Annexin A2 is involved in the production of classical swine fever virus infectious particles

Chun Sheng, Xiaoxiang Liu, Qiuyue Jiang, Bin Xu, Chenhao Zhou, Yujing Wang, Jun Chen and Ming Xiao

College of Life and Environment Sciences, Shanghai Normal University, Shanghai, PR China

Annexin A2 (ANXA2) is an important host factor regulating several key processes in many viruses. To evaluate the potential involvement of ANXA2 in the life cycle of classical swine fever virus (CSFV), an RNA interference (RNAi) approach was utilized. Knockdown of ANXA2 did not impair CSFV RNA replication but significantly reduced CSFV production. A comparable reduction of extracellular and intracellular infectivity levels was detected, indicating that ANXA2 might play a role in CSFV assembly rather than in genome replication and virion release. Furthermore, ANXA2 was found to bind CSFV NS5A, an essential replicate component. Amino acids R338, N359, G378 of NS5A were revealed to be pivotal for the ANXA2–NS5A interaction. Substitutions of these amino acids had no effect on viral RNA replication but substantially reduced CSFV production, which might partly be due to these mutations destroying the ANXA2–NS5A interaction. These results suggested that ANXA2 might participate in CSFV production process by binding NS5A.

Classical swine fever virus (CSFV) is an important livestock pathogen and is a member of the genus *Pestivirus*, along with bovine viral diarrhea virus 1 (BVDV-1), BVDV-2, and Border disease virus (BDV). They belong to the family *Flaviviridae*, which also contains the closely related human hepatitis C virus (HCV) (Heinz et al., 2000; Becher & Thiel, 2002). CSFV is an enveloped positive-strand RNA virus. The virus contains a single-strand, positive-sense RNA genome, which is composed of a 5′UTR, an ORF and a 3′UTR. The 3′UTR and the 5′UTR are thought to regulate pestivirus genome replication (Isken et al., 2003, 2004; Xiao et al., 2004; Pankraz et al., 2005). The 5′UTR includes an internal ribosome entry site that is responsible for translation initiation of viral genomes (Fletcher & Jackson, 2002; Xiao et al., 2011). The ORF codes for a resulting polyprotein that is subsequently processed into mature proteins (N-pro, C, E-pro, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) by cellular and viral proteases (Moennig & Plagemann, 1992). The CSFV NS5B protein has RNA-dependent RNA polymerase (RdRp) activity and is able to bind its cognate 3′UTR and initiates genome replication (Steffens et al., 1999; Xiao et al., 2004). NS5A is a component of the replication complex. CSFV NS5A comprises 497 aa and is able to regulate viral RNA replication and translation through binding to NS5B and 3′UTR (Chen et al., 2012; Xiao et al., 2009).

The viral life cycle requires various viral non-structural proteins as well as host cellular proteins. Annexin A2 (ANXA2) is known as a lipid raft-associated scaffold protein. It is calcium dependent and cytosolic and mediates essential biological processes, including membrane trafficking, endosome formation, and aggregation of vesicles (Drust & Creutz, 1988). ANXA2 has been identified as an important host factor in the regulation of several key processes in cytomegalovirus (Wright et al., 1995), influenza virus (LeBouder et al., 2008), calcivirus (González-Reyes et al., 2009), bluetongue virus (Beaton et al., 2002), human immunodeficiency virus (Ryzhova et al., 2006) and HCV (Backes et al., 2010). Recently, a proteomic analysis revealed that CSFV infection led to a significant increase of ANXA2 protein in host cells (Sun et al., 2008). Haem oxygenase 1, another cellular protein and ANXA2 were found to be colocalized with the CSFV E2 protein (Shi et al., 2009). The expression of annexin A1, another member of the annexin family, was also elevated in PK-15 cells following CSFV infection (Sun et al., 2010). However, the role of many cellular proteins in the life cycle of CSFV remains poorly understood. In this paper, we show that ANXA2 is involved in CSFV production.

To evaluate the potential involvement of ANXA2 in the life cycle of CSFV, an RNA interference (RNAi) approach and CSFV subgenomic replicons and genomic replicons (Sheng et al., 2010) (Fig. 1a) were utilized. A small interfering RNA (siRNA) molecule targeted to porcine ANXA2 sequence (GenBank accession no. NM_001005726) and a negative control siRNA molecule that has no matches either in the viral or the porcine genome were synthesized (Shanghai Gene Chem). An anti-CSFV siRNA directed to the CSFV NS5B gene served as a positive control (Xu et al., 2008). Results showed that the ANXA2-specific siRNA reduced the amount of ANXA2 without a significant influence on the cell viability (results not shown) while the control siRNA, anti-CSFV siRNA and the mock transfection without siRNA addition did not (Fig. 1b). Silencing ANXA2 did not reduce viral protein expression.
The amount of intracellular Core protein is comparable to the cells transfected with anti-ANXA2 siRNA, while the lower levels of extracellular Core protein were detected for the cells transfected with anti-ANXA2 siRNA. Lower levels of specific infectivity (SI) of intracellular and intracellular infectivity levels and a comparable level for control siRNA treatment (Fig. 2a, b). The anti-ANXA2 siRNA resulted in a significant reduction of extracellular Core proteins by about 70% compared to that with the control siRNA (Fig. 2b). These data indicated that ANXA2 is important for Core protein release from extracellular CSFV particles.
virions made in the presence of anti-ANXA2 siRNA were detected while release of virus RNA was comparable to the level for control siRNA treatment (Fig. 2d). Taken together, these results indicated that a decrease in the production of infectious CSFV particles might be attributable to defective virion assembly rather than to virus release or RNA replication. The anti-ANXA2 siRNA treatment might not impair virus RNA, but result in virions with lower infectivity.

Previous reports showed that HCV NS5A is necessary for HCV assembly and production and interacts with ANXA2 (Appel et al., 2008; Masaki et al., 2008; Saxena et al., 2012). To investigate whether ANXA2 interacts with CSFV NS5A, several NS5A mutants (Fig. 3a) formed in our previous work (Chen et al., 2012) were used for immunoprecipitation analysis. As shown in Fig. 3(b), ANXA2 specifically coprecipitated CSFV NS5A. Mutant D335–389 lost ANXA2 binding activity but other NS5A mutants retained

Fig. 2. Effect of siRNA-mediated knockdown of ANXA2 on the extracellular and intracellular amount of viral Core protein and infectivity. Seventy-two hours after the indicated siRNA transfection, the siRNA transfected PK-15 cells were retransfected with a second dose of the same siRNA together with a CSFV genomic replicon, SM- (Sheng et al., 2010). (a) At 72 h post-transfection, the cells were harvested and extracellular and intracellular Core proteins were detected by Western blot analysis with anti-Core antibody. β-Tubulin serves as an internal control. (b) Signal intensities quantified by Kodak ID 3.5 software were used to evaluate the levels of extracellular and intracellular Core. The relative values were shown by comparing densitometric values with the densitometric value for the SM replicon, which was set at 1. (c) Extracellular and intracellular infectivity levels were determined by TCID_{50} assay as described in Fig. 1(d). (d) Release of virus RNA from transfected cells 72 h post-transfection was determined by using reverse transcript-qPCR (RT-qPCR) as described in Fig. 1(c). Specific infectivity (SI) was calculated as TCID_{50} ml^{−1}/RNA copies. The SM was used to normalize the data. All quantified results are shown as means ± SD of at least three independent experiments. ** P<0.01.
the activity (Fig. 3b), indicating that amino acids 335–389 might be important for ANXA2 binding. As a comparison, we aligned the NS5A sequences of CSFV, BDV, BVDV and HCV. Three conserved amino acids R338, N359 and G378 were indicated (Fig. 3c), suggesting that these amino acids might be important for NS5A interactions with ANXA2. To corroborate this assumption, alanine or leucine substitutions were introduced into R338, N359 and G378 in CSFV NS5A. Four CSFV NS5A mutants, R338A, N359A, G378A and G378L were produced (Fig. 3d). Immunoprecipitation assays revealed that ANXA2 binding activity was lost for R338A, N359A and G378L and was substantially reduced for G378A (Fig. 3e). The results indicated that amino acids R338, N359 and G378 of CSFV NS5A are pivotal for the ANXA2–NS5A interaction. Furthermore, the same substitution mutations were incorporated into the CSM2 subgenomic replicon and the SM2 genomic replicon, respectively. These mutations had no substantial effect on viral RNA replication and viral protein expression (Fig. 3f) but substantially reduced CSFV production (Fig. 3g).

**Fig. 3.** ANXA2 interacts with CSFV NS5A. (a) Schematic of the CSFV NS5A protein and its mutant forms from previous reports (Chen et al., 2012). D335–389 indicates an internal deletion mutant lacking corresponding amino acids. The ANXA2, NS5B and 3′UTR binding activity of these proteins are shown on the right. (b) Immunoprecipitation analysis of the interaction between ANXA2 and CSFV NS5A. PK-15 cells were transfected with the pcDNA3.1/N-FLAG vector containing NS5A (WT or mutant). At 72 h post-transfection, cell lysates were immunoprecipitated using anti-ANXA2 antibody. Immunoprecipitation of NS5A–ANXA2 interaction was determined by Western blot analysis with anti-FLAG antibody (upper) or anti-ANXA2 antibody (below). Total lysates were fractionated by 10% SDS-PAGE and subjected to Western blot analysis with anti-FLAG antibody (middle). (c) The NS5A protein sequence of CSFV was aligned with that of BDV, BVDV and HCV using CLUSTAL_X (partially shown). The conserved amino acids are indicated by asterisks. (d) Schematic representation of NS5A and its substitution mutants used in this study. (e) Immunoprecipitation of the NS5A–ANXA2 interaction was determined by Western blot analysis with anti-FLAG antibody or anti-ANXA2 antibody as described in Fig. 3(b). (f) PK-15 cells were transfected with the CSM– subgenomic replicon carrying WT or mutant NS5A proteins (R338A, N359A, G378A or G378L). Quantification of viral RNA was determined as described in Fig. 1(c). The amounts of NS3 (below) were evaluated as described in Fig. 1(b). (g) PK-15 cells were transfected with the SM– subgenomic replicon carrying WT or mutant NS5A proteins (R338A, N359A, G378A or G378L). Titres were determined as described in Fig. 1(d). All quantified results are shown as means ± SD of at least three independent experiments. **P < 0.01.
Host proteins participate in many aspects of viral infection. Vesicle-associated membrane protein-associated proteins mediate the formation of HCV RNA placement complex on lipid rafts (Gao et al., 2004). RNA helicase A, another host protein, has been found to be involved in the expression and replication of CSFV and bind CSFV 5'UTR and 3'UTR (Sheng et al., 2013). Cellular NFAR proteins associate specifically with both the termini of the BVDV RNA genome involving regulatory elements (Isken et al., 2003). ANXA2 is an important host protein that plays a role in different steps of viral life cycle (Backes et al., 2010; Beaton et al., 2002; González-Reyes et al., 2009; LeBouder et al., 2008; Ryzhova et al., 2006; Wright et al., 1995). In the report, we identified ANXA2 as an important factor in the production of the CSFV infectious particles. ANXA2 also is a key host factor that influences HCV yield (Backes et al., 2010). For the role of ANXA2 in viral RNA replication, scientists reported contradictory findings. Some people showed that ANXA2 knockdown reduced viral RNA replication (Saxena et al., 2012) while another observed that ANXA2 knockdown did not have this effect (Backes et al., 2010). Our present results suggested that ANXA2 plays a role in CSFV assembly rather than in genome replication and virion release. The discrepancy may partly be due to the differences in virus species and in siRNA sequences.

The data for immunoprecipitation experiments showed that ANXA2 interacts with CSFV NS5A, in agreement with the previous reports in which ANXA2 binds HCV NS5A (Saxena et al., 2012). The NS5A protein of CSFV, BVDV and HCV has been shown to be an essential replicase component (Masaki et al., 2008; Tellinghuisen et al., 2006; Sheng et al., 2010), thus, ANXA2 might participate in the CSFV production process by binding to NS5A protein. In fact, ANXA2 has been found to be involved in the formation of HCV replication complex on the lipid raft (Saxena et al., 2012). Mutation analysis revealed that amino acids 335–389 of NS5A might be responsible for ANXA2 binding (Fig. 3c, d). NS5A is a multifunctional protein. The C terminus of CSFV NS5A contains several binding sites, such as a NS5B binding site and a 3'UTR binding site (Fig. 3a) (Chen et al., 2012). Recently, we have found that the C-terminal sequence from amino acids 478–487 of NS5A is important for the interaction between CSFV NS5A and Core protein, which also might be related to the production of infectious CSFV particles (Sheng et al., 2014). The amino acid fragment responsible for ANXA2 binding is adjacent to the NS5B binding site at the C terminus of CSFV NS5A (Fig. 3a) (Chen et al., 2012). Furthermore, R338, N359 and G378 of CSFV NS5A were observed to pivotal for ANXA2–NS5A interactions (Fig. 3c, e). Substitution of these amino acids had no effect on viral RNA replication but substantially reduced CSFV production (Fig. 3f, g), which might partly be due to this substitution destroying ANXA2–NS5A interactions. These results suggested that ANXA2 might participate in the CSFV production process by binding NS5A. This is attractive for further investigation of the role of ANXA2 in the life cycle of pestivirus and HCV.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (30870492, 31070671), the Natural Science Foundation of Shanghai (12ZR1422100), the Shanghai Municipal Science and Technology Commission (11JC1409300) and the Shanghai Municipal Education Commission (13ZZ102, 11YZ87, 14ZZ123).

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