792	◊
probable ATP-dependent RNA helicase DDX17	34.15	14	22	407	●
protein RCC2	35.58	14	19	520	●

ATP-binding cassette sub-family F member 2	22.72	13	16	625	●
ATP-dependent RNA helicase DDX1	25.68	13	17	740	●
cold shock domain-containing protein E1 isoform X1	20.14	13	18	844	●
heterogeneous nuclear ribonucleoprotein L isoform X1	26.00	13	19	623	●
insulin-like growth factor 2 mRNA-binding protein 3 iso. X1	33.16	13	17	579	●
la-related protein 1	18.47	13	18	1072	●
40S ribosomal protein S2-like	46.94	13	39	294	●
neuron navigator 1	10.29	13	14	2157	●
nuclear mitotic apparatus protein 1 isoform X1	8.76	13	14	2111	◊
structural maintenance of chromosomes protein 4	14.85	13	13	1286	●
tRNA (cytosine(34)-C(5))-methyltransferase	21.99	13	14	755	●
tRNA-splicing ligase RtcB homolog	31.88	13	17	505	●
ataxin-2-like protein isoform X1	14.92	12	16	1099	●
caprin-1 isoform X1	21.22	12	14	707	●
dnaJ homolog subfamily A member 1	47.61	12	12	397	●
heterogeneous nuclear ribonucleoprotein F	46.02	12	82	415	●
heterogeneous nuclear ribonucleoprotein Q isoform X1	24.88	12	15	623	●
kinectin	12.52	12	12	1350	●
nucleolar protein 58	32.97	12	14	543	●
serine/arginine-rich splicing factor 1	42.74	12	15	248	●
transcription intermediary factor 1-beta	21.25	12	15	833	●
elongation factor Tu, mitochondrial isoform X1	33.05	11	14	469	●
eukaryotic translation initiation factor 2 subunit 3	30.51	11	16	472	●
guanine nucleotide-binding protein-like 3-like protein	22.88	11	15	577	◊
heterogeneous nuclear ribonucleoprotein H isoform X1	37.92	11	21	472	●
heterogeneous nuclear ribonucleoproteins C1/C2 isoform X1	37.26	11	21	314	●
kinesin-like protein KIF2A isoform X1	21.67	11	11	743	●
nuclease-sensitive element-binding protein 1	53.42	11	18	322	●
nucleolar GTP-binding protein 1	17.19	11	12	634	◊
protein PRRC2C isoform X1	5.31	11	12	2865	●
ras GTPase-activating-like protein IQGAP1	9.47	11	11	1657	●
rho guanine nucleotide exchange factor 2 isoform X1	14.17	11	12	1030	◊
ribosomal RNA processing protein 1 homolog B	26.88	11	11	744	●
serine-threonine kinase receptor-associated protein	47.14	11	12	350	●
signal recognition particle subunit SRP72	23.07	11	14	672	●
ADP/ATP translocase 2	32.55	10	14	298	●
aspartyl/asparaginyl beta-hydroxylase isoform X1	17.22	10	13	755	●
ATP-dependent RNA helicase A	8.51	10	12	1387	●
cleavage and polyadenylation specificity factor subunit 7 iso. X1	28.87	10	14	471	●
eukaryotic initiation factor 4A-III	30.41	10	14	411	●
nuclear pore complex protein Nup50	33.69	10	13	469	●
nuclear valosin-containing protein-like isoform X1	17.23	10	11	853	◊
ribonucleases P/MRP protein subunit POP1	13.12	10	11	1006	◊
small subunit processome component 20 homolog	7.47	10	10	1861	◊
sulfide:quinone oxidoreductase, mitochondrial	34.38	10	10	352	●
U2 snRNP-associated SURP motif-containing protein	9.43	10	10	1029	◊
UPF0568 protein C14orf166 homolog	55.74	10	13	244	◊
zinc finger RNA-binding protein isoform X1	14.14	10	11	1082	◊

* Protein interactors unique to WEEVcapsid-V5 IP/MS output are indicated with '◊' and those noted in the control but with higher peptide numbers are indicated with '●'.

Table 3. Protein groups identified from WEEVcapsid-V5 transfected BSR-T7 by IP/MS for validation studies following literature search.

Dead Box and Helicases	Coverage	Peptides	PSM	Control
ATP-dependent RNA helicase A	8.51	10	12	●
nucleolar RNA helicase 2	51.72	40	61	●
DDX3X	43.05	26	40	●
RNA helicase Pl10 isoform X1	35.91	23	32	●
DDX5	42.93	25	46	●
DHX15	27.42	18	27	●
DDX1	25.68	13	17	●
DDX17	34.15	14	22	●
regulator of nonsense transcripts 1 isoform X1	18.67	17	19	●
Nuclear Pore Proteins				
nuclear pore complex protein Nup214 isoform	16.19	22	28	◊
nuclear pore complex protein Nup50	33.69	10	13	●
Nuclear Importers and Exporters				
exportin-1	19.70	14	18	●
importin subunit beta-1	33.90	22	33	●
importin subunit alpha-1	18.90359	6	7	●
Heterogenous Ribonucleoproteins				
hnRNP A3	43.27	18	30	●
hnRNP F	46.02	12	82	●
hnRNP H isoform X1	37.92	11	21	◊
hnRNP K	42.89	19	31	●
hnRNP L isoform X1	26.00	13	19	●
hnRNP M isoform X1	51.10	36	78	●
hnRNP Q isoform X1	24.88	12	15	●
hnRNP U	44.43	31	62	●
hnRNP C1/C2 isoform X1	37.26	11	21	◊
U2 snRNP-associated SURP motif-containing p	9.43	10	10	●
U5 small nuclear ribonucleoprotein 200 kDa he	10.25	16	17	●
Fragile X Proteins				
fragile X mental retardation syndrome-related p	28.51	14	23	●
nuclear fragile X mental retardation-interacting	30.06	16	25	●
WEEV capsid	60.47	21	284	◊

* Protein interactors unique to WEEVcapsid-V5 IP/MS output are indicated with '◊' and those noted in the control but with higher peptide numbers are indicated with '●'.

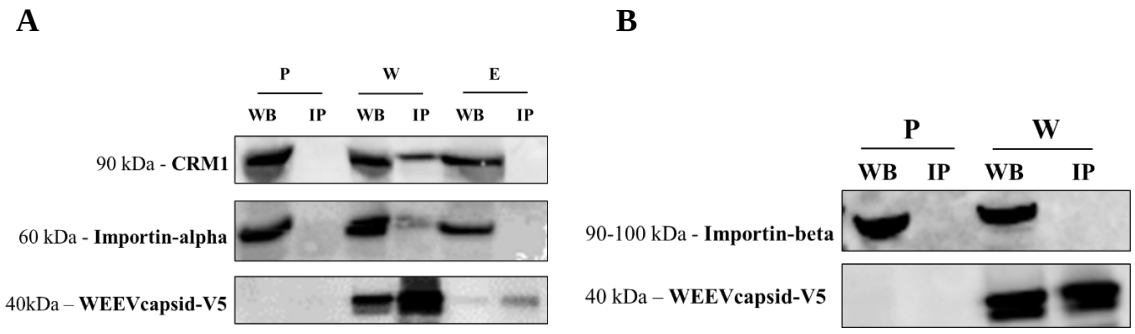

Figure 10.

Western blot detection of potential protein interactors of WEEV capsid protein, exportin-1 (CRM1), importin-alpha and importin-beta. Following immunoprecipitation of WEEVcapsid-V5, both IP samples and non-IP protein lysates were subjected to SDS-PAGE and western blotting for the various nuclear import and export proteins. Following WB, CRM1 was detected in interaction with immunoprecipitated WEEVcapsid-V5 and was present in WB samples. Additionally, importin-alpha was co-immunoprecipitated with WEEVcapsid-V5.

DISCUSSION

The current study addresses the design and validation of alphavirus capsid molecular constructs for use in studies evaluating protein interactions between the host-system and capsid. The study specifically focuses on the validation and use of WEEV capsid construct for proteomic interaction studies. The study identified specific localization differences between -eGFP and -V5 tagged alphavirus capsid constructs. Additionally, an inhibition of EEEV capsid protein expression derived from molecular constructs was observed in Vero cells but not BSR-T7. The study also identifies specific WEEV capsid interactions with importin-alpha and CRM1, nuclear import and export proteins.

Following design of molecular constructs for alphaviruses VEEV, EEEV and WEEV capsids with reporter gene tags -eGFP and -V5, all three constructs were transfected, and protein expression was evaluated in Vero and BSR-T7 cell systems. In Vero cells, VEEV and WEEVcapsid-V5 constructs resulted in detectable protein expression by WB and IFA following transfection. It was observed that VEEV and WEEVcapsid-V5 was expressed in Vero and visualized by IFA to have relatively nuclear phenotypes (Fig. 3). In contrast, VEEV and WEEVcapsid-eGFP tagged constructs yielded capsid protein localization that was more cytoplasmic in distribution (Fig. 4). It is hypothesized that the difference in capsid localization of the two different types of molecular constructs may be due to difference in protein tag. Molecular constructs with -eGFP reporter genes yield capsid protein with an added 238 amino acid GFP protein tag.

In contrast, capsid-V5 constructs yield capsid proteins with a 14 amino acid protein tag. It is thought that the size of the protein tag may impact the ability of capsid to localize to the nucleus. The larger GFP protein tag may interfere with specific residues on capsid that permit localization to the nucleus or due to large size difference, does not permit access to the nucleus. Due to the difference in localization, we utilized capsid-V5 molecular constructs for proteomics studies in order to ensure that the protein tag would not alter capsid's interactions with proteins within the host-cell. All subsequent studies utilized alphavirus capsid-V5 constructs for optimization.

EEEVcapsid-V5 protein expression in Vero cells was minimally detectable under similar transfection conditions by WB and IFA (Fig. 2, 3). The lack of protein expression of EEEV capsid under similar transfection conditions is hypothesized to be result of transcriptional inhibition of CMV promoters and subsequent inhibition of EEEV capsid protein generation in Vero cells. Previous studies have observed reporter gene transcriptional inhibition by EEEV capsid when the sole source of transcription is CMV promoter based and RNA polymerase II reliant (9). These previous studies implicate EEEV capsid's role in inhibition of mammalian RNA Polymerase II-based transcription (9). The results of the current study show that the EEEV capsid molecular construct is not readily expressed in Vero and this could be due to the transcriptional repression of EEEV capsid's own expression through inhibition of transcripts generated from CMV promoters. The protein expression of EEEV capsid is enhanced in BSR-T7 when a CMV promoter is not utilized for generation of transcripts and protein expression as observed in this study (Fig. 5, 6). Through assessment of EEEV capsid protein expression in Vero

as compared to BSR-T7, EEEV capsid protein expression was greater in BSR-T7 as visualized by WB and IFA (Fig. 5, 6). Following observation that overall protein expression was enhanced in BSR-T7 for EEEVcapsid-V5, BSR-T7 was used to ensure capsid protein expression was sufficient for use in immunoprecipitation and MS experiments involving all three alphavirus capsid constructs. Although Vero cells were utilized in parallel to assess protein interactions of WEEV capsid and filtering protocols, the data will not be used in this study, but will be used in future studies investigating conserved protein interactions across cell lines. The current study focuses on proteomic output generated from BSR-T7 transfected cells.

Following IP of WEEVcapsid-V5 from BSR-T7 cells, a proteomic output was generated and subjected to filtering. Filtering parameters were applied for narrowing of protein output and assurance that proteins were accurately identified, abundantly present and had biological relevance. Following filtering of BSR-T7 proteomic output, 91 proteins were identified as potential interactors and considered for selection (Fig. Table 2, Fig. 9B). Following literature review of proteins and associated with viral pathogenesis, several protein categories were generated from the proteomic output.

Categories of interest included, DEAD-box proteins and helicases, Nuclear Import and Export Proteins, Nuclear Pore complex (NPC) proteins, Fragile X (FXR) proteins and Heterogenous Ribonucleoproteins (HnRNP's) (Table 3.). HnRNP's are proteins involved in various aspects of nucleic acid metabolism and are capable of binding coding and non-coding RNA's (31, 32). Recent areas of research have implicated hnRNP's in the development of neurodegenerative disease (31, 32). Additionally, these

RNA binding proteins are subject to many types of post-translational modifications which may modulate hnRNP activities (31, 32). HnRNP's were of interest due to the numerous members identified to be in association with WEEV capsid. DEAD-box proteins and helicases were of interest due to protein interaction studies identifying DEAD-box protein (DDX1) and (DDX3) to be in interaction with VEEV nsP3 (26). It was observed that inhibitors of DDX3 negatively impacted VEEV replication (26). Interestingly, both DDX1 and DDX3 (indicated as DDX3X in Table 2., Table 3.) were identified to be potential protein interactors of WEEV capsid as indicated in Table 2. Several FXR proteins were identified from MS output specifically, nuclear fragile mental retardation interacting protein 2 (FMRP-interacting protein 2) fragile x mental retardation syndrome-related protein 1 (FXR1). FXR proteins are involved in cognitive development and mutations in these proteins have been associated with neurodegenerative disease development, fragile x syndrome and autism (29, 30). FXR proteins have been previously identified to interact with VEEV and EEEV nsP3 protein hypervariable domains (HVD) (29, 30). Knockdown of several FXR members results in reduced infectivity of VEEV (29, 30).

Nuclear Pore proteins have been associated with viral pathogenesis and several viral proteins interact with FG-repeat containing nucleoporins (NUPs) contained in the host-nuclear pore complex (NPC). Specifically, nuclear pore protein 214 (Nup214) has been implicated in pathogenesis of several viruses. Adenovirus utilizes Nup214 for import of viral DNA and Herpesvirus capsid protein interacts with Nup214 to release viral DNA into the host-nucleus (27, 28). Nuclear import and export proteins were of

interest due to previous studies investigating the effect of nuclear import and export inhibition on the pathogenesis of VEEV. Specifically, importin-alpha subunit 1 (importin-alpha), importin-beta subunit 1 (importin-beta) and exportin-1 (CRM1) were identified. We were interested in identifying interactions between these proteins and WEEV capsid in order to assess if similar interactions are conserved across NW alphaviruses. While previous studies involving VEEV and these proteins proposed the formation of a tetrameric complex, a similar complex has not been implicated with WEEV (3). Relative conservation of NW capsid proteins may play a role in conserved protein interactions in host-cells.

Following selection of nuclear import and export protein category selection, interactions of WEEV capsid and members importin-alpha, importin-beta and CRM1 were validated through IP/WB (Fig. 10A). Using WB, detectable importin-alpha and CRM1 protein were detected following IP of WEEV capsid while importin-beta protein was not detected (Fig. 10B). The detection of these proteins in interaction with WEEV capsid suggest the association of WEEV capsid at the nuclear pore complex with importins, for the purpose of movement across or association with the nuclear pore. Additionally, the binding of CRM1, nuclear exporter could suggest association of WEEV capsid with CRM1 for purpose of export from the host-nucleus. Through the conducted IFA analysis of WEEV capsid protein localization, capsid is localized to both the nucleus and the cytoplasm, implying transport of capsid protein back to the nucleus following translation of the protein in the host-cell cytoplasm. Importin-alpha interactions could be the source of WEEV capsid localization to the nucleus through interactions of importin-

alpha with the NLS of WEEV capsid. Previous studies involving VEEV, have mapped NLS signals to importin alpha/beta binding. Additionally, WEEV capsid could be utilizing CRM1 to exit the nucleus through binding of capsid NES to CRM1. As previously elucidated with regard to VEEV, WEEV capsid could also be simultaneously binding both importin-alpha and CRM1.

Interestingly, importin-beta was not detected following IP/WB of WEEVcapsid-V5. There are several potential explanations for the detection of importin-beta in MS proteomic output but lack of detection by WB methods. Nuclear import mechanisms include transport of cargoes across the NPC through binding of NLS to importin-alpha (34, 35). Subsequently, importin-alpha classically binds importin-beta through an importin-beta binding motif (34, 35). Through the simultaneous binding process, cargo is transported across the nuclear pore (34, 35). One possible explanation for lack of detection of importin-beta is that during IP processing steps, importin-beta did not remain attached to importin-alpha and subsequently was lost in wash steps. It is possible that the interaction of these two import proteins is relatively weak and subsequently mechanical processing resulted in detachment.

Another possible explanation of the lack of detection of importin-beta detection by WB is that importin-alpha may be translocating from cytoplasm to the nucleus in an importin-beta independent manner (34, 35). Previous studies have observed that importin-alpha is transported across the NPC in an importin-beta and Ran-independent manner (34, 35). It is possible that WEEV capsid binds importin-alpha and subsequently importin-alpha interacts with protein members of the NPC such as nucleoporins to

traverse and deliver cargo to the nucleus. Previous studies have identified interactions between importin-alpha, Nup50, and Nup153 (34, 35). Nup50 contains a N-terminal binding domain that interacts with importin-alpha, and it also interacts with Nup153 within the nuclear basket to aid in transport of cargo (34, 35). Through interactions between these two Nup's, cargo is delivered across the nuclear pore (34, 35). In WEEV capsid MS data output, Nup153 was identified in proteins that were not considered for final validation studies. However, Nup50 and Nup214 were considered in the final list of proteins for validation. All three of these proteins are FG-repeat containing proteins that could aid in the docking of importin-alpha and WEEV capsid to the nuclear pore.

Ongoing studies for future understanding of these associations include observation of WEEV capsid localization with respect to nuclear importers and exporters by IFA. Previous studies involving VEEV have observed localization of capsid and the importin-alpha/beta complex and CRM1 and proposed a model of tetrameric complex formation at the nuclear pore (3). Observation of localization and possible co-localization of WEEV capsid and these proteins could be used to determine that a similar complex is formed with WEEV capsid. Additionally, ongoing studies include investigation of the effect of export and import inhibitors on the localization of WEEV capsid. Specifically, Leptomycin B, a CRM1 inhibitor will be utilized for observation of the effect of CRM1 inhibition on capsid localization. Previous studies conducted with VEEV, have observed that upon Leptomycin B treatment of VEEV TC-83, capsid is localized or remains confined to the nucleus (25). These studies implicate CRM1 as being a critical factor for nuclear export of capsid from the host-nucleus (25). It would be important to observe

localization patterns in context of WEEV capsid. Additionally, importin-alpha/beta inhibitors will be used to assess the localization of WEEV capsid upon treatment. An importin inhibitor, G231-1485 has been shown to reduce viral replication of VEEV in infected cells with low levels of cytotoxicity (33). This specific inhibitor will be used to assess the effect of inhibition of importins on the localization of WEEV capsid.

The current study does not address the potential for WEEV capsid to disrupt host-cellular processes such as transcription. Further investigations are necessary to determine whether WEEV capsid inhibits host-transcription upon nuclear association, like other NW alphaviruses. It would need to be further defined as to whether inhibition may be attributed to the formation of a complex at the nuclear pore.

Future studies will include generation of V5-tagged WEEV capsid virus to confirm that protein interactions between the host and capsid are conserved in the context of full-virus. Additionally, through use of tagged WEEV virus, the localization patterns can also be assessed and observe whether capsid localization is conserved in this context as well. Importin and exportin inhibitors will also be utilized for observation of localization differences in the tagged virus *in vitro*.

In addition to studies validating interactions of WEEV capsid, all three capsid-V5 molecular constructs, VEEV, EEEV and WEEV will be utilized in generation of a capsid-proteomic panel. Through IP and MS of NW alphavirus capsid proteins in parallel, common protein interactors will be identified and selected for validation. Following validation, further insight will be provided regarding conserved protein

interactions of NW alphavirus capsid proteins. Subsequently, therapeutic targets can be selected for disruption of conserved capsid-host protein interactions.

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