Structural Glycoproteins of Classical Swine Fever Virus: Implication for Vaccine Development

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ABSTRACT

Classical Swine Fever (CSF), also called Hog Cholera or European Swine Fever, is a highly contagious disease of pigs caused by CSF virus (CSFV). Intermittent CSF outbreaks in China and other parts of the world have led to significant economic losses. Infection with highly virulent CSFV strains causes acute CSF characterized by high mortality and morbidity, while moderate to low virulence induces a prolonged, chronic disease. The envelope of the CSF virion contains three glycoproteins, E^{rns}, E1 and E2. E^{rns} has RNase activity and E2 is the major antigenic protein exposed on the outer surface of the virion. *In vivo*, these viral proteins have been shown to play a major role in virulence and pathogenicity, to produce antibodies and induce protective immune response. An improved understanding of the genetic basis of E^{rns}, E1 and E2 glycoproteins will permit rational design of new CSF vaccines with enhanced safety, efficacy and utility. In this article, we focus on biochemical properties and their functions of CSFV glycoproteins, and further demonstrate the development of new vaccines based on these proteins.

Key Words: CSF; CSFV; glycoprotein; structure; property; function; vaccine.

INTRODUCTION

Classical Swine Fever Virus (CSFV), a small enveloped RNA virus, belongs to the genus pestivirus of the family Flaviviridae and causes a severe disease of domestic pigs and wild boar characterized by fever, severe leukopenia and hemorrhage (1). The 5-terminal part of the pestiviral RNA encodes the structural proteins, the capsid protein C and three envelope glycoproteins (E^{rns}, E1, and E2) (2). These envelope proteins are located in the lipid membrane of cellular origin, and processed from the polyprotein in a hierarchical way, starting with the translocation of the C-terminal signal peptide downstream of the capsid protein into the endoplasmic reticulum (ER), followed by the release of the E2 protein (3). E1 and E2 are the major envelope glycoproteins exposed on the outer surface of the virion. E^{rns} also called E0 lacks a typical membrane anchor and it is secreted from infected cells however associated with mature virions (3).

Live attenuated vaccines, such as the Chinese lapinized vaccine (C-strain), have been proven safe and efficient, and successfully used to control CSF around the world (4). However, these vaccines increase the possibility of viral recombination between the mutated vaccine strains and the wild-type CSFV strains. Furthermore, the humoral immune response induced by traditional live attenuated vaccines does

not differ from that elicited by wild-type viruses. Therefore, using these vaccines does not allow for the differentiation between infected and vaccinated animals. To overcome this drawback, some new vaccines need to be studied and developed, and the efficacies of these types of vaccines are required to be tested.

Glycoproteins of CSFV may provide a useful material for developing new CSFV vaccines. As illustrated in Figure.1, in addition to representing structural components of the CSFV virion (2), E^{rns}, E1 and E2 of CSFV are involved in the virulence and pathogenicity of CSFV (5). E^{rns} possess enzymatic activity which plays a significant role in the viral life cycle and viral replication (6). E2 is the essential protein in virus replication and infection, and it is also the major immunogenic protein that is responsible for inducing virus-neutralizing antibodies and elicits protective immunity in the natural host (7). Among these proteins, E^{rns} and E2 are two major targets for the design of the candidate vaccines. Serological diagnosis of CSF often aims to demonstrate the presence of antibodies to these two glycoproteins (8). The vaccines developed by E^{rns} and E2 glycoproteins are capable of conferring protective immunity in pigs and of inducing virus-neutralizing antibodies (7,9). In this article, structure, biochemical properties, functions of CSFV glycoproteins and vaccines developed from these proteins are reviewed.

BIOCHEMICAL PROPERTIES OF CSFV GLYCOPROTEINS

Glycosylation of CSFV E^{rns}, E1 and E2 proteins

The most important feature of E^{rns}, E1 and E2 proteins of CSFV are that they are heavily glycosylated. CSFV E1 glycoprotein contains 3 N-linked putative glycosylation sites which are highly conserved in CSFV strains (10), and CSFV E^{rns} contains seven putative N-linked glycosylation sites (11). The N-glycosylation has a complex mixture of neutral and monosialylated multi-antennary N-glycans which count for up to nearly half of the molecular mass of the mature glycoproteins (3,7). The monomers of E^{rns} have slightly differing molecular masses, probably due to variation in glycosylation
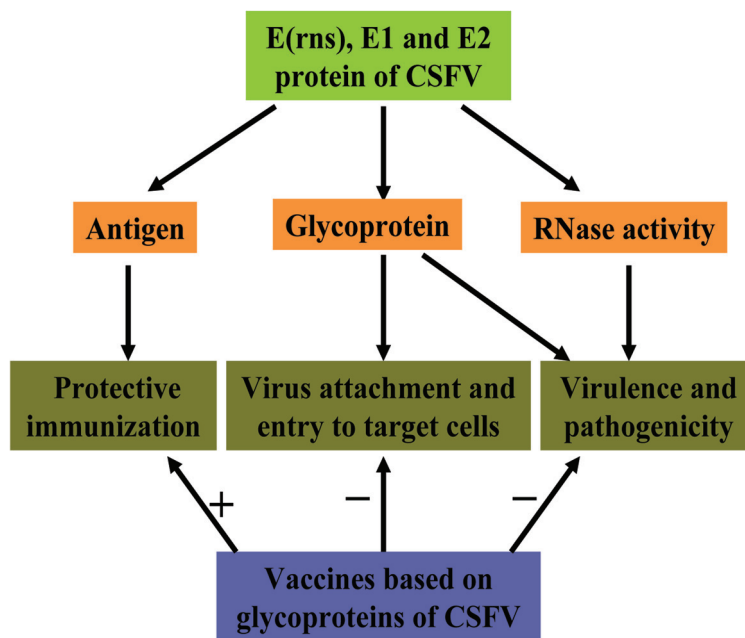

Figure1. Biochemical properties and functions of CSFV glycoproteins. Erns and E2 proteins of CSFV are heavily glycosylated with antigen property and RNase activity, respectively. E^{rns}, E1 and E2 glycoproteins are multifunctional, which are involved in virus attachment and entry to target cells, influencing virulence and pathogenicity, and inducing protective immunization. Vaccines based on glycoproteins of CSFV may provide a strong immune response and confer total protection against CSFV.

(3). However, N-glycosylation has no effect on dimer formation of E^{rns} but is essential to that of E2 (12).

A role of glycosylation of E^{rns}, E1 and E2 in virulence and pathogenicity is also strongly suggested by many facts: N-glycan of CSFV E^{rns} is essential for binding double-stranded RNA, preventing interferon-beta induction and influencing CSFV pathogenesis (11,13). Alteration of the N-linked glycosylation condition in envelope proteins E^{rns} (11,14), E1 (15), and E2 (16) of CSFV yields viruses as virulent as the parental CSFV Brescia, or viruses with a variable degree of attenuation. Additionally, E^{rns}, E1 and E2 can form biologically active N-glycosylated heterodimeric and homodimeric complexes needed to effectively infect host cells (12). Some glycosylation inhibitors, such as tunicamycin, which act at the early stages of glycan chain processing, can influence, not only glycosylation, but also the stability of E2 and E^{rns} glycoproteins, effectively inhibiting the formation of glycoprotein complexes and virus yield (17). Therefore, modification of glycosylation patterns of E^{rns}, E1 and E2 can be used for developing CSFV live attenuated vaccines (11,18).

RNase activity of E^{rns} protein

Subsequent analysis of E^{rns} protein purified from cells infected with CSFV identifies that E^{rns} has the unique feature of containing intrinsic RNase activity with a clear preference for uridine-rich sequences. Two short homologous regions of 8 amino acids in the sequence of E^{rns}, located in the N-terminal half of the protein, are responsible for the RNase activity (19).

Besides possessing neurotoxic activity and antihelminthic activity, the RNase activity of E^{rns} plays an important role in virulence, which has been further proven by mutation of the regions responsible for its activity. In these regions, histidine residues appear to be essential for catalysis and inactivation of RNase activity by a lysine substitution of histidine in CSFV strain C, resulting *in vitro* in a cytopathogenic virus (20). Amino acid exchanges or deletions introduced by site-directed mutagenesis into the putative active site of the RNase residing in the E^{rns} of CSFV abolish the enzymatic activity of this protein and lead to virus attenuation. However, neither carbohydrate moieties nor disulfide bonds are prerequisite for RNase activity (21). Furthermore, the RNase activity of E^{rns} has been proved to participate in virus attachment to or entry into host cell (22), to play an important role in the viral life cycle and to be responsible for the persistent infection of these viruses in their natural host (20). Another study demonstrates that E^{rns} and its enzyme activity contribute to regulation of RNA synthesis in infected cells and to weaken host immune defenses early in infection (23). Moreover, E^{rns} has been shown to be involved in the interaction of the virus with the immune system of the host or the host cells (24). An earlier indication of immunological activity induced by E^{rns} is the appearance of CSFV-specific gamma interferon (IFN-γ)-secreting cells in the peripheral blood as early as 6 days after vaccination (25). A novel function for CSFV E^{rns} glycoprotein is to counteract the IFN-beta induction pathway (26), reducing IFN-beta mRNA synthesis and blocking IFN-alpha/beta production at the transcriptional level (13). In addition, E^{rns} is involved in interference with the type I IFN response of cells to dsRNA, and this activity is dependent on the RNase activity and its capacity to bind dsRNA (24).

Antigenic properties of E2 glycoprotein

E2 is regarded as the major neutralizing antigen for CSFV infection (7,27,28). E2 protein contains four antigenic domains, A to D, located within the N-terminal half of the protein which are highly conserved in all CSFV strains and among different pestiviruses (29-31). Specific binding sites of E2 protein to mAbs are found at residues 690-714 in domain B, residues 715-740 and 741-765 in domain C, respectively (32,33). Asn207 site-specific micro-heterogeneity of the E2 most relevant antigenic and virulence site is determined by HPLC-mass spectrometry of glycopeptides (27).

E2 can elicit neutralizing antibodies and confers protection against lethal CSFV challenge when given alone, indicating that E2 glycoprotein of CSFV is a virulence determinant in swine (16,18). Partial or complete deletions of the E2 gene have proven to attenuate virulence of CSFV (21), indicating that glycoprotein E2 is potentially an ideal candidate that can be used to develop recombinant vaccines against CSFV. Vaccines developed based on E2 of CSFV are discussed later in this article.

DEVELOPMENT OF VACCINES

Because of the enormous economic losses, eradication or at least control of CSFV is an important task that would be facilitated by a suitable vaccine. The use of a vaccine against CSFV during a CSF outbreak should lead to a reduction in the horizontal or vertical transmission of CSFV (34). Intensive studies of the molecular biology of pestiviruses, the characterization of glycoproteins and recombinant DNA technology have opened a novel way to develop new vaccines for protection against CSFV. Different kinds of vaccines developed on the basis of these glycoproteins are shown in Table.1.

DNA-BASED VACCINES

DNA-based vaccines are circular DNA molecules which carry the gene encoding an antigenic protein. Once these DNA molecules enter the mammalian cell, they begin to express the carrier gene. The resulting protein ultimately enables the immune system to elicit an immune response against the antigen. DNA-based vaccines have many advantages, for instance, plasmid DNA can be highly purified and is easy to produce under standardized protocol. They are very stable at ambient temperatures and there is less opportunity for viruses to contaminate the vaccine preparation. Furthermore, DNA-based vaccines can be safety and efficacy used in animal models.

Table 1. Vaccines based on glycoproteins of CSFV

Types of vaccine	Treatment	References
DNA vaccine	The Semliki Forest virus (SFV) replicon-derived DNA vaccine expressing the complete E2 protein	(36,37)
	Alphavirus replicon-derived DNA vaccine expressing glycoprotein E2	(38,39)
Recombinant virus vaccines	Vaccinia virus recombinants (VVR) expressing E2 protein	(46,47)
	The recombinant fowlpox virus expressing Erns-E2	(48)
	Swinepox Virus (SPV) expressing E2 subunit	(49,50)
	The recombinant human adenovirus type 5 expressing the CSFV E2 gene	(52)
	The recombinant Adenovirus encoded the CSFV E2 gene fused with the UL49 gene from pseudorabies Virus (PRV)	(53)
	A recombinant strain rPAV-gp55 expressing E2 at a high level	(54,55)
	PRV vector expressing CSFV E2 protein	(56)
	The recombinant PRV strains expressing E2 gene	(57)
	A recombinant baculoviruses expressing Erns and/or E2	(59,60)
Epitope-based vaccines	Epitopes of E2 protein such as CKEDYRY (aa693-699)	(41)
	Epitopes of E2 protein such as N-terminal 90 residues (domains B/C)	(30,32,33,61)
	Epitopes of E2 protein such as CKEDYRYAISSTNEIGLLGAGGLT	(62)
	Linear neutralizing epitopes mapped to Erns	(42,63)
Marker vaccines	Deletion of the A-domain of E2	(31)
	Synthetic peptide vaccines using E2 N-terminal antigenic units B/C	(32,33,62,63)
	Synthetic peptide vaccines using E2 CKEDYRY (aa693-699)	(41)
	Using the eukaryotic expression plasmid with only 5' signal sequence of E2	(64)
	Deletion of E2-encoding region of CSFV	(65)

In recent years, DNA vaccines have become an attractive prospect taking into account their advantages, and numerous efforts are in progress to develop them. Among the structural components of CSFV, the E2 glycoprotein is an exceptionally potent antigen that can be targeted to ensure protection against clinical manifestations of CSF (35). Therefore, DNA-based vaccines expressing E2 protein can be used for vaccination against CSF.

Several DNA vaccines based on the E2 protein have been developed and have proven to confer varied protection of pigs against CSF. For instance, the Semliki Forest Virus (SFV) replicon-derived DNA vaccine expressing the complete E2 protein of CSFV confers total protection against a severe viral challenge in domestic pigs immunized three times with 600 mg or twice with 100 mg (36, 37), and an alphavirus replicon-vectored DNA vaccine encoding the E2 glycoprotein of CSFV can induce CSFV-specific cell-mediated immunity and can completely protect the immunized pigs from lethal challenge (38, 39).

SUBUNIT VACCINES

Subunit vaccines are usually composed of only one purified antigenic protein from the pathogen. CSFV peptide responses make it a candidate for a potential subunit vaccine. The membrane glycoprotein E2 is considered as one of the preferred target proteins in vaccine research for the development of a novel subunit vaccine (40,41). Previous research has shown that vaccination with the CSFV E2 subunit vaccine was an efficacious tool in a control program during an outbreak of CSF as from 10 days after vaccination (42,43), which could significantly reduce the vertical transmission of moderate-virulent strain of CSFV from the pregnant sow to its offspring and decrease the horizontal virus transmission of pigs (34). Furthermore, the anti-CSFV E2 neutralizing antibody was found to be induced in vaccinated pigs which subsequently developed resistance to the lethal virulence of CSFV challenge (7, 40). In cell cultures, pure preparations of envelope glycoproteins E^rns and E2 of CSFV synthesized in insect cells were used to study infection of porcine and bovine

cells with the pestiviruses CSFV and bovine viral diarrhea virus (BVDV). The results demonstrated that administration of 100 µg/ml E^{rns} or 10 µg/ml E2 could almost inhibit 100% infection of PK-15 cells with CSFV (22). Recently, a novel strategy for the large-scale production of a subunit vaccine expressing CSFV E2 has been investigated. It was found that the recombinant polyhedra obtained from the hemolymph in baculovirus-infected silkworm larvae could be used as an efficient strategy for the large-scale production of CSFV E2 subunit vaccine (44).

These promising results above suggest the feasibility of using subunit vaccines developed by E2-CSFV antigen which can be potentially used under outbreak conditions to stop CSFV spread and for eradication programs in CSF enzootic areas. However, the onset of immunity elicited by subunit vaccines occurs 2 weeks post-vaccination, limiting their efficacy relative to traditional live attenuated vaccines when animals are exposed to CSFV shortly after vaccination (40). Thus, subunit vaccines still require additional improvements aimed to ensure early protection, even when neutralizing antibodies are not detectable (36).

RECOMBINANT VIRUS VACCINES

Selection of a better vaccine vector that can stimulate a high immune response or enhance the immune response in vaccinated animals is therefore considered as an important factor for recombinant virus vaccine development. Up to date, Poxviruses, adenovirus (AdV), pseudorabies virus (PRV) and baculovirus have been constructed as a biologically safe CSF vaccine vector for this kind of vaccine development.

Poxvirus vector vaccine

Vaccinia virus (VV), ever used for immunization against smallpox, is a member of Orthopoxvirus genus, Poxviridae family. It can replicate within the cytoplasm of susceptible host cells and has a wide host range, such as humans, monkeys, swine, and cattle. Furthermore, VV has a large double-stranded DNA genome, in which many genes can be deleted without disturbing viral replication. Therefore it is considered as a good expression vector for heterologous genes in the laboratory investigations and the development of recombinant virus vaccines (45). In mice and pigs, neutralizing antibodies against CSFV are induced by using recombinant VV vaccine which expresses E2 protein. In addition, immunization with

the same recombinant vaccine protects pigs against a lethal challenge with CSFV, although CSFV-neutralizing antibodies are not detectable (46). Similar results have been obtained by other researchers, especially in connection with expression of envelope glycoproteins (47).

Except for VV, some other members of Poxviridae family also serve as vaccine vectors. Fowlpox virus and canarypox virus, two members of avipoxvirus genus, are excellent non-replicating expression vectors. Unlike VV with a broad host range, they replicate only in avian species. In a study, E^{rns} and E2 genes of CSFV are cloned and inserted into a fowlpox virus vector to construct a recombinant plasmid. After immunization with this vaccine, specific anti-CSFV antibodies are detected in experimental mice and pigs. Moreover, the immunized pigs can resist the attack of the virulent CSFV strain indicating that the recombinant fowlpox virus expressing E^{rns}-E2 represents a potential candidate for the development of a novel vaccine for the potential prevention of CSFV (48).

Le Potier *et al.* (2005) suggested a recombinant parapoxvirus strain which expressed E2 subunit against CSF. Swinepox virus (SPV), the only member of suipoxvirus genus, is known to infect only the porcine, and its infection in nature is usually mild and occasionally accompanies localized skin lesions that can heal naturally, indicating the possibility of another promising vector for CSFV vaccine (49,50).

Adenovirus vector vaccine (AdV)

In recent years, AdV has shown great promise as a good vector for recombinant vaccine development, because of its low virulence, efficacy and safety, and the ability to grow to high titers in cell cultures. Furthermore, AdV has been shown to induce effective humoral and cellular immune responses in experimental animals when it is delivered by the oral, intraperitoneal, intramuscular or intranasal routes (51).

The recombinant AdV is an attractive candidate vaccine for preventing CSF. Previous findings indicated that a recombinant human adenovirus type 5 expressing the CSFV E2 gene (rAdV-E2) developed high-level of CSFV-specific neutralizing antibodies in rabbits and pigs. The rAdV-E2-immunized rabbits were protected from fever induced by infection with C-strain, and the rAdV-E2-immunized pigs were protected from lethal challenge with highly virulent Shimen strain (52). To enhance the efficacy of the vaccine, Sun *et al.* (2011) found that the recombinant AdV encoded

the CSFV E2 gene fused with the UL49 gene from pseudo-rabies virus (PRV) induced the strongest CSFV-specific humoral immune response and the highest level of lymphocyte proliferation and interferon (IFN) in pigs (53). Therefore, this kind of vaccine might be an attractive candidate vaccine for preventing CSFV infection. In another study, by inserting CSFV E2 gene into the right hand end of the porcine AdV-3 genome, Hammond *et al.* (2000, 2001) obtained a recombinant strain rPAV-gp55 which remained stable and continued to express E2 at a high level. Although only low levels of neutralizing antibodies emerged in immunized pigs after a single subcutaneous vaccination, all the pigs were completely protected from lethal challenge (54,55).

Pseudorabies Virus vector vaccine

The incorporation of CSFV E2 into the gX locus of PRV does not change cell or host tropism, nor does it enhance the virulence of non-virulent PRV. So this recombinant PRV is safe to its hosts. Results of vaccination studies in pigs with a PRV vector expressing E2 protein showed that an antibody response directed against E2 was sufficient to protect pigs from CSF (56). Subsequently, Hooft *et al.* (1996) constructed another four recombinant PRV strains (M401, M402, M403 and M404) based on a strain M143 which contained deletions in the genes encoding thymidine kinase (TK) by using three overlapping cosmids (C-179, C-27 and C-443) and the HindIII-B fragments from plasmids pN3HBdelgE. The E2 gene inserted in the gE locus was able to archive detectable expression under the control of a proper promoter. All pigs vaccinated with one dose of M402 were fully protected from the challenge by the virulent CSFV strain Brescia (57).

Baculovirus vector vaccine

A recombinant baculovirus expressing E2 protein of CSFV elicited high-level CSFV-specific neutralizing antibodies and resisted a lethal challenge infection in the immunized mice and piglets (58), providing an easy and economical way to produce vaccines. Moreover, a recombinant baculoviruses expressing E^{rns} and/or E2 could be used potentially against CSFV infections (59, 60).

EPITOPE-BASED VACCINES

Based on the previous studies, the neutralizing epitopes corresponding to different regions of the A or BC domains of E2 were proposed and used as vaccines against CSFV in either mono-or multi-peptide formulations (31,42,61).

Up to date, many studies have indicated possibilities for developing epitope-based vaccines against CSF. Epitopes of E2 protein are mainly located at N-terminus such as CKEDYRY (aa693-699) (41), CFRREKPFPHRMDCVTTTVENED and CKEDYRYAISSTNEIGLLGAGGLT (62), N-terminal 90 residues (domains B/C) (30,32,33,61), and linear neutralizing epitopes mapped to E^{rns} of CSFV (42,63) are useful for development of diagnostic assays and epitope-based vaccines against CSFV.

MARKER VACCINES

Marker vaccines against CSFV infection is type of vaccine that enables easy but accurate serological differentiation between infected and vaccinated animals. As a candidate marker vaccine, it must be stable, low cost, highly sensitive, without potential danger to immunized animals or other species after possible genetic recombination and prevention of vertical or horizontal transmission.

CSFV marker vaccines with differentiation between infected and vaccinated animal capabilities have been developed using CSFV E2 envelope protein (64). The observations demonstrated that a deletion of the A-domain of E2 or E2-encoding region of CSFV, and using the eukaryotic expression plasmid with only 5' signal sequence of E2 (31) could be used as non-transmissible, modified, live-attenuated marker vaccines which protected pigs from a lethal challenge dose of the highly virulent strain and differentiate between infected and vaccinated animals (46,65). Additionally, epitope vaccines such as synthetic peptide vaccines using E2 N-terminal antigenic units B/C, which retained correct immunogenicity and was able to induce a protective immune response against CSFV infection, could be used as a potential marker vaccine to differentiate infected from vaccinated animals (32,33,62,63). Furthermore, a few studies suggested that the E^{rns} protein which possessed enzymatic activity and retained antigenicity might provide useful material for developing a marker vaccine (41).

CONCLUSION

E^{rns}, E1 and E2 are three important envelope glycoproteins of CSFV with antigen property and RNase activity, which are indispensable for viral attachment and entry of pestiviruses

into susceptible cells. Immunization of domestic pigs, mice or rabbits with a vaccine expressing only one or more than one viral glycoproteins of CSFV could induce a strong cellular immune response and confer total protection against a severe viral challenge. Therefore, it is possible to use glycoproteins of CSFV to develop marker vaccines against CSF. Many aims have been achieved through the efforts of research, however, there is still much work need to be done: 1) Comparative, studies mainly focusing on E^{rns} and E2 of CSFV, much less is known about E1; more investigations are required to study properties, functions and applications of E1; 2) the relationship between these glycoproteins needs to be studied; 3) the functions and the mechanism of heterodimeric and homodimeric complexes formed by glycoproteins of CSFV needs to be understood; 4) the locations of antigenic domains on glycoproteins and their application in CSF prevention and 5) the potential development of the peptide vaccines approach used to develop marker vaccines against CSF.

CONFLICT OF INTEREST

The authors declare that they have no conflict interests.

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