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The interaction of Rotavirus A pig/China/NMTL/2008/G9P[23] VP6 with cellular beta-actin is required for optimal RV replication and infectivity

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ABSTRACT

VP6 forms the intermediate layer of the rotavirus (RV) capsid, and it plays important roles after RV penetration and uncoating. These functions rely on its ability to interact with host cell proteins. To gain further insights into the role of VP6 in porcine RV (PoRV) infection, a glutathione S-transferase pull-down assay was utilized to find unknown cellular factors that interact with VP6. In this study, beta-actin, tropomyosin 1, and 40S ribosomal protein S16 were identified as interaction partners of VP6 by mass spectrometry and co-immunoprecipitation. The interaction with beta-actin was further studied. By immunoelectron microscopy, we observed VP6 proteins that labeled with colloidal gold localized on the actin microfilaments at the early stage of PoRV infection, we also found VP6 distributed in the ribosome, mitochondria, endoplasmic reticulum and nucleus in the infected cells. Actin binding protein spin-down assays verified PoRV double-layered particles (DLPs) bound to F-actin in vitro, but didn't have actin polymerization enhancement activity. After a small interfering RNA (siACTB) was used to knock down beta-actin expression, PoRV VP6 expression and the infection rates of newly synthesized virions releasing into culture supernatants decreased dramatically. Our results confirm and extend previous reports indicating that the interaction between PoRV VP6 and beta-actin plays vital roles in the PoRV lifecycle.

1. Introduction

Porcine rotavirus (PoRV) belongs to the genus *Rotavirus* in the family *Reoviridae*, and it is the most common viral causative agents of neonatal diarrhea in suckling and weaned piglets. PoRV particles are non-enveloped, icosahedral, triple-layered particles (TLPs), and the viral genome is formed by 11 segments of double-stranded RNA (Patton et al., 2006), which encode six structural proteins (VP1 to VP4, VP6, and VP7) and five to six nonstructural proteins (NSP1 to NSP5/6) depending on strain (Martella et al., 2010). VP4 and VP7 make up the outermost layer of the TLPs. Once inside a cell, TLPs lose the surface proteins VP4 and VP7 then deliver double-layered particles (DLPs) into the cytoplasm, which are covered by VP6 (Mattion et al., 1988). The core of the virion is composed of VP2 and encloses the viral genome (Patton et al., 1997), the RNA-dependent RNA polymerase VP1, and the viral capping enzyme VP3.

In recent years, VP4, VP6, and VP7 of PoRV have been intensively studied because of their unique biological functions (Zhang et al., 2015), and their association of host cell proteins in the RV replication cycle has been analyzed (Silva-Ayala et al., 2013). VP4 and VP7 are responsible for the initial interactions of RV with host cells (Crawford et al., 2001). These interactions are mostly involved in receptor binding (Arias et al., 2001) and a post-attachment step (Graham et al., 2003), which frequently triggers cellular signaling cascades that facilitate virus entry or replication (Arias et al., 2015). VP6 is the most abundant PoRV structural protein, and it plays important roles after virus penetration and uncoating; thus, interactions between VP6 and viral/cellular proteins will involve in several major steps of RV infection after DLPs formation in the cytoplasm. To date, certain interactions between VP6 and other RV proteins, as well as their associated functions, have been reported. For example, VP6 residues located at the interface with VP2 are essential for capsid assembly and transcriptase activity (Charpilienne et al., 2002). Each VP7 trimer clamps onto an underlying VP6 trimer, while the interaction is involved in RV assembly (Gilbert et al., 2001). The VP5* foot interacts both with VP6 and VP7, and

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this interaction recruits cytosolic VP4 onto the DLPs and presumably templates its trimerization (Settembre et al., 2011). Additionally, NSP4 serves as an intracellular receptor for the nascent DLPs by interacting with VP6, and participates in the budding of the particles into the lumen of the ER (Desselberger, 2014). Furthermore, several cellular factors have been shown to interact with VP6. VP6 has been implicated in heat shock cognate protein Hsc70-mediated RV infection (Gualtero et al., 2007). Calmodulin was also found to directly interact with VP6 in the presence of Ca^{2+}, and this interaction positively regulates RV propagation (Chattopadhyay et al., 2013). However, in view of its multifunctional nature, VP6 may associate with plenty of other cellular factors during RV infection.

Our lab had isolated Rotavirus A pig/China/NMTL/2008/G9P[23] (abbreviated as NMTL strain) from China (Shi et al., 2012), which is an unusual genotype constellation and combination for PoRVs, for G9 strain is often found in humans group A rotavirus (GARV) (Silva et al., 2013). GARV is considered the most important due to high prevalence and pathogenicity (Chandler-Bostock et al., 2015). In addition to the economic importance in pigs, there is potential for zoonotic transmission to humans, as pigs may act as critical reservoirs for new RV strains with variable genotypes (Nagai et al., 2015). In this study, we used NMTL strain to find unknown interaction partners of PoRV VP6 in the MA104 rhesus monkey kidney cell line, to investigate the functions of these interactions in PoRV replication and pathogenicity. Our results provide additional insights into the PoRV infection process, and increase our understanding of the critical role of the VP6 protein in the complex process of sequential virus-cell interactions.

2. Materials and methods

2.1. Cells and virus

The rhesus monkey kidney cell line MA104 was grown in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), at 37 °C in a 5% CO_2 incubator. The NMTL strain was isolated in our laboratory (Shi et al., 2012). PoRV was propagated in MA104 cells as previously described (Arnold et al., 2009).

2.2. Antibodies

Mouse monoclonal antibodies (mAbs) 1F4 and 2E2 against PoRV VP6 were prepared in our laboratory, by fusing SP2/0 cells with spleen cells from BALB/c mice immunized with recombinant group A PoRV VP6-His protein, and the titers in cell culture medium of the hybridoma and ascites were 1:12800 and $1:10^6$, respectively. In addition, these MAbs were positively reacted with authentic VP6 protein of group A PoRV detected by western blot and indirect immunofluoresence assay. Anti-beta actin antibodies (ab8226, ab6276), anti-tropomyosin 1 (TPM1) antibodies (ab133292, ab155260) and anti-RPS16 antibodies (ab177951) were purchased from Abcam (Cambridge, UK). Colloidal gold-labeled goat anti-mouse IgG were purchased from Sigma-aldrich (St. Louis, MO, USA). Mouse anti-tubulin mAb and 4′,6-diamidino-2-phenylindole (DAPI) staining solution were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA).

2.3. Construction of recombinant expression plasmid

The VP6 gene of the PoRV NMTL strain (nucleotide sequence accession number: JF781162) was amplified using primers F-PoRV-VP6 (5′–GCGGATCC**ATGGAGGT TCTGTATTCATTGTC**–3′) (Nucleotide position No.: 1–23) and R-PoRV-VP6 (5′–GGCTCGAG**TCACTTAATCAACATGCTTCTAAT**–3′) (Nucleotide position No.: 1171–1194), which contain BamHI and XhoI restriction endonuclease sites (underlined), respectively. The VP6 polymerase chain reaction (PCR) products were ligated into the glutathione S-transferase (GST) fusion vector pGEX-6P-1 (GE Healthcare, Little Chalfont, UK), and the GST tag was ligated to the 5′ of VP6 gene in the fusion protein. The resulting recombinant plasmid pGEX-PoRV-VP6 was verified by DNA sequencing. GST and the GST-VP6 fusion protein were expressed in the *Escherichia coli* BL21 (DE3) strain by induction with 1 mM isopropyl-β-D-thiogalactopyranoside. After ultrasonication and centrifugation at 8000 rpm for 10 min, soluble supernatants were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the GST-VP6 fusion protein was purified using a GST affinity column and identified by western blotting.

2.4. GST pull-down assay

Purified GST-VP6 fusion protein was conjugated to glutathione-Sepharose beads at 4 °C overnight. MA104 cells were washed three times with phosphate-buffered saline (PBS, pH 7.4), and then lysed in 1 mL of radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate) containing the protease inhibitor phenylmethanesulfonyl fluoride (PMSF, 1 mM). After centrifugation at 12,000 × *g* for 15 min, total cell supernatants were incubated with the GST-VP6-loaded glutathione-Sepharose beads as described previously (Zhang et al., 2014). The binding reaction was performed at 4 °C overnight, and then the beads were washed four times with ice-cold buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% NP-40). Bound proteins were resolved by SDS-PAGE. A pull-down using GST alone served to identify proteins that bound non-specifically to GST or the glutathione-Sepharose beads. GST-VP6-loaded glutathione-Sepharose beads that were not incubated with cell lysates were used as a negative control. After comparison with the control samples, differential protein bands bound to the GST-VP6-loaded glutathione-Sepharose beads were excised from the gel and identified using matrix-assisted laser desorption/ionization dual time of flight (MALDI-TOF/TOF) mass spectrometry (MS).

2.5. Co-immunoprecipitation (co-IP) assay and western blotting

MA104 monolayer cells were infected with the PoRV NMTL strain at a multiplicity of infection (MOI) of 1. The lysates of MA104 cells infected for 24 h were prepared with RIPA lysis buffer containing 1 mM PMSF. After centrifugation at 12,000 × *g* for 15 min, lysate supernatants were pretreated with protein A + G agarose (Beyotime Biotechnology Co., Ltd.) for 30 min at 4 °C to eliminate proteins that non-specifically bound to the agarose beads. Subsequently, lysate supernatants were added to 50 uL of fresh protein A + G agarose, and the mixtures were incubated with anti-PoRV VP6 (2E2) or anti-beta-actin mAb (ab8226), TPM1 RabmAb (ab133292), RPS16 RabmAb (ab177951) at 4 °C overnight, respectively. After washing four times with lysis buffer, the isolated, co-immunoprecipitated proteins were then analyzed by western blotting using antibodies against beta-actin, TPM1, RPS16 and PoRV VP6. The lysates of PoRV mock-infected MA104 cells were used as a control.

2.6. Immunoelectron microscopy

To observe the distribution of PoRV VP6 at the early stage of PoRV infection, MA104 cells were infected with the PoRV NMTL strain at an MOI of 50. At 20 min, 40 min, 60 min post-infection, infected cells were collected and fixed, then dehydrated by