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Rotavirus NSP4 Interacts with Both the Amino- and Carboxyl-Termini of Caveolin-1

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Abstract

Rotavirus NSP4 plays multiple roles in viral pathogenesis, morphogenesis and replication. We previously reported a direct interaction between full-length NSP4 and the enterotoxin peptide composed of NSP4 residues 114–135 with full-length caveolin-1, the structural protein of caveolae. Caveolin-1 forms a hairpin loop in the cytoplasmic leaflet of plasma membrane caveolae. This unique orientation results in both termini of caveolin-1 exposed to the cytoplasm. The goal of this study was to map the caveolin-1 residues that interact with NSP4 to obtain a more complete picture of this binding event. Utilizing reverse yeast two-hybrid analyses and direct peptide binding assays, the NSP4 binding site was localized to caveolin-1 residues 2–22 and 161–178, at the amino- and carboxyl-termini, respectively. However, NSP4 binding to one of the termini was sufficient for the interaction.

Keywords

NSP4; enterotoxin; caveolae; caveolin-1; binding assay; yeast two-hybrid

1. INTRODUCTION

1.1 Rotavirus and Rotavirus NSP4

Rotavirus (RV) is the major etiologic agent of virally induced gastroenteritis in children and infants worldwide, contributing to 440,000 pediatric deaths annually (Parashar et al., 2003). RV non-structural protein 4 (NSP4) has been shown to be a multifunctional protein with key roles in viral assembly, replication and pathogenesis. During RV maturation, NSP4 functions as an intracellular receptor at the ER to bind and deliver immature double-layered progeny into the ER lumen. A specific interaction between the C-terminus of NSP4 and the structural viral protein, VP6, facilitates the budding of immature particles into the ER (Au et al., 1989; Bergmann et al., 1989). Recent studies show silencing NSP4 in RV-infected cells results in a 75% reduction in viral progeny and the redistribution of VP6 throughout the cell (Lopez et al., 2005). These data demonstrate the critical role of NSP4 in virus maturation. NSP4 also displays

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enterotoxigenic activity pertinent to RV pathogenesis. Purified NSP4 or the enterotoxigenic peptide, amino acids (aa) 114–135, induce diarrhea in neonatal mice by a Ca^{2+} -mediated signaling event (Ball et al., 1996; Dong et al., 1997). Further, NSP4 promotes plasma membrane (PM) Ca^{2+} permeability, resulting in influx of Ca^{2+} from extracellular sources (Dong et al., 1997). During subsequent cycles of RV replication, an NSP4 cleavage fragment (aa 112–175) is secreted from infected cells, presumably to interact with uninfected neighboring cells to exert its enterotoxigenic effects (Zhang et al., 2000). Thus NSP4 contributes to RV pathogenesis through a complex interplay with cellular components and other viral proteins.

1.2 NSP4 bypasses the Golgi apparatus

Recent reports have shown increasing evidence that NSP4 fails to traverse the Golgi while trafficking to the PM. First, high-mannose glycosylated NSP4 is found in Triton-X 100 resistant lipid rafts at the apical surface of polarized Caco-2 cells indicating NSP4 bypasses the endomannosidase-rich Golgi (Jourdan et al., 1997). Second, the NSP4_{112–175} fragment found in the media of RV-infected cultured cells appears to be secreted by a Golgi-independent mechanism (Zhang et al., 2000). Third, NSP4 is known to bind microtubules, causing a redistribution of COP1 and ERGIC-53, two markers of trafficking vesicles that cycle between the ER and Golgi apparatus (Xu et al., 2000). NSP4 colocalizes with these markers and redistributes them throughout the cell, which can be visualized as radial projections extending from the ER toward the PM and away from the Golgi (Xu et al., 2000). Fourth, co-localization of the NSP4-GFP fusion protein with the autophagosome marker LC3 has been reported (Wang and Klionsky, 2003). Autophagosomes typically traffic via a cytoplasm to vacuole or lysosomes pathway and by-passes the Golgi (Abeliovich et al., 2000). Taken together, these studies suggest that NSP4 utilizes an unconventional vesicular trafficking pathway upon exiting the ER and likely utilizes multiple mechanisms. One potential mechanism for such transit is the cell's caveolae membrane system.

1.3 Caveolae and caveolin-1

Caveolae are a subset of lipid raft PM microdomains. Traditionally described as flask-shaped PM invaginations, caveolae are rich in cholesterol, sphingomyelin, glycosphingolipids, signaling molecules, and caveolin proteins (Anderson, 1998). These unique lipid domains function in endocytosis, signal transduction, calcium homeostasis, and cholesterol transport. Caveolin-1 is the major structural protein of caveolae and functions in caveolae biogenesis such that expression of caveolin-1 in cells lacking detectable caveolae suffices to induce formation of PM caveolae (Campbell et al., 1999; Parton et al., 2006). Caveolin-1 has an unusual membrane orientation with a central hydrophobic domain (aa 102–134) predicted to form a hairpin loop in the PM cytoplasmic leaflet resulting in cytoplasmic exposure of both the N- and C- termini (Martin and Parton, 2005). Stable caveolin-1 oligomers form at the cytofacial leaflet via caveolin-1 monomer interactions at the oligomerization domain (aa 60–100). These oligomers comprise distinct filaments along the cytoplasmic face, and regulate caveolae vesicle formation by altering membrane curvature (Li et al., 1998; Monier et al., 1995); (Fernandez et al., 2002; Schlegel et al., 1999). The caveolin-1 scaffolding domain (CSD) at residues 80–100 has been shown to interact with multiple proteins, including G proteins and other signaling molecules (Scherer et al., 1996). It has been proposed that the CSD organizes the signaling molecules and events at the plasma membrane (Li et al., 1995).

Although caveolin-1 can traffic via the conventional secretory pathway, it also bi-directionally transports cholesterol from the ER to PM (Parton et al., 2006). Because NSP4 functions both at the ER and the cell periphery, and likely travels via a Golgi-bypassing pathway, the caveolae system is a reasonable candidate for the intracellular transport of NSP4. This is strengthened by the preferential interaction of NSP4 with model membranes that are anionic, rich in cholesterol and resemble caveolae (Huang, H. et al., 1999; Huang, H. et al., 2004; Huang, H.

et al., 2001). Direct interaction between caveolin-1 and NSP4 at residues 114–135 further suggests NSP4 could traffic with the caveolae membrane system (Bowman et al., 2000; Parr et al., 2006). NSP4 and caveolin-1 have been shown to interact via a hydrophobic interaction (Parr, et al., 2006) dependent on the amphipathic character of the C-terminus of NSP4 (Bowman et al., 2000). The goal of this study was to identify the NSP4 binding site(s) on the caveolin-1 protein to obtain a more complete picture of the NSP4-caveolin-1 binding event.

2. MATERIALS AND METHODS

2.1 Cloning

The Gateway™ cloning technology (Invitrogen, Carlsbad, CA), a universal cloning system that transfers gene segments between vectors via site-specific recombination (Hartley et al, 2000) was utilized to determine a protein-protein interaction between caveolin-1 and NSP4. The cloning of full length caveolin-1, full length NSP4, and NSP4_{80–140} into the Invitrogen Gateway™ shuttle vector, pENTR-11, and the yeast two-hybrid vectors pDEST-22 (encoding the GAL4 DNA binding domain) and pDEST-32 (encoding the GAL4 activation domain) has been previously described (Parr et al, 2006). Five caveolin-1 mutant clones were generated to test the scaffolding and oligomerization domains for binding to NSP4: Cav_{1–156}, Cav_{60–178}, Cav_{Δ60–100}, Cav_{Δ83–123}, and Cav_{60–156} (Fig. 1). Briefly, the mutant genes were amplified with True Fidelity DNA Polymerase (CLP, San Diego) from the template pENTR11-Cav-1 employing the oligonucleotides listed in Table 1. PCR products were ligated into XmnI/XhoI-digested pENTR-11 using T4 DNA ligase (Stratagene, La Jolla). A recombination event was used to subclone the mutant caveolin-1 genes from pENTR-11 to the yeast two-hybrid expression vectors pDEST22 and pDEST32 (Table 2) (Gateway™ manual). For *in vitro* binding assays, caveolin-1, NSP4, NSP4_{80–140}, and the new caveolin-1 deletion mutants were subcloned into the yeast expression vector pYES-DEST52 via a Gateway recombination reaction (Table 1) (Gateway™ manual). All plasmids were manipulated according to standard protocols in *E. coli* DH5α as described in the Gateway™ manual and sequence verified prior to use (Gene Technologies Lab, Texas A&M, College Station, TX).

2.2 Yeast Two-Hybrid Assay

The ProQuest™ Two-hybrid System with Gateway™ Technology (Invitrogen, Carlsbad, CA) was utilized as previously described (Parr et al, 2006). In brief, the Cav_{1–156}, Cav_{60–178}, Cav_{Δ60–100}, Cav_{Δ83–123}, and Cav_{60–156} plasmids were expressed as bait, fused to the GAL4 DNA binding domain (DBD) encoded by the plasmid pDEST22 and tested for binding with the prey, NSP4_{80–140} expressed as a fusion partner to the GAL4 activation domain (AD) encoded by the plasmid pDEST32 and vice versa. Selection of NSP4_{80–140} for these studies was based on previous two-hybrid data in which NSP4_{80–140} shows the strongest positive interaction with full length caveolin-1 (Parr et al, 2006). Caveolin-1 deletion mutants were selected based on exposure to the cytoplasm and the bias that NSP4 would interact at the CSD or oligomerization domain. The *Saccharomyces cerevisiae* strain MaV203 (used in two-hybrid assays) was cotransformed with bait and prey plasmids via a modified lithium acetate (LiAc) protocol (Geitz and Woods, 2002). Positive transformants were grown for 72 h at 30°C on complete synthetic medium lacking leucine and tryptophan (CSM Leu⁻Trp⁻) and then replica plated onto the following selective media: CSM Leu⁻Trp⁻Ura⁻, CSM Leu⁻Trp⁻ + 0.2% 5FOA, and CSM Leu⁻Trp⁻His⁻ + 3-Aminotriazole (AT, 25mM, 50mM, and 100mM). β-galactosidase activity qualitatively was detected in the presence of X-gal (5-bromo-5-chloro-3-indoyl-β-D-galactoside) and quantitatively by the addition of chlorophenolred-β-D-galactopyranoside (CPRG). Interaction of candidate protein pairs was determined by the induction of three reporter genes, *URA3*, *HIS3*, and *lacZ*, based on growth on selective media and β-galactosidase assays. For each caveolin-1 mutant construct (bait or prey), 75 clones were screened for

interaction with NSP4_{80–140}. Full length caveolin-1 was included as a positive control in addition to the panel of controls provided by the manufacturer.

2.3 Confirmation of Fusion Protein Expression by Western Blot Analyses

Yeast two-hybrid fusion protein expression was verified by western blot analyses of yeast lysates. Yeast expressing both bait and prey plasmids were grown overnight in CSM Leu⁻Trp⁻ at 30°C, the cells were pelleted, resuspended in YPAD [yeast extract, peptone (Difco), 0.01% adenine sulfate, 2% dextrose (Sigma)] and grown for an additional 48 h at 30°C. Yeast protein extracts were prepared with the Zymolase Yeast Protein Extraction kit (Zymo Research, Orange, CA) following the manufacturer's protocol. Equivalent protein concentrations as determined by BCA (Pierce, Rockford, IL) were separated by 12% SDS-PAGE, electroblotted onto nitrocellulose membranes and probed with rabbit anti-Cav_{2–22}, rabbit anti-Cav_{65–78}, or rabbit anti-NSP4_{114–135}. Reactive bands were visualized by the addition of goat HRPO-anti-rabbit IgG and SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL), and exposure to x-ray film.

2.4 Generation of Untagged Caveolin-1 Proteins

For over expression of caveolin-1 mutants and NSP4 in a different yeast strain (InVSc1), the expression vectors pYES-DEST52NSP4 (full length), pYES-DEST52NSP4_{HydroMut}, pYES-DEST52Cav_{1–178}, pYES-DEST52Cav_{1–156}, pYES-DEST52Cav_{60–178}, pYES-DEST52Cav_{Δ60–100}, pYES-DEST52Cav_{Δ83–123}, and pYES-DEST52Cav_{60–156} were transformed into the *Saccharomyces cerevisiae* strain InVSc1 (Invitrogen, Carlsbad, CA) by the LiAc protocol (Geitz and Woods, 2002). Transformants were selected on complete synthetic medium containing 2% (w/v) glucose and lacking uracil (CSM Ura⁻). Individual colonies from the CSM Ura⁻ plates were grown in 5ml liquid CSM Ura⁻ for 16–18 h at 30°C. The cultures were pelleted and resuspended in 5ml YPAG [yeast extract, peptone (Difco), 0.01% adenine sulfate, 2% galactose (Sigma)]. Induction of proteins expressed by the pYES-DEST plasmids in the presence of galactose continued for 24 h at 30°C. Protein extracts from yeast expressing full length and deletion mutants of caveolin-1 were prepared by the Zymolase Yeast Protein Extraction kit (Zymo Research, Orange, CA). Yeast expressing NSP4 and NSP4_{HydroMut} were lysed with YeastBuster (Novagen, San Diego) according to the manufacturer's protocol. All lysates were examined by western blot to verify protein expression. Primary antibodies included rabbit anti-caveolin_{2–22}, rabbit anti-Cav_{65–78} and rabbit anti-NSP4_{150–175}.

2.5 Peptide synthesis

Caveolin-1 peptides (Cav_{2–22} [SGGKYVDSEGHLYTVPIREQG], Cav_{68–80} [FEDVIAEPEGTHS], Cav_{76–101} [EGTHSFDGIWKASFTTFTVTKYWFFR], Cav_{161–178} [EAVGKIFSNNRINLQKEI]) were synthesized by standard fluorenylmethoxycarbonyl (Fmoc) solid-phase chemistry (Texas A&M University Peptide Synthesis Core Facility). Cleavage of the final peptide product from the solid polymer support and removal of side chain protecting groups was facilitated by the addition of 90% trifluoroacetic acid (TFA) in the presence of ethanedithiol (3%), thioanisole (5%) and anisole (2%). Following 2 h incubation at room temperature, the cleavage mixture was filtered into a polypropylene tube containing cold diethyl ether and extracted with cold diethyl ether an additional two times before drying under compressed nitrogen gas. Crude peptide was subjected to large scale, gravimetric gel filtration chromatography (Sephadex-G25, Amersham Biosciences, Arlington Heights, IL) and purification by reverse-phase HPLC (Delta Pak C4, Waters, Milford, MA). Plasma desorption mass spectrometry (PDMS) was performed on each of the purified peptides (Laboratory for Biological Mass Spectrometry, Department of Chemistry, Texas A&M University) to confirm the correct theoretical mass corresponded to 90% or greater of the final peptide product.

2.6 Antibodies

Rabbit antibodies were generated against synthetic peptides as described previously (Parr and Ball, 2003; Parr et al., 2006). Rabbit anti-caveolin-1 (68–75) was purchased from BD Transduction laboratories (San Diego, CA) and goat anti-rabbit-HRP was purchased from KPL (Gaithersburg, MD).

2.7 In Vitro Binding Assay

Synthetic peptides corresponding to NSP4_{114–135}, caveolin_{2–22}, caveolin_{161–178}, or caveolin_{76–101} were coupled to cyanogen bromide (CnBr)-activated Sepharose 4B (Amersham Biosciences, Piscataway) following the manufacturer's recommendation. Yeast lysates containing either NSP4 or caveolin-1 proteins (prepared as described above) were mixed with 50 µl of a 50% slurry of caveolin-1 or NSP4 peptide couple to sepharose beads, respectively, and rotated overnight at 4°C. The beads were pelleted, thoroughly washed, and resuspended in 5X SDS loading buffer. Equivalent amounts of each sample were analyzed by western blot analysis. The primary antibodies included peptide antisera prepared against Cav_{2–22}, Cav_{68–75}, and NSP4_{150–175}. Each experiment included InVSc1 lysates and uncoupled sepharose beads as negative controls.

3. RESULTS

3.1 Both the N- and C- termini of caveolin-1 interact with NSP4 in a yeast two-hybrid assay

A panel of caveolin-1 (cav) deletion mutants were expressed as fusion partners to both the activation domain (AD) and DNA binding domain (DBD) of the GAL4 transcription factor with the complimentary GAL4 factor fused to NSP4_{80–140} for yeast two-hybrid analyses. AD-cav-1 and DBD-cav-1 fusion proteins yielded essentially identical results when assayed with DBD- or AD-NSP4_{80–140}, respectively (Table 2). As noted above, NSP4_{80–140} was selected for these studies based on previous two-hybrid data showing a stronger interaction with caveolin-1 when the hydrophobic N-terminus is removed (Parr et al, 2006). The caveolin-1 deletion mutants were prepared based on the bias that NSP4 would interact at the CSD or oligomerization domain. Protein pairs were screened for interaction as determined by induction of transcription of three stably integrated reporter genes in the chromosome of MaV203, *URA3*, *HIS3*, *lacZ*. An overall score for the interaction phenotype of each protein pair was achieved by assimilating the data from each reporter gene. Initially, four caveolin-1 mutants were assayed for binding with NSP4_{80–140}: Cav_{1–156}, Cav_{60–178}, Cav_{Δ60–100} and Cav_{Δ83–123} (Fig. 1). These mutants lacked the C-terminus, the N-terminus, the oligomerization and CSD domain, and the CSD with a portion of the membrane traversing domain.

Transcription from the *URA3* gene was detected by growth on CSM Leu⁺Trp⁺Ura⁺ and lack of growth on CSM Leu⁺Trp⁺ + 5-Fluoroorotic acid (5FOA). Expression of *URA3* results in 5FOA toxicity and cell growth inhibition. Hence a lack of growth on CSM Leu⁺Trp⁺Ura⁺ and CSM Leu⁺Trp⁺ + 5FOA is possible with weak protein-protein interactions, (ProQuest™ manual). This interaction is illustrated by the protein pair RB-E2F1 (Table 2), which is integrated into control strain B of the ProQuest™ yeast two-hybrid system. Cav_{1–156}, Cav_{60–178}, Cav_{Δ60–100}, and Cav_{Δ83–123} failed to display growth on CSM Leu⁺Trp⁺Ura⁺ and demonstrated partial growth on CSM Leu⁺Trp⁺ + 5FOA when paired with NSP4_{80–140} (Table 2). While growth in the presence of 5FOA was not completely inhibited, these mutants displayed inhibition greater than the negative control A (no inserts) and comparable to the positive control protein pair Cav-1/NSP4_{80–140} (Table 2). Thus, each of these pairs induced transcription from the *URA3* promoter.

Two-hybrid dependent induction of the *HIS3* gene was monitored by growth on CSM Leu⁺Trp⁺His⁺ + 3-AT. MaV203 displays a basal level of *HIS3* activity that is specifically

inhibited in a dose-dependent manner by 3AT, allowing the detection of two-hybrid dependent *HIS3* induction above the basal level (ProQuest™ manual). Three concentrations of 3AT (12.5mM, 50mM, 100mM) were included in CSM Leu⁻Trp⁻His⁻ +3AT plates to both verify growth above basal levels and to trace the degree of inhibition with increased concentrations of 3AT. Cav₁₋₁₅₆, Cav₆₀₋₁₇₈, Cav_{Δ60-100}, Cav_{Δ83-123}/NSP₄₈₀₋₁₄₀ protein pairs showed growth on 12.5mM 3AT plates, and partial growth on plates containing 50mM 3AT, where the basal level of *HIS3* activity is inhibited. None of the caveolin-1/NSP₄₈₀₋₁₄₀ pairs was able to grow on plates containing 100mM 3AT, which suggested that the *HIS3* activity induced by each pair was inhibited by the high concentration of 3AT. Taken together, these data revealed that each of the four Cav/NSP₄₈₀₋₁₄₀ pairs induced *HIS3* transcription above the basal level of MAV203.

The third reporter gene, *lacZ*, was assayed for two-hybrid-induced activity via the addition of X-gal (5-bromo-5-chloro-3-indoyl-β-D-galactoside). As with other reporter gene phenotypes, the DBD- or AD-Cav mutants Cav₁₋₁₅₆, Cav₆₀₋₁₇₈, Cav_{Δ60-100} and Cav_{Δ83-123} when paired with AD- or DBD-NSP₄₈₀₋₁₄₀ appeared as the positive controls, producing a blue color in the presence of X-gal (Table 2). To confirm the two-hybrid induced *lacZ* transcription, the quantitative CPRG assay was employed. The average β-galactosidase units (BGU) for each DBD- or AD- Cav₁₋₁₅₆, Cav₆₀₋₁₇₈, Cav_{Δ60-100} and Cav_{Δ83-123} when reacted with AD- or DBD-NSP₄₈₀₋₁₄₀ were higher than that of the negative control strain A (average BGU 0.08), while consistent with the positive control strain B (average BGU 0.13) and the positive control pair caveolin-1/NSP₄₈₀₋₁₄₀ (average BGU 0.18) (Table 2). Thus all of the tested caveolin-1 mutant/NSP4 pairs induced *lacZ* transcription.

The three reporter gene activities acquired with the caveolin-1 mutants/NSP₄₈₀₋₁₄₀ were integrated to obtain positive or negative interaction phenotypes. All of the interaction phenotypes were positive indicating all of the caveolin-1 mutants bound NSP4. These data initially were confusing as we anticipated a negative phenotype for one or more of the reaction pairs. Alignment of all the cav-1 mutants indicated all of the mutants contained either or both termini. Therefore an additional caveolin-1 mutant was constructed to delineate the binding domain and to confirm the positive phenotype pattern of the Cav₁₋₁₅₆, Cav₆₀₋₁₇₈, Cav_{Δ60-100} and Cav_{Δ83-123}/NSP₄₈₀₋₁₄₀ pairs. In this mutant, both termini were deleted and the mutant was designated Cav₆₀₋₁₅₆ (Fig. 1). Yeast two-hybrid analysis was repeated and the Cav₆₀₋₁₅₆/NSP₄₈₀₋₁₄₀ pair showed a negative interaction phenotype (Table 2). The negative phenotype primarily was distinguished by the lack of qualitative and quantitative *lacZ* activity (Xgal and CPRG) as well as a lack of 5FOA associated inhibition, as evidenced by vigilant growth on CSM Leu⁻Trp⁻ +5FOA plates (Table 2). Each pair of constructs was tested in 5–10 separate yeast two-hybrid assays with consistent results.

3.2 Verification

To verify that the two-hybrid phenotypes resulted from the presence of caveolin-1 and NSP4, yeast extracts were prepared from yeast expressing the GAL4 fusion proteins and probed with NSP4- or caveolin-1-specific antibodies. All of the caveolin-1 mutant fusion proteins were detected as dimers in yeast transformed with the AD-Cav vectors (Fig. 2, panel A and B), but were absent in untransformed MaV203 (Fig. 2, lanes 1 and 3). With the AD fusion protein the Mr of the dimers ranged from slightly below to slightly above the 72 kD marker dependent on the mutant protein (Fig. 2, Panels A and B). Likewise, NSP₄₈₀₋₁₄₀ was detected in yeast transformed with DBD-NSP₄₈₀₋₁₄₀, but not in untransformed MaV203 (Fig. 2, panel C). These data confirmed that both NSP₄₈₀₋₁₄₀ and the caveolin-1 mutant plasmids were expressed in the yeast two-hybrid samples and were responsible for the observed interaction phenotypes.

To ensure that the negative phenotype observed with Cav₆₀₋₁₅₆ was not due to lack of protein expression, the co-transformed lysates similarly were examined by Western blot (Fig. 2D).

The Cav₆₀₋₁₅₆ fusion protein was observed as a monomer (~27 kD) and dimer (~54 kD) (Fig. 2D, lane 11) when probed with anti-Cav₆₅₋₇₈. The same lysates were probed with anti-NSP4₁₁₄₋₁₃₅ and showed the expression of the NSP4 fusion protein (Fig. 2E, lane 13). Infected cell lysates (2E, lane 14) were used as a positive control and untransformed yeast lysates as the negative control (2E, lane 12). Hence both Cav₆₀₋₁₅₆ and NSP4₈₀₋₁₄₀ fusion proteins were expressed in the co-transformed yeast but failed to associate. These data indicated at least one of the caveolin-1 termini must be present to bind NSP4 and both termini can interact with NSP4. We recognize that with two-hybrid data that a third protein may participate in the interaction of NSP4 and caveolin-1, i.e. a third protein could interact with both NSP4 and caveolin-1 rather than NSP4 and caveolin-1 directly binding. However this possibility should be detected in the verification binding assay.

3.3 Both termini of caveolin-1 bind NSP4

To confirm the yeast two-hybrid data, an *in vitro* binding assay was employed that utilized CnBr-activated sepharose beads coupled to NSP4 or caveolin-1 synthetic peptides to capture full-length or mutant caveolin-1 or NSP4, respectively, from transformed yeast cell extracts. Peptide-coupled sepharose beads were incubated with yeast lysates overnight at 4°C, thoroughly washed, and the bound proteins examined by western blot. NSP4₁₁₄₋₁₃₅ captured full length caveolin-1 (Fig. 3A, lane 2) as well as the Cav_{Δ83-123}, Cav_{Δ60-100} and Cav₁₋₁₅₆ mutants from the yeast extracts (Fig. 3A, lanes 4–6). In addition, full-length caveolin-1 was captured from MDCK mammalian cell lysates by the NSP4 114–135 peptide (Fig. 3A, lane 3). MDCK lysates were utilized because of their high expression of caveolin-1 (Parr et al, 2006), and to provide a link between induced and intrinsic experimental proteins. When the peptide-bound beads were reacted with untransformed InVSc1 yeast lysates, no caveolin-specific bands were detected (Fig. 3A, lanes 1, 7, 13), nor were caveolin bands observed in samples incubated with unbound beads (no peptide) (Fig 3A, lanes 7–12). Unreacted lysates were included to verify expression of the caveolin-1 mutant proteins (Fig. 3A, lanes 16–18) or full-length caveolin-1 (lanes 14–15). Because Cav₆₀₋₁₇₈ lacked the N-terminal residues 2–22, caveolin-1 antibodies generated to caveolin-1₆₈₋₇₅ were used to detect protein expression in the Western blot (Fig. 3B). No bands were observed in samples incubated with unbound beads (Fig. 3B, lane 2) and the positive control (lysates only) showed expression of Cav₆₀₋₁₇₈ (Fig. 3B, lane 1). When lysates were reacted with the NSP4₁₁₄₋₁₃₅ peptide linked to sepharose beads, a caveolin-1-specific band was seen at the same Mr as the lysates (Fig. 3B, lane 3). In addition, multimers of the caveolin-1 mutants were bound by the NSP4₁₁₄₋₁₃₅-linked beads. Two examples of multimer binding are shown in figure 3C. Taken together, these data confirmed the yeast two-hybrid results showing that NSP4 binds both termini of caveolin-1. The precise stoichiometry of binding remains unknown and will be pursued in another study.

Likewise, the same binding assay was performed with lysates expressing Cav₆₀₋₁₅₆. As anticipated, the NSP4₁₁₄₋₁₃₅ peptide linked to sepharose beads failed to associate with Cav₆₀₋₁₅₆ as no bands were visible by Western blot (data not shown).

To verify that NSP4 bound both termini in a separate binding assay, 3 synthetic peptides corresponding to the N-terminus (Cav₂₋₃₂), C-terminus (Cav₁₆₁₋₁₇₈) or central region (Cav₇₆₋₁₀₁) of caveolin-1 were synthesized, characterized, and linked to sepharose beads to repeat the peptide binding experiment in reverse. Sepharose-bound peptides were reacted with lysates expressing NSP4 or NSP4_{HydroMut}. As depicted in figure 4, both Cav₂₋₂₂ (N-terminus, lane 2) and Cav₁₆₁₋₁₇₈ (C-terminus, lane 5) captured full-length NSP4 from yeast lysates, whereas Cav₇₆₋₁₀₁ (central region) failed to capture NSP4 (Fig. 4, lane 8). NSP4 failed to interact with unbound beads (Fig. 4, lanes 10–12), and there was a lack of NSP4 in untransformed yeast (Fig 4, lanes 1, 4, 7). The NSP4_{HydroMut} mutant was used as a negative control as it has been shown that this mutant no longer binds caveolin-1 (Parr et al, 2006) (Fig.

4, lanes 3, 6, 9). Yet both proteins were expressed when lysates were directly evaluated (Fig. 4, lanes 14–15). These data agreed with the *in vivo* yeast two-hybrid results which showed that both the N- and C- termini of caveolin-1 bound NSP4, but either terminus was sufficient for binding.

4. DISCUSSION

The direct binding of NSP4 to full-length caveolin-1 initially was mapped to NSP4 residues 114–135 and subsequently to 3 hydrophobic aa in the same region (Parr, et al., 2006). The current study extends these findings by identifying the binding site for NSP4 at both termini of caveolin-1 that are localized in the cytoplasm. Using *in vivo* yeast two-hybrid analyses and *in vitro* peptide-based binding assays, we showed both the N- and C-termini of caveolin-1 were sufficient to bind full-length NSP4 and NSP4_{80–140}, and failed to bind untransformed yeast and NSP4_{HydroMut}. The data presented herein localized NSP4 interacting domains to caveolin-1 residues 2–22 at the N-terminus and aa 161–178 at the C-terminus. To our knowledge, these data are the second report of a protein interacting with both termini of caveolin-1 and the first report of a viral protein interacting with both termini. Dyn2 isoform of the membrane scission enzyme dynamin binds caveolin-1 residues 22–33 at the N-terminus as well as 21 residues of the C-terminus (Yao et al., 2005). Although unusual, these findings support our data and show caveolin-1 participates in complex protein-protein interactions beyond the scaffolding domain.

Because the CSD (aa 80–100) is reported as the protein interaction domain of caveolin-1, we initially hypothesized that NSP4 would bind within this region (Garcia-Cardena et al., 1997). However, caveolin-1 mutants that lacked significant portions of the CSD (Cav Δ 60–100 and Cav Δ 83–123) bound NSP4 in the yeast two-hybrid studies and *in vitro* binding assays (Table 2 and Fig. 2). These results were unexpected, as most caveolin interacting proteins bind residues within the CSD (aa 80–100) (Carman et al., 1999; Couet et al., 1997; Garcia-Cardena et al., 1997; Li, S. et al., 1996; Li et al., 1995; Li, Shengwen, Takashi Okamoto, Miyoung Chun, Massimo Sargiacomo, James E. Casanova, Steen H. Hansen, Ikuo Nishimoto, Michael P. Lisanti, 1995).

The hairpin topology of caveolin-1 in the membrane, cholesterol stabilization of both NSP4 helical structure and caveolin-1 integrity (Anderson, 1998), and the specific binding of NSP4 to caveolae-like membranes (Huang, H. et al., 2004; Huang, H. et al., 2001) indicate the membrane environment of caveolin-1 likely contributes to NSP4 binding. We propose the interacting, hydrophobic residues within the NSP4 C-terminal amphipathic helix interact with one or more hydrophobic amino acids in the cytoplasmic termini of caveolin-1 leaving the charged face of the NSP4 helix free to associate with the anionic phospholipids within the caveolae membrane. This would result in a complex of caveolin-1 and NSP4 along the cytofacial leaflet of PM caveolae that is stabilized by cholesterol. This model would account for the NSP4-caveolin-1 co-localization staining at the cell periphery, and the reported *in vivo* and *in vitro* binding events (Parr et al., 2006).

Since NSP4 bound to caveolin-1 outside of the CSD, we now propose this interaction induces a conformational change that influences CSD structure and prevent or disrupt CSD interactions with other signaling molecules. Alternatively, NSP4 binding to the N- and C-termini of caveolin-1 may cause inactive signal transducers to be released from the CSD, resulting in downstream signal activation as the CSD frequently holds signaling molecules in an inactive state (Williams and Lisanti, 2004). Perhaps the cytoplasmic arms of caveolin-1 act to ‘embrace’ NSP4, promoting NSP4 transport to the CSD, where the enterotoxin can access relevant signaling molecules and directly induce signaling pathways resulting in calcium mobilization and diarrhea. Given that one terminus of caveolin-1 was sufficient to bind NSP4 it is possible

differential binding to the N- or C-terminus results in different biological outcomes. Clearly additional studies are needed to elucidate the dynamic interplay between NSP4 and caveolin-1 and the biological effects.

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Figure 1.

Linear schematic of full-length (FL) and deletion mutants of caveolin-1. The N-terminus, C-terminus, caveolin-1 scaffolding domain (CSD, aa 80–100), hydrophobic domain that traverses the cytofacial leaflet of the plasma membrane (aa 102–134), and the oligomerization domain (60–100) were deleted to determine their role in binding NSP4.

Figure 2.

Western blot analyses of NSP4₈₀₋₁₄₀-, and full-length- and mutant caveolin-1-GAL4 fusion proteins expressed in MaV203 strain. Yeast were co-transformed with each of the fusion protein pairs and the lysates probed with NSP4- or caveolin-1-specific antibodies. **Panel A.** Untransformed and yeast transformed with the Cav₆₀₋₁₇₈-GAL4 fusion protein were probed with rabbit antibodies to Cav₆₅₋₇₈. A ~65 kD dimer of the Cav₆₀₋₁₇₈-GAL4 fusion protein was detected (lane 2); no reactivity was seen in the untransformed yeast lysates (lane 1). **Panel B.** The other caveolin-1 mutant-Gal4 fusion proteins were similarly tested for expression by Western blot analyses. Rabbit antibodies to Cav₂₋₂₂ were utilized to detect the Gal4-full length and -caveolin-1 deletion mutants in which the N-terminus remained intact. The full-length caveolin-1 fusion protein (lane 7) and all of the Cav-1 mutant-GAL4 fusion proteins (lanes 4–6) were detected as dimers. No band was observed in the untransformed yeast (lane 3). **Panel C.** To verify NSP4₈₀₋₁₄₀ fusion expression, Western blot analysis similarly was utilized to detect the NSP4 fusion protein in the same yeast lysates. The membrane was probed with anti-NSP4₁₅₀₋₁₇₅ and the expected Mr of the NSP4 fusion protein was seen (lane 9) that was absent from untransformed yeast (lane 8). **Panel D.** Western blot analysis of co-transformed yeast lysates showing the Cav₆₀₋₁₅₆ fusion protein was expressed as a monomer (~27 kD) and dimer (~54 kD) (lane 11). **Panel E.** The same co-transformed lysates analyzed in panel D were probed with anti-NSP4₁₁₄₋₁₃₅ and showed the expression of the NSP4 fusion protein (lane 13). The positive control (lane 14) was RV infected cell lysates and the negative control (lane 12) was untransformed yeast lysates.

Figure 3.

The NSP4₁₁₄₋₁₃₅ peptide binding assay confirms the yeast two-hybrid data. NSP4₁₁₄₋₁₃₅ was coupled to CnBr-activated sepharose beads and reacted with the transformed InVsc1 yeast lysates expressing full-length or mutant caveolin-1. Bound proteins were pelleted, washed and examined by Western blot analyses. **Panel A.** Yeast were transformed with an empty plasmid (InVsc1) (lanes 1, 7, 13) or the indicated caveolin-1 mutants. Yeast lysates were reacted with NSP4₁₁₄₋₁₃₅-bound sepharose beads (lanes 1–6), beads only (lanes 7–12), or lysates only were added directly to the gel to control for the presence of the mutant proteins (lanes 13–18). The bound proteins were separated by SDS-PAGE, transferred to nitrocellulose, and the membrane was probed with rabbit anti-Cav₂₋₂₂. To ensure antibody reactivity, MDCK lysates that normally express caveolin-1 were also tested in the binding assay (lanes 3, 9, 15). **Panel B.** Western blot analyses of the Cav₆₀₋₁₇₅ reacted with NSP4₁₁₄₋₁₃₅-bound beads. Antibodies to Cav₆₈₋₇₅ were utilized for detection of the caveolin-1 mutant. Lysates only (lane 1), reactivity with beads only (lane 2) and reactivity of Cav₆₀₋₁₇₅ reacted with NSP4₁₁₄₋₁₃₅-bound beads (lane 3) are shown. **Panel C.** The binding experiments were repeated several times with the

same results. The blot in panel C shows the multimeric forms of two of the mutant caveolin proteins that were bound by NSP4₁₄₄₋₁₃₅-linked beads. InVSC1 only served as the negative control and MDCK lysates were used as a positive control..

Figure 4. NSP4 is captured by peptides corresponding to the N- and C- termini of caveolin-1, but not by a peptide corresponding to the central region of caveolin-1. Untransformed yeast (lanes 1, 4, 7, 10, 13) and NSP4_{HydroMut} mutant that has been shown to not bind caveolin-1 (Parr, et al., 2006) (lanes 3, 6, 9, 11) served as the negative controls. Yeast lysates only showed both proteins were expressed (lanes 14–15). The N-terminal, Cav₂₋₂₂, and the C-terminal, Cav₁₆₁₋₁₇₈, peptides both captured NSP4 from yeast lysates expressing full-length NSP4 (lanes 2 and 5), whereas the peptide corresponding to the centrally located caveolin-1 residues, Cav₇₆₋₁₀₁, failed to capture NSP4 (lane 8).

Table 1

Oligonucleotides Used for Cloning Caveolin-1 Mutants, and Expression Vectors Used for Two-Hybrid and Peptide Binding Assays.

Clone	Forward Primer(s)	Reverse Primer(s)	AD-Y ^a	DBD-X pYES-DEST52 ^{b,c}
Cav _L -156	ATGTCTGGGGGCAAATA	atatctcgagttaca GAC GGT GTG GAC GTA	pDEST22Cav _L -156	pDEST32Cav _L -156 pYES-DEST52Cav _L -156
Cav ₆₀₋₁₇₈	atg AAC GAT GAC GTG GTC AA	atatctcgagTTATATTCTTTCTTGCAAGTTGAT	pDEST22Cav ₆₀₋₁₇₈	pDEST32Cav ₆₀₋₁₇₈ pYES-DEST52 Cav ₆₀₋₁₇₈
Cav _{Δ60-100}	TAAACACCTCCGCTTGCTG TCTGCC; ATGCTCTGGGGGCAAATA	AGACAGCAAGCGGAGGTGTTAGGGTCGC; atatctcgagTTATATTCTTTCTTGCAAGTTGAT	pDEST22Cav _{Δ60-100}	pDEST32Cav _{Δ60-100} pYES-DEST52Cav _{Δ60-100}
Cav _{Δ83-123}	GTT TTG ACT TCC TGC ACA TC; ATGCTCTGGGGGCAAATA	GTCAGGAAAGTCAAAACTGTG; atatctcgagTTATATTCTTTCTTGCAAGTTGAT	pDEST22Cav _{Δ83-123}	pDEST32Cav _{Δ83-123} pYES-DEST52Cav _{Δ83-123}
Cav ₆₀₋₁₅₆	atg AAC GAT GAC GTG GTC AA	atatctcgagttacACAGCGGTGTGGACGT	pDEST22Cav ₆₀₋₁₅₆	pDEST32Cav ₆₀₋₁₅₆ pYES-DEST52 Cav ₆₀₋₁₅₆

^a AD-Y denotes the activation domain of the GAL4 transcription factor fused to a known protein, Y, each of which are encoded by the yeast two-hybrid expression vector pDEST-22.

^b DBD-X denotes the DNA-binding domain of the GAL4 transcription factor fused to a known protein, X, each of which are encoded by the yeast two-hybrid expression vector pDEST-32.

^c pYES-DEST52, was used to over-express transformed proteins in *Saccharomyces cerevisiae*; also utilized was pYES-DEST52NSP4 and pYES-DEST52NSP480–140.

Table 2
Interaction and Reporter Gene Phenotypes for DBD-NSP480-140 and AD-caveolin-1 mutants.

AD/DBD ^a	-L-T-U ^b	5FOA ^c	-L-T-H + 3AT ^d			Xgal ^e	CPRG ^f	Interaction Phenotype ^g
			12.5mM	50mM	100mM			
			+	+	+			
No insert/No insert (Control A)	-	+	+	-	-	-	-0.08	Negative
RB/E2F1 (Control B)	-	-	+	+	-	+	+0.13	Positive
Cav-1/N ₈₀₋₁₄₀	-	+/+	+	+/+	-	+	+0.18	Positive
Cav ₁₋₁₅₆ /N ₈₀₋₁₄₀	-	+/+	+	+/+	-	+	+0.20	Positive
Cav ₆₀₋₁₇₈ /N ₈₀₋₁₄₀	-	+/+	+	+/+	-	+/+	+0.20	Positive
Cav ₁₆₀₋₁₀₀ /N ₈₀₋₁₄₀	-	+/+	+	+/+	-	+	+0.17	Positive
Cav ₁₈₃₋₁₂₃ /N ₈₀₋₁₄₀	-	+/+	+	+/+	-	+	+0.18	Positive
Cav ₆₀₋₁₅₆ /N ₈₀₋₁₄₀	-	+	+	+/+	-	-	-0.09	Negative

^aMaV203 were co-transformed with shown AD/DBD pairs and grown on CSM-Leu-Trp plates at 30°C for 72 h. Transformants were replica plated onto a panel of selective media and reporter gene phenotypes were scored as growth (+), slight growth (+/-) or no growth (-).

^bControl and test colonies were replica plated onto CSM-Leu-Trp-Ura.

^cControl and test colonies were replica plated onto CSM-Leu-Trp+ 0.2% 5FOA.

^dControl and test colonies were replica plated onto CSM-Leu-Trp-His +3AT (12.5mM, 50mM, and 100mM).

^eControl and test colonies were replica plated onto YPAD, overlaid with a nitrocellulose membrane and incubated with Xgal.

^fControl and test colonies were evaluated by CPRG assay for quantitative measurement of β-galactosidase activity. β-galactosidase units (BGU) exceeding those of RB/E2F1 (control B) were considered positive.

^gInteraction phenotypes were determined by assimilating the reporter gene activities of each AD/DBD pair.