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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 enterotoxic 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 enterotoxic activity pertinent to RV pathogenesis. Purified NSP4 or the enterotoxic 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 enterotoxic 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

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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 et al., 1999, 2004, 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 and Ball, 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: $Cav_{1–156}$, $Cav_{60–178}$, $Cav_{\Delta 60–100}$, $Cav_{\Delta 83–123}$, 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

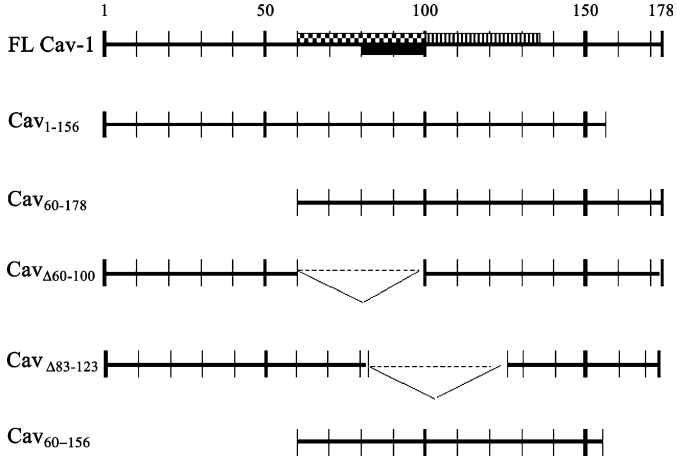

Fig. 1. Linear schematic of full-length (FL) and deletion mutants of caveolin-1. The N-terminus, C-terminus, caveolin-1 scaffolding domain (CSD, aa 80–100), hydrophobic domain that traverses the cytofacial leaflet of the plasma membrane (aa 102–134), and the oligomerization domain (60–100) were deleted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