Virus Research 200 (2015) 56–63

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Identification of the interaction between vimentin and nucleocapsid protein of transmissible gastroenteritis virus

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ARTICLE INFO

Article history:
Received 17 October 2014
Received in revised form
11 December 2014
Accepted 11 December 2014
Available online 20 December 2014

Keywords:
Vimentin
Nucleocapsid
Transmissible gastroenteritis virus

ABSTRACT

Nucleocapsid (N) protein of transmissible gastroenteritis virus (TGEV) packages viral RNA genome to form a ribonucleoprotein complex. In addition to its function as a structural protein, N protein is involved in cell apoptosis or cell-cycle regulation. N protein possibly interacts with host factors to modulate cellular functions. To identify cellular proteins that interacted with N protein of TGEV, methods of GST pull-down and Co-IP were utilized to precipitate cellular proteins of swine testicular (ST). Bound cellular proteins were resolved by SDS-PAGE. Analysis of interacting proteins by mass spectrometry allowed identification of 15 cellular protein bands representative of 12 cellular proteins including vimentin that bound to N protein. Furthermore, the function of vimentin cytoskeleton in ST cells during TGEV infection was examined. Vimentin cytoskeleton was required for virus replication. The present study thus provides protein-related information about interaction of TGEV N protein with host cell that should be useful for understanding host cell response to coronavirus pathogenesis infection and the underlying mechanism of coronavirus replication.

1. Introduction

Coronaviruses (CoVs), a genus in the *Coronaviridae* family, are pleomorphic, enveloped viruses (Perlman and Netland, 2009). CoVs have been clustered in the *Cornavirinae* subfamily, which includes three approved genera, *alpha*-, *beta*- and *gammacoronavirus*, as well as a tentative new genus, the *deltacoronavirus* (de Groot et al., 2011; Reguera et al., 2012). Transmissible gastroenteritis virus (TGEV) is a representative CoV in the *alphacoronavirus* genus; severe acute respiratory syndrome-related coronavirus (SARS-related CoV) is a representative of the *betacoronavirus* genus; infectious bronchitis virus (IBV) is a representative of the *gammacoronavirus* genus; and Bulbul-CoV is a representative of the *deltacoronavirus* genus (de Groot et al., 2011). The *Coronaviridae* are involved in respiratory, enteric, hepatic and neuronal infectious disease in animals and humans (Perlman and Netland, 2009).

TGEV infection causes severe diarrhea in suckling piglets (about 2 weeks old), which results in enormous economic loss in swine-producing areas in the world (Kim and Chae, 2001; Sestak et al., 1996). About two-thirds of the TGEV genome (28.5 kb) encodes the replicase gene (rep) at the 5′ end, and one-third of the genome encodes other viral genes at the 3′ end in the order 5′-S-3a-3b-E-M-N-7-3′ (Penzes et al., 2001). The genome of the TGEV encodes four structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N). N proteins of CoVs are highly basic with a molecular mass ranging from 40 to 63 kDa, depending on the species and strains. N protein binds to the RNA genome, forming a helical nucleocapsid (Escors et al., 2001; Sturman et al., 1980).

Recently, some reports showed that N protein of TGEV play an important role in host cell for virus replication. N protein of TGEV facilitates template switching and is required for efficient transcription (Zuniga et al., 2010). N protein underwent proteolysis in parallel with the activation of caspases within host cell and N protein of TGEV is a substrate for caspases (Eleouet et al., 2000). TGEV N protein nucleolus localization was found in transfection experiments and might induced a cell cycle delay or arrest to facilitate virus replication (Wurm et al., 2001). In contrast, TGEV N protein was not accumulated in the nucleus in the infection context (Calvo et al., 2005). In addition, the role of TGEV N protein in cell cycle arrest has been recently reported (Ding et al., 2014). N protein of TGEV may function through direct or indirect interaction with cellular proteins.

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http://dx.doi.org/10.1016/j.virusres.2014.12.013
0168-1702/

For better understanding of the mechanisms associated with pleiotropic functions of N protein, cellular proteins of swine testicular (ST) cells were pulled down associated with N protein using glutathione (GST)-tagged full-length N proteins immobilized on GST agarose. And cellular proteins of ST cells were precipitated using N protein mAb. By SDS-PAGE coupled with mass spectrometry (MS), a total of 12 cellular proteins interacting with N protein were successfully identified. Information on the expanded repertoire of cellular proteins interacting with N protein will provide a framework for future biochemical analyses of these protein functions in TGEV infection.

2. Materials and methods

2.1. Cells and virus

ST cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum under standard culture conditions (5% CO_2, 37 °C). TGEV infectious strain H (Accession No. FJ755618) (Wang et al., 2010) was propagated on an ST cell monolayer.

2.2. Antibodies

Mouse mAb to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab9484) and Rabbit poloclonal antibody vimentin (ab92547) were purchased from Abcam. FITC-labeled goat anti-mouse IgG was purchased from Kirkegaard and Perry Laboratories (KPL). TRITC-labeled goat anti-rabbit IgG was purchased from Sigma. The mAb to N protein of TGEV were donated from Mr. Wang Shao (Institute of Animal Husbandry and Veterinary Science, Fujian Academy of Agricultural Science, China).

2.3. Cell infection

ST cells were plated in six-well plates 1 day prior to infection with TGEV infectious strain H at a multiplicity of infection (MOI) of 1. TGEV not-infected samples were exposed to culture medium alone. After adsorption for 1 h, cells were washed twice and incubated in fresh RPMI-1640.

2.4. GST pull-down assay

The prokaryotic expression plasmid pGEX-TGEV-N was constructed previously (Zhang et al., 2014). *Escherichia coli* BL21 (DE3) strain containing pGEX-TGEV-N plasmid was expressed under induction of 1 mM isopropyl-β-D-thiogalactopyranoside. GST pull-down assay was performed as previously described (Zhang et al., 2014). Expressed GST protein was used as a control.

2.5. Co-immunoprecipitation (Co-IP) assay

The lysate of TGEV-infected ST cells was prepared with RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% deoxycholate) containing a protease inhibitor phenylmethanesulfonyl fluoride (PMSF) (1 mM). After centrifugation at 12,000 × *g* for 15 min, lysate supernatant was pretreated with 2 μL mouse IgG control (Beyotime) and protein A/G plus-agarose (Santa Cruz Biotechnology) for 30 min at 4 °C to eliminate non-specific binding to agarose gel. The lysate supernatant (500 μg) was incubated with 1 μg of mAb to N protein of TGEV for overnight at 4 °C. Then, 20 μL resuspended protein A/G plus-agarose was added to this mixture and incubated at 4 °C on a rocker platform for 2 h. After washing four times with lysis buffer, isolated immunoprecipitated proteins (boiling 10 min with PAGE sample loading buffer) were then analyzed by 12% PAGE analysis. The lysate of TGEV not-infected ST cells was used as a control.

2.6. Protein identification by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF/TOF) MS

The gels described above stained with PhastGel Blue R (GE Healthcare). Protein bands of interest were manually excised from gels. MALDI-TOF/TOF was performed as previously described (Zhang et al., 2009). Data were searched by GPS Explorer (ver. 3.6) with the search engine MASCOT (ver. 2.1). The search parameters were as follows: National Center for Biotechnology Information non-redundant (NCBInr) database (release date, July 2011), and the database *Sus* (41,373 sequences; 16,019,616 residues); a trypsin digest was performed with one missing cleavage, MS tolerance was set at 100 ppm and MS/MS tolerance at 0.6 Da. Known contaminant ions (tryptic autodigest peptides) were excluded. MASCOT protein scores (based on combined MS and MS/MS spectra) >59 were considered statistically significant ($p \leq 0.05$). Individual MS/MS spectrum, with a statistically significant (confidence interval ≥ 95%) ion score (based on MS/MS spectra), was accepted. To eliminate redundancy of proteins that appeared in database under different names and accession numbers, single protein member belonging to species *Sus* or with the highest protein score (top rank) was separated from multi-protein family.

2.7. Western blotting

Equivalent amounts of cell lysates were subjected to 12% PAGE and then transferred to 0.22 μm nitrocellulose membranes (Hybond-C Extra, Amersham Biosciences). After blotting, membranes were incubated with mouse pAb to vimentin or with mouse mAb to N protein (1:2000) at 37 °C for 1 h. After washing three times with PBST, membranes were inoculated with DyLight™ 800-labeled antibody to mouse IgG (H+L) (1:10,000, KPL, USA) or DyLight™ 800-labeled antibody to rabbit IgG (H+L) (1:5000, KPL, USA) at 37 °C for 45 min. Images were visualized by Odyssey Infrared Imaging System (LI-COR).

2.8. Immunofluorescence assay

ST cells inoculated with TGEV were cultured for 0, 1, 2, 4, 8, and 16 h. Cells were washed twice with PBS and fixed with paraformaldehyde (4%) for 30 min at 4 °C, and then allowed to air dry. After blotting with 5% skimmed milk powder, the fixed cells were incubated with mAb to TGEV N protein (1:200) and rabbit pAb to vimentin (1:100, Abcam) for 1 h at 37 °C in a humidified chamber. After washing three times with PBST, the fixed cells were incubated with FITC-labeled goat anti-mouse IgG (1:100, KPL) and TRITC-labeled goat anti-rabbit IgG (1:200, Sigma). Additional nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) was performed as described previously (Jungmann et al., 2001). The triple-stained cells were washed three times with PBST and subsequently examined under a Leica TCS SP5 laser confocal microscopy.

2.9. Transfection of siRNA against vimentin

siRNA against vimentin (GenePharma) was used for transfection. Sequence of the siRNA strands (two rounds of silencing) were as follows: 5′-GCUAACUACCAAGACACUATT-3′ (sense) and 5′-UAGUGUCUUGGUAGUUAGCTT-3′ (antisense); 5′-CCUCUGGUUGACACCCAUUTT-3′ (sense) and 5′-AAUGGGUGUCAACCAGAGGTT-3′ (antisense). Negative control siRNA strands were as follows: 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense), 5′-ACGUGACACGUUCGGAGAATT-3′ (antisense). Transfection with siRNA was performed with Lipofectamine 2000 reagent (Invitrogen) by following the manufacturer's instructions. ST cells were cultured overnight in six-well tissue culture plates. The siRNA (20 nM) was complexed with Lipofectamine 2000 reagent by incubating