Virus Research 201 (2015) 16–23

Contents lists available at ScienceDirect

Virus Research

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

Annexin 2 is a host protein binding to classical swine fever virus E2 glycoprotein and promoting viral growth in PK-15 cells

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ARTICLE INFO

Article history:
Received 13 November 2014
Received in revised form 9 February 2015
Accepted 11 February 2015
Available online 19 February 2015

Keywords:
Classical swine fever virus
E2
Interaction
Annexin 2

ABSTRACT

Glycoprotein E2 of classical swine fever virus (CSFV) is a key determinant and major immunogen for viral entry and immunity, but little is known about its interaction with host proteins. In a previous study, we showed by proteomic analysis that cellular membrane protein annexin 2 (Anx2) was up-regulated in PK-15 cells following CSFV infection, but its function in CSFV replication remains unknown. In the present study we observed the interaction of Anx2 with CSFV E2 following infection of PK-15 cells by co-immunoprecipitation (Co-IP), mass spectrometry, Western blot and confocal laser scanning microscopy. The interaction between CSFV E2 and Anx2 was further confirmed in an E2-expressing PK-15 cell line, in which up-regulation of Anx2 was also observed, indicating that E2 alone can interact with, and increase, the expression of Anx2 protein. Further studies showed that siRNA-mediated knock-down and plasmid-mediated over-expression of Anx2 in PK-15 cells inhibited and increased CSFV replication and proliferation respectively. Remarkably, treatment of PK-15 cells with Anx2-specific polyclonal antibody prior to virus infection significantly inhibited CSFV multiplication, indicating that Anx2 is a cellular membrane protein likely associated with CSFV entry into cells. In conclusion, Anx2 is the novel host protein identified to interact with CSFV E2 and promote CSFV multiplication. These observations provide support for the potential use of Anx2 as a cellular target for the development of novel anti-CSFV therapies.

1. Introduction

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a notifiable disease of OIE causing substantial economic losses for the pig industry worldwide (Thiel and Moennig, 1996). CSFV has a positive-stranded RNA genome that contains a single large open reading frame encoding a 3898 amino acid polyprotein which undergoes co- and post-translational processing to generate 12 viral proteins, including structural proteins C, E^{rns}, E1, E2 and nonstructural proteins N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5 (Lindenbach, 2007; Meyers et al., 1989; Ruggli et al., 1996; Rumenapf et al., 1993). E2 is the major envelope glycoprotein exposed on the outer surface of the CSFV virion, and mediates viral adsorption to the cell, determines virulence, and induces the major neutralizing antibodies (Reimann et al., 2004; Risatti et al., 2005; Gavrilov et al., 2011; Weiland et al., 1990; Zhou et al., 2011).

A number of host proteins have been identified to be involved in CSFV replication and pathogenesis. Of these, CSFV N^{pro} protein interacts with several cellular proteins involved in apoptosis, the immune response, IFN production and viral growth, including interferon regulatory factor 3 (IRF3) (Bauhofer et al., 2007), IκBα (an inhibitor of NF-κB) (Doceul et al., 2008), anti-apoptotic protein HAX-1 (Johns et al., 2010) and poly(C)-binding protein 1 (Li et al., 2013a). Glycoprotein E^{rns} interacts with cellular membrane-associated heparan sulfate (HS) to facilitate viral entry (Hulst et al., 2000). The C protein interacts with small ubiquitin-like modifier (SUMO-1) and a SUMO-1 conjugating enzyme, and disruption of its binding to these two proteins attenuates its virulence in swine (Gladue et al., 2010). The interaction of C protein with a cytoskeleton regulator IQGAP1 is essential for completion of virus growth *in vitro* and for its virulence *in vivo* (Gladue et al., 2011), but its interaction with hemoglobin subunit-β inhibits CSFV growth and replication by triggering the IFN signaling pathway (Li et al., 2013b). However, little is known about the interaction between E2 and

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http://dx.doi.org/10.1016/j.virusres.2015.02.013
0168-1702/

host proteins. Our previous studies have shown that CSFV infection up-regulates Anx2 expression in both PK-15 cells *in vitro* and in peripheral blood leukocytes *in vivo*, and co-localization of CSFV E2 and Anx2 was observed in the cytoplasm of infected PK-15 cells (Shi et al., 2009; Sun et al., 2008). Anx2, as a Ca^{2+}-dependent cytosolic protein binding to cellular membranes through association with cholesterol, has diverse cellular functions including membrane trafficking, endosome formation and aggregation of vesicles (Drust and Creutz, 1988; Zeuschner et al., 2001). It can also interact with viruses to mediate viral entry, replication, assembly, budding and release. In HIV-1 infection Anx2 plays important roles during viral entry, assembly and budding of viral particles and its down-regulation leads to a significant decline in viral replication in monocyte-derived macrophages (MDMs) (Ma et al., 2004; Ryzhova et al., 2006). Anx2 has been identified as the receptor in human umbilical vein endothelial cells for human cytomegalovirus (CMV) (Wright et al., 1994) and binds to CMV envelope glycoprotein B (Pietropaolo and Compton, 1997). Anx2 is also a potential receptor for respiratory syncytial virus (RSV) in human epithelial cells (Hep2) and is up-regulated after RSV infection (Malhotra et al., 2003). In the present study the interaction of CSFV E2 with Anx2 has been identified, revealing the important role of this host factor in CSFV replication and proliferation in PK-15 cells.

2. Materials and methods

2.1. Cell culture and CSFV infection

PK-15 cells in MEM (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin G, 100 mg/mL Na streptomycin sulfate and 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA) were added to 6-well plates (Corning, NY, USA) and incubated for 12 h at 37 °C in a 5% CO_2 incubator. At 80–90% confluency, each well was infected with 10^3 $TCID_{50}$ of CSFV Shimen strain and further cultured in 2 mL MEM supplemented with 2% FBS and antibiotics.

2.2. siRNA treatment

Based on published data (Ryzhova et al., 2006), Anx2-specific siRNA (siAnx2) with sequence GAUCUGAAUUCAAGAGGAA targeting porcine Anx2 gene (GenBank accession AY706383) and non-targeting negative control siRNA (siScr) consisting of a scrambled sequence (GCCUGUGUUACAGCGUGUA, non-homologous to pig and CSFV genomes) were chemically synthesized by Dharmacon Research Inc. (Lafayette, CO, USA). Freshly trypsinized PK-15 cells were added in MEM supplemented with 10% FBS to 6-well plates and incubated at 37 °C in 5% CO_2. At 80–90% confluency, cells were transfected with 100 nM siRNA in 1 mL MEM and FuGENE HD Transfection Reagent (Roche, Indianapolis, IN, USA) without FBS and antibiotics. After 12 h incubation at 37 °C in 5% CO_2, the siRNA-treated cells were infected with 10^3 $TCID_{50}$ CSFV strain Shimen. Cells at 24 h, 48 h and 72 h post-infection (p.i.) were collected for SDS-PAGE, real-time RT-PCR and $TCID_{50}$ assay.

2.3. Establishment of PK-15 cell lines expressing E2 and Anx2

To establish cell lines expressing CSFV E2 and Anx2, total RNA was extracted from CSFV and PK-15 cells, and reverse transcribed with oligo (dT) using SuperScript First-Strand Synthesis System for RT-PCR according to the manufacturer's instructions (Takara Bio Inc., Dalian, China). The E2 cDNA of Shimen strain (GenBank accession AU775178) was amplified by PCR with E2-specific primers containing appropriate restriction sites, while porcine Anx2 was amplified with Anx2-specific primers containing a hexahistidine tag sequence and appropriate restriction sites. The primer sequences for both E2 and Anx2 are available upon request. Both cDNAs were then cloned separately into eukaryotic expression vector pLNHX (Takara), which has a neomycin resistance gene, to construct plasmids pLNHX-E2 and pLNHX-Anx2. For stable expression, PK-15 cells were transfected with these plasmids using FuGENE HD Transfection Reagent (Roche) according to the manufacturer's protocol, with transfection by empty vector pLNHX as a control. Transfected cell clones were screened by cultivation in MEM containing 500 ng/mL G418 and confirmed for the expression of E2 or Anx2 by indirect immunofluorescence assay (IFA) and Western blot (Shi et al., 2009). The expression of foreign Anx2 and its differentiation from intrinsic Anx2 was detected by Western blot using mouse anti-his-tag monoclonal antibody (mAb) (Abcam Ltd., HK, China). A cell line over-expressing Anx2 was then cultured in 6-well plates and infected with 10^3 $TCID_{50}$ CSFV Shimen strain per well. Cells collected at 24 h, 48 h and 72 h p.i. were subjected to real-time RT-PCR to detect the replication levels of the CSFV genome. Infectious virus titers were determined by measuring $TCID_{50}$.

2.4. Co-immunoprecipitation (Co-IP)

PK-15 cells were infected with CSFV as described above. The infected cells were harvested at 72 h p.i. and washed three times with cold PBS (pH 7.4) prior to Co-IP using Roche Immunoprecipitation Kit/Protein G (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Briefly the washed cells were lysed with lysis buffer and incubated with protein G-agarose beads to remove residual immunoglobulin. The beads were then removed by centrifugation, and the supernatant was incubated overnight at 4 °C in a rotator with 10 μg goat anti-porcine Anx2 polyclonal antibody (pAb) (Abcam Ltd., HK, China) or mouse anti-CSFV E2 mAb WH 303, or normal goat/mouse IgG (Hangyuan Ltd., Shanghai, China) as a negative control. Thirty microliters fresh protein G beads were then added and incubation was continued for another 12 h at 4 °C with constant rocking. The beads were then gently spun down and washed three times with PBS, then boiled in 2× loading buffer for 3 min and pelleted by centrifugation. Proteins in the supernatant were separated by SDS-PAGE and the individual bands were subjected either to MALDI-TOF mass spectrometry after staining with Coomassie Brilliant Blue R-250, or to immunoblotting analysis. Following transference, the PVDF membrane was blocked for 1.5 h with LI-COR Odyssey Blocking Buffer (LI-COR Biosciences, Nebraska, LI, USA), then incubated with primary antibodies WH303 (CSFV antibodies) or anti-Anx2 for 1 h at room temperature (RT), and probed with Alexa-tagged donkey anti-mouse (Alexa Fluor 680) or anti-goat (Alexa Fluor 800) secondary antibodies (Sigma, St. Louis, MO, USA). After washing the membrane was scanned at 700 nm or 800 nm using the Odyssey Infrared Imaging System (LI-COR Biosciences).

2.5. Confocal laser scanning microscopy (CLSM)

Coverslip cultures of PK-15 cells infected with 10^3 $TCID_{50}$ CSFV strain Shimen were collected at 72 h p.i. and fixed with 4% paraformaldehyde for 30 min at RT. The fixed cells were washed three times in PBS and permeabilized with 0.1% Triton X-100 for 5 min at RT. To quench free aldehyde groups, the cell sheets were incubated with 0.1 M glycine for 10 min at RT, and then blocked in PBS containing 10% goat serum albumin at RT for 2 h, following which the coverslips were incubated sequentially with anti-Anx2 pAb and anti-E2 mAb WH 303 for 2 h. After washing three times (5 min each) at RT, the coverslips were incubated sequentially with FITC-conjugated rabbit anti-mouse IgGs and Texas Red-conjugated rabbit anti-goat IgGs (Sigma) for 30 min at RT, then stained with DAPI (4′,6′-diamidino-2-phenylindole) (Invitrogen, Carlsbad, CA, USA) in PBS/0.1% BSA. After several washes with PBS, the coverslips