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Interaction between Core protein of classical swine fever virus with cellular IQGAP1 protein appears essential for virulence in swine

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ABSTRACT

Here we show that IQGAP1, a cellular protein that plays a pivotal role as a regulator of the cytoskeleton interacts with Classical Swine Fever Virus (CSFV) Core protein. Sequence analyses identified residues within CSFV Core protein (designated as areas I, II, III and IV) that maintain homology to regions within the matrix protein of Moloney Murine Leukemia Virus (MMLV) that mediate binding to IQGAP1 [EMBO J, 2006 25:2155]. Alanine-substitution within Core regions I, II, III and IV identified residues that specifically mediate the Core-IQGAP1 interaction. Recombinant CSFV viruses harboring alanine substitutions at residues ^{207}ATI^{209} (I), ^{210}VVE^{212} (II), ^{213}GVK^{215} (III), or ^{232}GLYHN^{236} (IV) have defective growth in primary swine macrophage cultures. *In vivo*, substitutions of residues in areas I and III yielded viruses that were completely attenuated in swine. These data shows that the interaction of Core with an integral component of cytoskeletal regulation plays a role in the CSFV cycle.

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Introduction

Classical swine fever virus (CSFV) is a small, enveloped virus with a positive, single-stranded RNA genome that causes classical swine fever (CSF), a highly contagious disease of swine. CSFV, along with Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV), are members of the genus *Pestivirus* within the family *Flaviviridae* (Fauquet et al., 2005). The CSFV genome is approximately 12.3 kb and contains a single open reading frame encoding a polyprotein of 3898-amino-acids. Co- and post-translational processing of the polyprotein by cellular and viral proteases ultimately yields 11 to 12 final cleavage products (NH_2-Npro-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) (Rice, 1996). Structural components of the CSFV virion include the glycoproteins E^{rns}, E1, E2, and a nucleocapsid of unknown symmetry, the Core protein.

Within the *Flaviviridae* family, Core is encoded as the second product of the polyprotein. The Core protein of pestiviruses is a small, highly basic polypeptide that is cleaved at its N-terminus by Npro, an event critical for infectious particle production. The Core protein of BVDV has additionally been characterized as lacking significant secondary structures (Murray et al., 2008). It is known that the C-terminal of Core is cleaved by signal peptide peptidase. (Heimann et al., 2006; Rümenapf et al., 1998; Meyers et al., 1989), and it has been suggested that the CSFV Core protein influences regulation of cellular transcription (Liu et al., 1998) and also interacts with host SUMOylation proteins (Gladue et al., 2010).

Analysis of the Core protein of Hepatitis C Virus (HCV), another member of the *Flaviviridae* family, provides further insight into the possible functions of the Core protein of CSFV. HCV Core self-assembles into nucleocapsid-like particles in the presence of nucleic acids (Kunkel et al., 2001) and can directly interact with HCV RNA (Fan et al., 1999; Shimoike et al., 1999; Tanaka et al., 2000). Core can bind other HCV proteins such as NS5A and E1 (Goh et al., 2001; Lo et al., 1996; Masaki et al., 2008) and interacts with host cellular proteins (Jin et al., 2000; Mamiya and Worman., 1999; Otsuka et al., 2000; Yoshida et al., 2002; You et al., 1999), influencing HCV pathogenesis by modulation of signaling pathways, cell transformation and proliferation, regulation of cellular and viral gene expression, apoptosis, and alteration of lipid metabolism. (Giannini and Brechot, 2003; Levrero, 2006; Tellinghuisen and Rice, 2002; Lai and Ware., 2000; McLauchlan, 2000; Ray and Ray, 2001). HCV Core protein is capable of impairing the host's immune response, by interacting with cell molecules that results in suppression of IL-12 synthesis in human macrophages (Eisen-Vandervelde et al., 2004), T cell dysfunction (Yao

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et al., 2007), and inhibition of T-lymphocyte activation and proliferation (Chen et al., 1994; Kittlesen et al., 2000; Yao et al., 2001).

Although the role of CSFV structural glycoproteins in virus virulence has been studied in detail (Meyers et al., 1999; Risatti et al., 2005a, 2005b, 2006, 2007a, 2007b; van Rijn et al., 1994; van Gennip et al., 2004), knowledge about the role of Core protein on the outcome of CSFV infection in swine is limited. Recently, we have shown that CSFV Core protein interacts with proteins of the cellular SUMOylation pathway, SUMO-1 (small ubiquitin-like modifier) and UBC9, a SUMO-1 conjugating enzyme (Gladue et al., 2010). Substitution of Core residues in CSFV involved with binding to SUMO1 and UBC9 resulted in virus attenuation in swine. To further elucidate the role of Core protein in CSFV virulence, we expanded this previous study to identify additional host factors interacting with the Core protein during virus infection. Here we report that the cellular IQGAP1 protein interacts specifically with the CSFV Core protein. IQGAP1 is involved in a diverse set of protein–protein interactions and is a prominent regulator of the cytoskeleton (for a review, see Noritake et al., 2005; Brandt and Grosse, 2007). This protein binds monomeric G proteins: Cdc42 and Rac, likely mediating their effects in reorganizing the cytoskeleton (Fukata et al., 2002; Watanabe et al., 2004).

Specific interaction of IQGAP1 with the Moloney murine leukemia virus (MMLV) matrix (M) protein suggests its involvement in intracellular trafficking of the virus, an interaction that is essential for virus replication (Leung et al., 2006). Mutational studies performed in that report defined residues of MMLV M protein, critical for its interaction with IQGAP1. Based on sites within the MMLV M protein that recognize IQGAP1, four areas (I–IV) within the CSFV Core protein that potentially recognize IQGAP1 were identified. Substitution of Core native amino acid residues with alanine in areas I and III completely abolished the Core–IQGAP1 protein–protein binding whereas substitutions in areas II and IV only partially affected the interaction. CSFV mutants harboring substitutions in these four areas were developed to assess the importance of the Core–IQGAP1 protein interaction for virulence in swine. Remarkably, substitutions in areas I and III of the Core protein, significantly affecting IQGAP1 recognition, correlate with complete absence of virulence in swine. Therefore, the ability of CSFV Core protein to bind cellular IQGAP1 protein during infection plays a critical role in virus virulence within the swine host.

Results

CSFV structural Core protein binds swine IQGAP1 protein

To identify host cellular proteins that interact with CSFV Core protein, we constructed an N-terminal fusion of the Gal4 protein binding domain to the Core protein as 'bait' for the yeast two-hybrid system. Approximately 1×10^7 independent yeast colonies derived from a swine primary macrophage cDNA library containing 3×10^6 independent clones were screened. These colonies were selected for growth using -Leu/-Trp/-His/-Ade media. Plasmids were isolated from positive colonies and sequenced. In-frame proteins were retested for specificity to the Core protein. As a negative control proteins were tested for binding the Lam-BD protein. Several proteins were identified as specific binding partners for the CSFV Core protein (data not shown). One of these proteins, IQGAP1, was selected for further study since IQGAP1 functions as a prominent regulator of the cytoskeleton which likely plays a role in viral pathogenesis. IQGAP1 specifically bound Core protein when compared to binding of Lam-BD protein (Fig. 1).

Mapping areas of the CSFV Core protein critical for IQGAP1 recognition

Previous studies have described the binding of Moloney murine leukemia virus (MMLV) matrix (M) protein with IQGAP1 protein (Leung et al., 2006). Mutational studies defined residues of the MMLV M protein critical for interaction with IQGAP1, revealing a correlation between binding and virus replication. Based on the sites of MMLV M protein that recognize IQGAP1 (Leung et al., 2006), four (I–IV) homologous areas (Fig. 2) were identified in CSFV Core protein using the ClustalW software program (Thompson et al., 1994). CSFV mutants harboring alanine substitutions in place of native amino acid residues within these four areas were developed to assess whether these regions are important for IQGAP1 binding (Figs. 1 and 2). We produced the following mutant proteins containing Ala substitutions within the Core protein: CoreΔIQ.I (^{207}ATI^{209}), CoreΔIQ.II (^{210}VVE^{212}), CoreΔIQ.III (^{213}GVK^{215}), and CoreΔIQ.IV (^{232}GLYHN^{236}) (Table 1). These CoreΔIQ proteins were tested in the yeast two-hybrid system against the swine IQGAP1 protein. Interestingly, substitutions within areas I and III resulted in loss of ability to bind IQGAP1 (Fig. 1). In contrast, substitutions in areas II and IV only caused a decrease in the ability of the Core protein to bind the swine IQGAP1 protein. All CoreΔIQ mutated proteins maintained their ability to bind swine clathrin in the yeast two-hybrid at similar levels (data not shown), indicating that these mutated areas within the CoreΔIQ mutated proteins are areas specific for IQGAP1 binding.

Sequence analysis of the 100 amino acid residues of Core protein from geographically and temporally different CSFV isolates revealed a high degree of sequence similarity and conservancy at putative IQGAP1 target sites (Fig. 2), suggesting these sites play a critical role in the biology of CSFV. Additionally, the areas predicted in CSFV to bind IQGAP1 are highly conserved in BVDV and BDV, further suggesting a critical role for these residues in other pestiviruses (data not shown).

Replication of CoreΔIQ mutant viruses in vitro

To further evaluate the role of CSFV Core IQGAP1 binding sites in the biology of the virus, recombinant viruses based on virulent strain Brescia (BICv) were constructed, containing alanine substitutions in the previously described four critical IQGAP1 binding sites of the Core protein. Mutant viruses referred to as CoreΔIQ.Iv, CoreΔIQ.IIv, CoreΔIQ.IIIv, and CoreΔIQ.IVv represent each of the four putative IQGAP1 binding sites within the CSFV Core protein (Table 1 and Fig. 2). Viruses were rescued from transfected cells by 4 dpi (days post-infection). Nucleotide sequences of viable rescued virus genomes were identical to parental DNA plasmids, confirming that only mutations at predicted mutated sites were reflected in rescued viruses.

In vitro growth characteristics of these mutant viruses were evaluated relative to parental BICv in a single-step growth curve. Primary swine macrophage cell cultures were infected at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$ per cell. Virus was adsorbed for 1 h (time zero), and samples were collected at 72 h post-infection (hpi). All mutant viruses exhibited about one log10 decrease in titer when compared with parental BICv (Fig. 3), suggesting that all mutant viruses have an *in vitro* growth defect when compared to parental BICv.

Effect of Core–IQGAP interactions on the cell cytoskeleton

The effects of viral infection on γ-tubulin and vimentin were examined to compare the distribution of microtubules and intermediate filaments in cells infected with BICv, CoreΔIQ.Iv, CoreΔIQ.IIv, CoreΔIQ.IIIv, CoreΔIQ.IVv, to that seen in uninfected cells (Fig. 5). In uninfected cells, the microtubules (visualized with antibodies recognizing γ-tubulin) as well as the intermediate filaments (visualized with antibodies recognizing vimentin), were arranged in a filamentous network running throughout the cytoplasm. In BICv-infected cells, γ-tubulin was rearranged into a ring surrounding the nucleus. When vimentin was examined a similar rearrangement was observed in BICv infected cells. Examining the appearance of γ-tubulin and vimentin in infected cells also revealed differences between the effects of BICv and CoreIQ viruses. Infection with CoreΔIQ.IIv and CoreΔIQ.IVv led to a rearrangement of both markers, γ-tubulin and vimentin, into a ring