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Classical Swine Fever Virus: Independent Induction of Protective Immunity by Two Structural Glycoproteins

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To study which proteins of classical swine fever virus (CSFV) are able to confer protective immunity in swine, N-terminal autoprotease, viral core protein, and the three structural glycoproteins were expressed via vaccinia virus recombinants (VVR). CSFV proteins synthesized in cells infected with VVR showed migration characteristics on sodium dodecyl sulfate gels identical to those of their respective CSFV counterparts. Apparently authentic dimerization of the recombinant glycoproteins was observed. The glycoproteins E0 and E2 were detected on the surfaces of VVR-infected cells. In protection experiments, swine were immunized with the different VVR, and the generation of humoral immune response was monitored. Only animals vaccinated with VVR expressing E0 and/or E2 resisted a lethal challenge infection with CSFV. Glycoprotein E0 represents a second determinant for the induction of protective immunity against classical swine fever.

Classical swine fever (CSF), a highly contagious infection of swine, is caused by a small enveloped RNA virus named classical swine fever virus (CSFV) that is classified as belonging to the genus *Pestivirus* of the family *Flaviviridae* (36). The vast economic importance of CSF initiated various efforts to extinguish the disease. Vaccination with attenuated live virus is safe and effective but interferes with serodiagnosis and the maintenance of a virus-free status. In order to avoid trade restrictions, eradication programs are mostly based on the destruction of infected and serologically positive animals. In spite of continued attempts to control CSF, outbreaks have occurred intermittently in several European countries. In some areas, eradication is complicated by the prevalence of CSFV in the wild boar population. Furthermore, the enormous cost of eradication programs stimulated the search for alternative strategies. New tools for vaccine development were offered by recombinant DNA technology. Intensive studies of the molecular biology of pestiviruses (reviewed in reference 15) and the characterization of virus-encoded proteins (2, 25) enabled the rational design of vector or subunit vaccines which contain only selected viral polypeptides. Such new vaccines might combine efficacy, safety, and the opportunity for serological discrimination between vaccinated and infected animals.

Development of new vaccines requires comprehensive knowledge concerning the host's immune response toward the respective pathogen. Antibodies neutralizing CSFV are regarded as the most important specific defense against disease (27); however, the cellular immune response has not been thoroughly investigated. Pestiviruses encode four structural proteins, namely, the nucleocapsid protein C and the glycoproteins E0, E1, and E2 (2, 25, 28). CSFV glycoproteins E0 and E2 are known to induce virus-neutralizing antibodies (nAb) in mice (34, 35). Pigs vaccinated with vaccinia virus recombinants (VVR) (20) or pseudorabies virus (PRV) recombinants expressing E2 (32) developed nAb against CSFV and resisted a lethal challenge infection. However, the expression of E2 alone excludes the formation of E1-E2 heterodimers, which are present in major amounts in both cells infected with CSFV and virions (28). Dimerization of viral proteins may be important for authentic antigen presentation to the host's immune system and the induction of a stable, long-term immunity. With the exception of E2, the role of CSFV proteins in the context of antiviral host response is not defined. Resistance of swine vaccinated with a VVR, which expressed all CSFV structural proteins except for E2, against a lethal challenge strongly suggested that another CSFV protein(s) besides E2 is able to mediate protection (20).

Recently, the N termini of CSFV structural glycoproteins were determined by protein sequencing (21). This knowledge allowed the expression of essentially authentic CSFV proteins with VVR. In this paper, we demonstrate that E0 represents the second glycoprotein capable of protecting swine against CSF.

MATERIALS AND METHODS

Cells and viruses. The cell line MAX B4Z was derived from the kidney cells of a National Institutes of Health minipig (major histocompatibility complex $I^{d/d}$ haplotype) by transformation with simian virus 40 large T antigen (17) and can be infected with both CSFV and vaccinia virus. CVI, PK15, and 143tk$^-$ cells were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and nonessential amino acids.

Vaccinia virus WR was obtained from G. L. Smith (Cambridge, United Kingdom). CSFV Alfort Tübingen is identical to CSFV Alfort used in previous studies (14).

Recombination plasmids. Recombination plasmids were constructed by standard procedures (22). Vaccinia virus recombination vector pGS62 was kindly provided by G. L. Smith (3).

(i) pGS62-E0. A 0.7-kbp fragment (corresponding to nucleotides 1114 to 1838 of CSFV Alfort Tübingen [14]) which encompassed the coding sequence for all but 5 amino acids (aa) of CSFV E0 was isolated from plasmid pHCK11 (20) by digestion with *Bgl*I and *Ban*I. The missing codons were substituted for with synthetic adaptor oligonucleotides. The 5′ adaptor BBA (5′GATCCACCAT GGGGGCCCTGT3′) linked the *Bgl*I site of the 0.7-kbp fragment to the *Bam*HI site of pGS62 and contained a sequence according to Kozak's rules (11). Besides the initial methionine, BBA coded for 3 aa: glycine (not found in the CSFV sequence) and alanine and leucine (corresponding to CSFV aa 250 and 251). The 3′ adaptor BEA (5′GTGCCTATGCCTGAGTTA3′) connected the *Ban*I site of the 0.7-kbp fragment to the *Eco*RI site of pGS62 and encoded amino acids corresponding to glycine 491 to alanine 494 of CSFV as well as a stop codon.

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After ligation with adaptors BBA and BEA, the 0.7-kbp fragment was introduced into recombination vector pGS62 to derive plasmid pGS62-E0.

(ii) pGS62-E1/2 and pGS62-E1. A 2.1-kbp *Ban*I-*Hpa*I DNA fragment (representing nucleotides 1835 to 3971 of CSFV Alfort Tübingen) was subcloned from plasmid pHCK11 into the vector pBR02-16/4 containing a sequence coding for PRV gX signal peptide (PRV-SP) (18). The PRV-SP E1/2 fragment was excised by digestion with *Avi*I-*Sca*I and ligated into the *Sma*I site of pGS62 to give rise to pGS62-E1/2.

For the subsequent construction of a vector coding exclusively for E1, a 1-kbp *Nde*I-*Hin*dII fragment (including nucleotides 1835 to 2471 of CSFV Alfort Tübingen) resembling PRV-SP and CSFV E1 coding sequences was transferred into plasmid pGS62-Core (20) predigested with *Nde*I-*Stu*I to generate pGS62-E1. This construction positioned a TGA stop codon in frame downstream of the integrated CSFV sequence.

(iii) pGS62-E2. A 1.5-kbp DNA fragment (representing nucleotides 2433 to 3971 of CSFV Alfort Tübingen) isolated from clone pHCK11 by *Nhe*I-*Hpa*I digestion was ligated into plasmid pBR02-16/4. The resulting construct contained the nucleotide sequences for CSFV E2 and PRV-SP. Isolation of the PRV-SP E2 sequence and introduction into vector pGS62 gave rise to pGS62-E2.

Generation of VVR. VVR were generated essentially as described by Rümenapf et al. (20). Briefly, CVI cells were infected with vaccinia virus strain WR (VAC-WR) at a multiplicity of infection (MOI) of 0.05 and after 1 h were transfected with the respective recombination plasmids by using a mammalian transfection kit according to the supplier's protocol (Stratagene, Heidelberg, Germany). After 48 h of incubation, virus progeny was harvested by repeated freezing and thawing. Selection for a thymidine kinase-negative phenotype on human 143tk$^-$ cells was carried out with medium containing 1% agarose and 100 μg of bromodeoxyuridine per ml. VVR were isolated from thymidine kinase-negative virus plaques and were plaque purified twice.

Two VVR used in this study have been described: VAC-N^{pro}/C (formerly termed VACcore) expressing the N-terminal autoprotease (N^{pro}) together with the core protein and VAC-3.8 expressing all structural proteins and N^{pro} (20).

Immunoblot analyses. MAX B4Z cells infected with VVR (at an MOI of 2 for 12 to 24 h) or CSFV (at an MOI of 0.1 for 48 h) were harvested with a cell scraper after being washed repeatedly with phosphate-buffered saline (PBS). Aliquots representing identical cell numbers were diluted with 5 volumes of loading buffer containing 2% sodium dodecyl sulfate (SDS) with or without 5% β-mercaptoethanol and were heat denatured. Samples were subsequently separated by SDS–10% polyacrylamide gel electrophoresis (PAGE) (23) and transferred to nitrocellulose filters (Schleicher & Schuell, Dassel, Germany). Prestained molecular weight markers were from Bethesda Research Laboratories. Filters were blocked with 2.5% nonfat dry milk dissolved in PBS containing 0.05% Tween 20, and this was followed by overnight incubation with tissue culture supernatants from hybridomas 24/16 (anti-E0) (34) or a18 (anti-E2) (35) (both monoclonal antibodies [MAb] were diluted 1:2 in PBS, 1% bovine serum albumin, and 0.05% Tween 20) or rabbit anti-U1 serum (diluted 1:5,000 in the same buffer). The rabbit serum was prepared against bacterial fusion protein U1 encompassing aa 486 to 540 of CSFV. After several washes and incubation with alkaline phosphatase conjugated to goat anti-mouse or goat anti-rabbit immunoglobulin (Ig) antibodies (Ab) (Dianova, Hamburg, Germany), filters were bathed in the premixed components of an enhanced chemiluminescence system (Amersham/Buchler, Braunschweig, Germany) and subsequently exposed to Kodak XAR-5 X-ray film.

Radioimmunoprecipitation analyses. Metabolic labeling of CVI cells and radioimmunoprecipitation were carried out as described previously (20).

Immunofluorescence. MAX B4Z cells were infected with VVR (at an MOI of 1 for 12 h) or CSFV Alfort Tübingen (at an MOI of 1 for 24 h). Cells were washed in PBS and fixed in 2% paraformaldehyde buffered in PBS without $MgCl_2$ and $CaCl_2$ for 20 min at 4°C. For detection of intracellular antigens, cells were subsequently permeabilized by treatment with 1% *n*-octyl glucopyranoside (Boehringer, Mannheim, Germany) for 5 min at 4°C. Immunofluorescence staining was performed with MAb anti-E0 or anti-E2 and a goat anti-mouse IgG Cy3 conjugate (Dianova); the MAb against simian virus 40 large T antigen was also from Dianova.

Animal protection experiments. Pigs were vaccinated with a single dose of VVR or VAC-WR by three different routes simultaneously (intradermally, intraperitoneally, and intravenously). A total of 5×10^7 PFU of VVR was given by each route. Clinical reactions after vaccination were monitored by daily examination. Sera taken 4 weeks after vaccination were tested for nAb against vaccinia virus (VAC-WR) and CSFV (strain Alfort Tübingen) as described previously (20). The pigs were challenged intranasally 5 weeks after immunization with 2×10^7 50% tissue culture infective doses of CSFV Alfort Tübingen. Clinical symptoms were monitored daily. Blood samples were taken at days 5 and 12 postinfection (p.i.) and at the slaughter of the animals (days 12 to 27 p.i.).

Peripheral blood leukocytes from heparinized blood were counted with a hemocytometer. Plasma and buffy coat cells were prepared from heparinized blood and tested for the presence of infectious CSFV by inoculation of PK15 cells. CSFV in PK15 cell cultures was detected by indirect immunofluorescence (MAb a18).

Enzyme-linked immunosorbent assay (ELISA). (i) E0 Ab ELISA. Microtiter plates (Nunc, Roskilde, Denmark) were coated with rabbit IgG anti-E0 (29), blocked with 2.5% nonfat dry milk (in Tris-buffered saline containing 0.05% Tween 20), and subsequently incubated with serial dilutions of swine sera and antigen. Antigen was prepared from SF158 insect cells infected with a recombinant *Autographa californica* nuclear polyhedrosis virus which expresses CSFV E0 (12). After several washing steps with Tris-buffered saline with 0.05% Tween 20, immobilized nonblocked antigen was detected with biotinylated rabbit IgG anti-CSFV E0 and horseradish peroxidase conjugated to streptavidin (Dianova) with *o*-phenylene diamine as substrate. Microtiter plates were read for optical density at 492 nm (OD_{492}). A serum dilution was regarded as positive (anti-E0 antibodies) if the mean OD_{492} (from duplicates) was less than the mean OD_{492} of the respective preimmune sera (from eight replicates) minus three standard deviations.

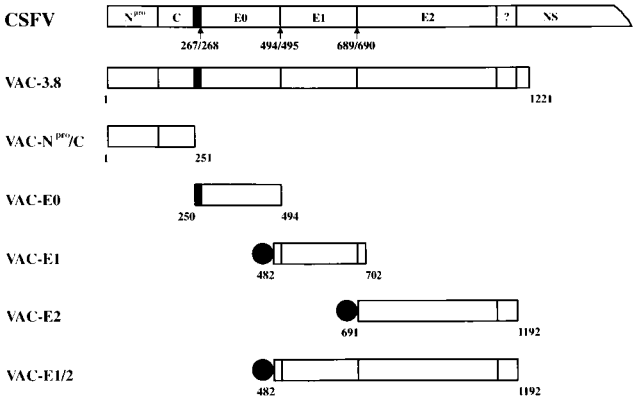

FIG. 1. Schematic presentation of VVR expressing different parts of the CSFV polyprotein. On the basis of cDNA clones derived from CSFV Alfort Tübingen, VVR expressing the core protein C together with the N^{pro} and the three viral glycoproteins (E0, E1, and E2) separately as well as in combinations (E1-E2 and N^{pro}-C-E0-E1-E2) were generated. Translocation of the glycoproteins into the endoplasmic reticulum was ensured by the authentic CSFV signal peptide (indicated by a solid bar) (VAC-E0 and VAC-3.8) or the PRV-SP (indicated by a solid circle) (VAC-E1, VAC-E2, and VAC-E1/2). Arrows indicate the cleavage sites of the CSFV polyprotein as determined by protein sequencing. Numbers refer to CSFV amino acid positions (strain Alfort Tübingen). NS, nonstructural proteins.

(ii) E2 Ab ELISA. Microtiter plates were coated with serum-free cell culture supernatant of hybridoma a18 (anti-CSFV E2). Blocking of plates was followed by addition of supernatant from SF158 cells infected with a CSFV-*Autographa californica* nuclear polyhedrosis virus recombinant that expresses a soluble form of CSFV E2 (12). After the plates were washed, serial dilutions of swine sera were added. Detection of bound Ab was carried out with a peroxidase-conjugated goat anti-swine Ig serum (Dianova) and *o*-phenylene diamine. The OD_{492} test serum/OD_{492} preimmune serum ratio was determined at a serum dilution of 1:200.

RESULTS

Generation of VVR. The regions of the CSFV polyprotein expressed by the different VVR are schematically shown in Fig. 1. For the expression of E0, the putative internal signal sequence localized immediately upstream of the E0 N terminus was included. While the exact start of this signal peptide is not known, sequence comparison with other signal sequences (33) suggested the use of a construct starting with aa 250 (alanine) of the CSFV polyprotein. VVR encoding E1 or E2 contained the PRV-SP upstream of the respective CSFV sequences to assure translocation of recombinant CSFV proteins into the lumen of the endoplasmic reticulum. When PRV-SP was used, effective translocation of CSFV glycoprotein E2 occurred in cells infected with PRV-CSFV recombinants (18). In order to obtain heterodimerization, an additional VVR encoding E1 together with E2 was generated. Translational stop codons at the 3′ ends of inserted CSFV nucleotide sequences were introduced to prevent the synthesis of fusion proteins.

Demonstration of CSFV proteins expressed by VVR. Expression of CSFV proteins by VVR was analyzed by immunoblotting, radioimmunoprecipitation, and immunofluorescence.

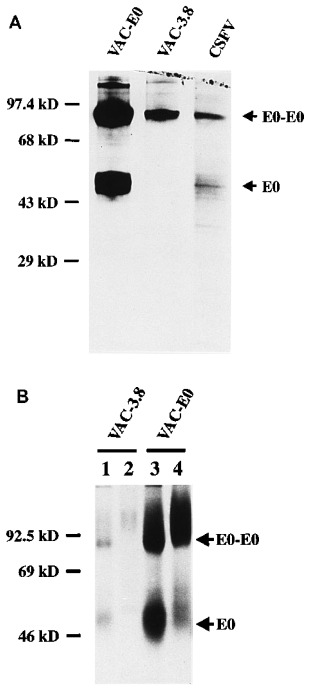

FIG. 2. Immunoblot analysis of E0 expression by VVR. (A) MAX B4Z cells were infected with VVR for 12 h or CSFV for 48 h. Cell lysates were separated by SDS–10% PAGE under nonreducing conditions, electrotransferred to nitrocellulose membrane, and subsequently immunostained with a MAb (24/16) against E0. Molecular masses are given in kilodaltons. E0 monomers and E0-E0 homodimers are indicated. (B) Radioimmunoprecipitation analysis of cell-associated and secreted E0 expressed by VAC-3.8 and VAC-E0. CVI cells were infected with VAC-3.8 or VAC-E0 and labeled metabolically with [^{35}S]cysteine-methionine. Cell extracts and cell culture supernatant were incubated with E0-specific MAb 24/16. Precipitates were separated under nonreducing conditions by SDS–10% PAGE. Precipitates from cell extracts (lanes 1 and 3) and cell culture supernatants (lanes 2 and 4) are shown. Molecular masses in kilodaltons are indicated at the left of the autoradiograph. E0 monomers and E0-E0 homodimers are indicated. Identical results were obtained with MAX B4Z cells as well.

N^{pro} and core protein C synthesized in cells infected with VAC-N^{pro}/C or VAC-3.8 revealed identical apparent molecular weights when compared with the respective CSFV polypeptides by immunoblotting (data not shown).

Glycoprotein E0 has been described as a 44- to 48-kDa protein that forms homodimers in both cells infected with CSFV and virions (28, 34). With respect to migration on polyacrylamide gels and homodimerization, E0 found after infection with VAC-E0 or VAC-3.8 was indistinguishable from authentic CSFV E0 (Fig. 2A). The highest level of expression was observed with the VVR expressing only E0.

Substantial amounts of glycoprotein E0 are present in virus-free supernatant of cells infected with CSFV (21); soluble E0 may either originate from extracellular virions or be secreted from infected cells. After infection with VVR, the secreted form of E0 could not be detected by immunoblotting. Cells infected with VVR VAC-E0 or VAC-3.8 were therefore metabolically labeled, and cell extracts as well as supernatants were incubated with an E0-specific MAb. E0 was precipitated from supernatants mostly as a dimer. The increased apparent molecular weight in comparison with that of the intracellular E0 is probably due to a modified carbohydrate composition (Fig. 2B). Glycoproteins E1 and E2 were not detected in tissue culture supernatants (data not shown).

After infection of tissue culture cells with CSFV, E1 forms

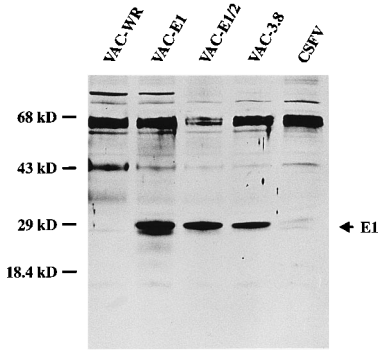

FIG. 3. Immunoblot analysis of E1 expression by VVR. MAX B4Z cells were infected with VVR and CSFV. Cell lysates were separated under reducing conditions by SDS–10% PAGE, transferred to nitrocellulose membrane, and incubated with rabbit anti-U1 serum. The serum is directed against a bacterial fusion protein comprising CSFV aa 486 to 540. The additional protein migrating faster than authentic E1 (lane VAC-E1) has not been further characterized. Molecular masses are given in kilodaltons.

intermolecular disulfide bridges with E2 (35). In order to demonstrate monomeric E1, the immune precipitates obtained from cells after infection with VAC-E1, VAC-E1/2, VAC-3.8, or CSFV were analyzed under reducing conditions. The migration pattern of E1 generated by the VVR was not different from that of the authentic CSFV glycoprotein of 33 kDa (Fig. 3).

For CSFV glycoprotein E2, three different forms, namely, E2 monomers (55 kDa), E2 homodimers (100 kDa), and E1-E2 heterodimers (75 kDa), have been described; the dimeric forms were found in infected cells as well as in virions (28). In extracts from cells infected with CSFV, mainly heterodimers were detected (Fig. 4). All three different forms of E2 occurred in cells infected with VVR VAC-3.8 and VAC-E1/2. The appearance of doublets or triplets may be due to different E2 protein backbones and/or to different glycosylation. We have recently demonstrated that alternative processing at the carboxy terminus generated E2 molecules with different apparent molecular weights (4). Whereas only minor amounts of monomeric and homodimeric E2 were detected after infection with VAC-3.8, substantial quantities of both forms were detected after expression by VAC-E1/2. In cells infected with VAC-E2, apparently similar amounts of monomer and homodimer were found.

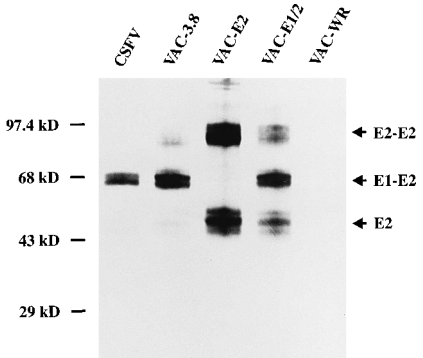

FIG. 4. Immunoblot analysis of E2 expression by VVR. MAX B4Z cells were infected with VVR and CSFV. Cell lysates were separated by SDS–10% PAGE under nonreducing conditions, transferred to nitrocellulose membrane, and immunostained with E2-specific MAb a18. Molecular masses are given in kilodaltons. E2 monomers, E1-E2 heterodimers, and E2-E2 homodimers are indicated.

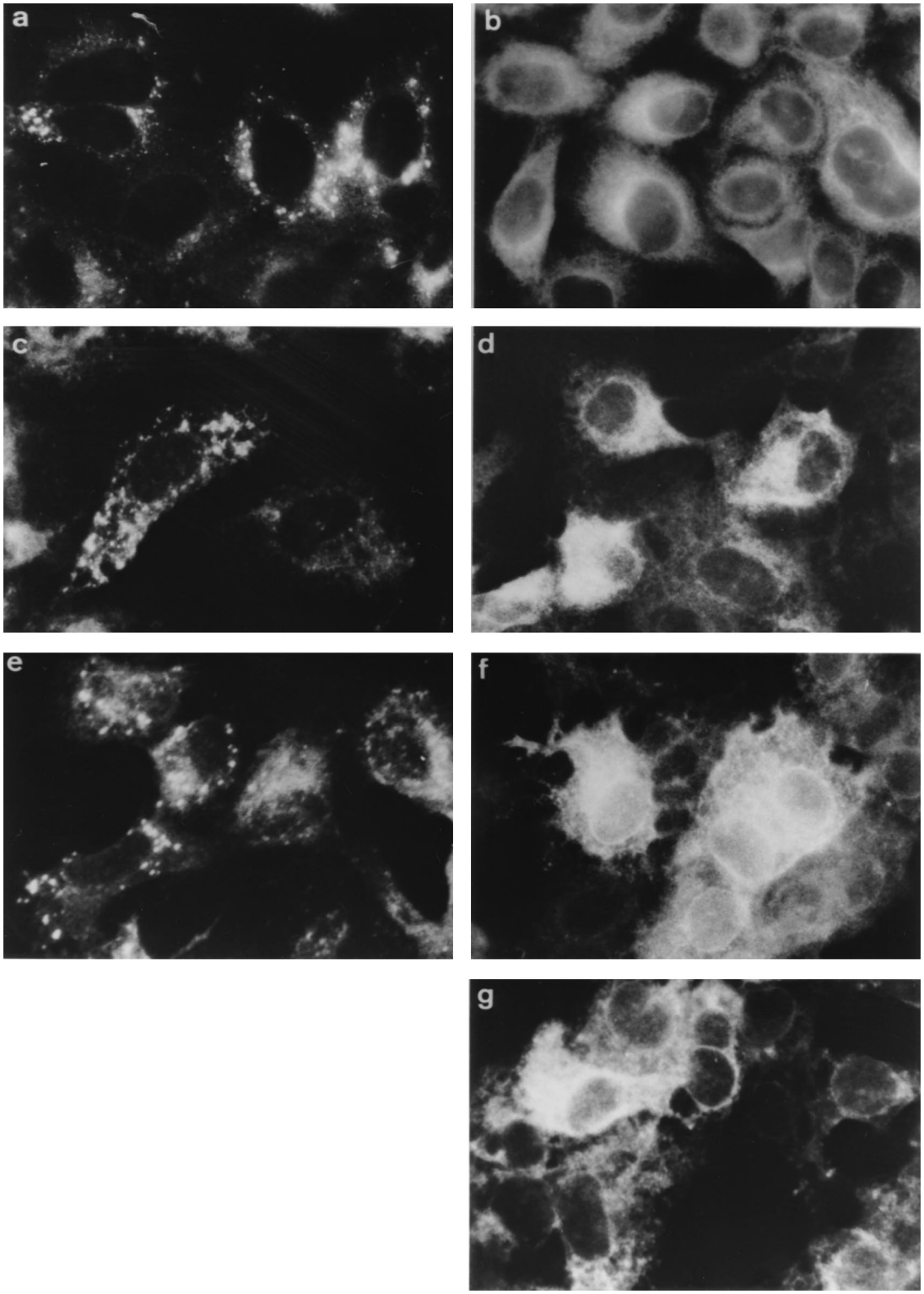

FIG. 5. Intracellular localization of CSFV proteins E0 and E2. MAX B4Z cells were infected with CSFV (a and b), VAC-3.8 (c and d), VAC-E0 (e), VAC-E2 (f), and VAC-E1/2 (g). Cells were fixed in 2% paraformaldehyde at 12 h p.i. (24 h for CSFV), permeabilized, and incubated with MAb anti-E0 (a, c, and e) or MAb anti-E2 (b, d, f, and g). Immunostaining was performed with a goat anti-mouse Cy3 conjugate. Pictures were taken with a fluorescence microscope.

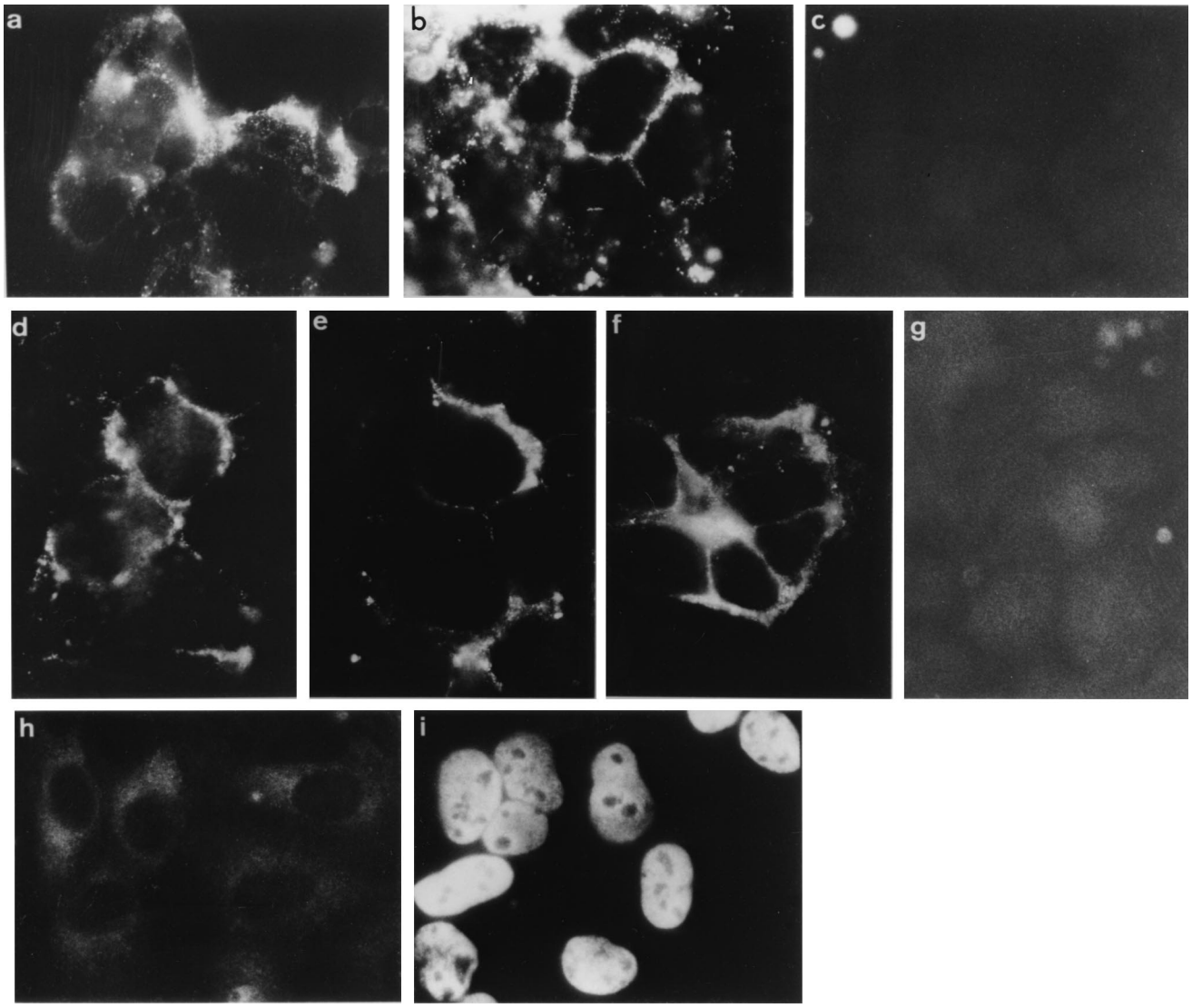

FIG. 6. Surface localization of CSFV proteins E0 and E2. In an experiment paralleling that shown in Fig. 5, staining was performed without permeabilization. Cells were infected with VAC-3.8 (a and d), VAC-E0 (b), VAC-E2 (e), VAC-E1/2 (f), and VAC-WR (c and g) and stained with MAb anti-E0 (a to c) or MAb anti-E2 (d to g). Nonpermeabilized (h) and permeabilized (i) MAX B4Z cells were infected with VAC-WR and stained with a MAb against simian virus 40 T antigen.

The localization of recombinant CSFV glycoproteins was investigated by immunofluorescence in MAX B4Z cells infected with VAC-E0, VAC-E1/2, VAC-E2, or VAC-3.8 (Fig. 5 and 6). Cells were fixed and permeabilized before a visible cytopathic effect had occurred. After the cells were immunostained, intracellular distribution patterns for E0 and E2 that resembled the situation described for cells after infection with CSFV were observed (34). Accordingly, E0 appeared in large granules with a distinct, mostly perinuclear localization, whereas E2 occurred in small granules distributed uniformly throughout the cytoplasm (Fig. 5). Interestingly, both glycoproteins were detected on the cell surface when cells infected with VVR were labeled with MAbs against E0 or E2 without permeabilization (Fig. 6). A remarkably strong surface expression of E0 after infection with VAC-E0 and of E2 after infection with VAC-E2 was observed. In contrast, only weak surface expression occurred on cells infected with VAC-3.8 (Fig. 6). The distribution patterns of E0 and E2 were similar in the two cell lines tested. In order to affirm the integrity of the cellular membrane after infection with VVR, MAX B4Z cells were stained with a control MAb against simian virus 40 large T antigen. In permeabilized cells, staining was observed in the cell nucleus, whereas no signal occurred in nonpermeabilized cells (Fig. 6).

Protection of swine against CSFV. In order to assess the ability of different CSFV proteins expressed by VVR to mediate protection in the natural host, animal experiments were performed. Two pigs were each inoculated with VVR or VAC-WR and subsequently monitored for clinical reactions and seroconversion; for VAC-3.8, only one animal was used. A significant rise in body temperature, observed 5 to 6 days after immunization, and nAb titers against vaccinia virus, determined 4 weeks after inoculation, demonstrated the successful vaccination of the animals (Table 1).

In sera taken prior to the challenge infection, the presence of Ab reacting with CSFV E0 or E2 was determined by employing two different ELISA systems. Sera from the two pigs inoculated with VAC-E0 and the animal that received VAC-

TABLE 1. Results of swine vaccination against CSFV

Pig no.	VVR	Titers for nAb against VAC[a]		Titers for nAb against CSFV[b]			Result for anti-E0 Ab[c]			Result for anti-E2 Ab[d]			Disease[e]	Titers for CSFV[f]		Day of death[g]
		S0	S1	S0	S1	S2	S0	S1	S2	S0	S1	S2		Day 5 p.i.	Day 12 p.i.	
1	VAC-N^{pro}/C	−	2.5	−	−	3.0	−	−	−	−	−	+	+	1.6	2.7	19
2		−	2.5	−	−	3.5	−	−	+	−	−	++	+	4.1	2.8	19
3	VAC-E0	−	2.8	−	−	3.7	−	++	+++	−	−	++	−			
4		−	2.5	−	−	3.1	−	+	+++	−	−	+	−			
5	VAC-E1	−	2.8	−	−	−	−	−	−	−	−	−	+	+[h]	3.9	12
6		−	2.2	−	−	>4.0	−	−	+++	−	−	++	+	+[h]		
7	VAC-E2	−	2.5	−	3.2	4.2	−	−	+	−	++	+++	−			
8		−	2.8	−	3.2	5.2	−	−	+	−	+	+++	(+)			
9	VAC-E1/2	−	2.8	−	3.9	4.8	−	−	+	−	++	+++	−			
10		−	2.5	−	3.4	5.4	−	−	+	−	++	+++	−			
11	VAC-3.8	−	2.5	−	3.4	4.2	−	+	+++	−	+	++	−			
12	VAC-WR	−	1.9	−	−	2.5	−	−	+	−	−	++	−		2.7	14
13		−	2.8	−	−	1.9	−	−	−	−	−	+	+	+[h]		14

[a] Titers are given as −log of the dilution that led to 50% plaque reduction. Serum samples were taken before vaccination (S0) and before challenge infection (S1). VAC, vaccinia virus; −, no neutralization detected.

[b] Titers are given as −log of the dilution neutralizing 100 50% tissue culture infective doses of CSFV in 50% of the inoculated microcultures (log 50% neutralizing dose per milliliter of serum). −, no neutralization detected (titer, <1.4 log 50% neutralizing dose per milliliter); S0, before vaccination; S1, before challenge infection; S2, at slaughter of the animals.

[c] Ab against E0 were detected by ELISA. S0, before vaccination; S1, before challenge infection; S2, at slaughter of the animals; −, negative; +, positive at a serum dilution of 1:8 to 1:16; ++, positive at a serum dilution of 1:32 to 1:64; +++, positive at a serum dilution of ≥1:128.

[d] Ab against E2 were detected by ELISA. S0, before vaccination; S1, before challenge infection; S2, at slaughter of the animals; −, OD_{492} test serum/OD_{492} preimmune serum ratio of ≤1.5; +, ratio of 1.5 to 1.9; ++, ratio of 2.0 to 2.4; +++, ratio of ≥2.5.

[e] Fever, inappetence, conjunctivitis, and leukopenia were regarded as signs of disease. (+), mild fever without other symptoms; +, presence of disease; −, absence of disease.

[f] CSFV viremia was demonstrated by virus isolation from plasma and buffy coat cells on days 5 and 12 p.i. Titration of virus was performed from plasma samples only (titers are given as log 50% tissue culture infective doses per milliliter).

[g] Moribund animals were killed at indicated day p.i.

[h] Virus was detected only in the buffy coat fraction.

3.8 reacted positive in the E0 Ab ELISA. Ab against E2 were demonstrated in sera from all animals inoculated with VVR expressing E2, i.e., VAC-E2, VAC-E1/E2, and VAC-3.8. Sera from swine vaccinated with VAC-N^{pro}/C, VAC-E1, and VAC-WR gave negative results in both ELISA tests. By a standard assay, nAb against CSFV were only detected in swine inoculated with VVR expressing glycoprotein E2 (Table 1).

Five weeks after vaccination, all animals were challenged with a lethal dose of CSFV Alfort Tübingen (26). Subsequent to the challenge infection, such marked clinical symptoms as fever, inappetence, and conjunctivitis were observed in swine immunized with VAC-N^{pro}/C, VAC-E1, or VAC-WR. In contrast, no clinical reactions occurred in the animals vaccinated with VAC-E0, VAC-E1/E2, or VAC-3.8. In the VAC-E2 group, one pig developed moderate fever for about 4 days without showing other symptoms.

A severe decrease of peripheral blood leukocytes is regarded as an early and significant parameter of acute CSFV infection (26, 30). After challenge with CSFV, marked depressions of peripheral blood leukocyte numbers were observed only in animals with severe clinical symptoms, i.e., swine vaccinated with VAC-N^{pro}/C, VAC-E1, or VAC-WR (data not shown).

Blood samples were analyzed to monitor the appearance of infectious virus in the animals after challenge. Infectious CSFV was isolated from plasma or buffy coat cells of swine obviously affected by the disease (i.e., pigs vaccinated with VAC-N^{pro}/C, VAC-E1, and VAC-WR) from day 5 p.i. onwards. Plasma titers ranged between 1.6 and 4.1 50% tissue culture infective doses per milliliter. No viremia was detected in the remaining animals (Table 1).

With the exception of one swine (VAC-E1) that recovered after a 2-week period of dramatic illness, all animals with severe clinical symptoms had to be sacrificed in a moribund state between days 12 and 19 p.i. Postmortem examinations revealed typical gross lesions of acute swine fever; viral antigens and viral RNA were demonstrated in lymphatic and nonlymphatic tissues (data not shown).

Examination of sera taken at the time of slaughter demonstrated nAb against CSFV in all animals, with the exception of one belonging to the VAC-E1 group that was sacrificed at day 12 p.i. Pigs vaccinated with VAC-E0 and VAC-3.8 developed a strong anti-E0 response, indicating a booster effect. The other animals showed only a weak or nondetectable reaction in the E0 Ab-ELISA. By the E2 Ab ELISA, a positive seroreaction was observed in all animals except the swine immunized with VAC-E1 that also did not show nAb against CSFV (Table 1). Following infection with a lethal dose of CSFV, specific serum Ab can generally be demonstrated at 2 to 3 weeks p.i. (30).

DISCUSSION

The main objective of the study was to determine which of the CSFV structural proteins are able to induce a protective immunity in the natural host. Glycoprotein E2 has been demonstrated to protect swine against a challenge infection with virulent CSFV when either PRV-CSFV-E2 recombinants or purified E2 is used (7, 32). Evidence that other CSFV proteins can induce protection was provided when swine resisted a challenge infection after vaccination with a VVR expressing CSFV-encoded structural proteins but not E2 (20). From this experiment, it could be concluded that N^{pro}, core protein C, E0, and E1 are additional candidates for the induction of protective immunity. Testing of individual CSFV structural proteins in swine was performed after the generation of VVR.

In order to study biosynthesis of the CSFV proteins expressed in the vaccinia virus system, different parameters were

examined: (i) apparent molecular weights of the proteins, (ii) reactivity with specific Ab, (iii) dimerization, and (iv) subcellular localization. Since cell types may differ with regard to posttranslational modifications, antigens for immunoblotting assays were derived from one cell line (MAX B4Z) infected with either VVR or CSFV. With respect to gel migration and dimerization, CSFV proteins expressed by VVR showed the same characteristics when compared with their CSFV equivalents. Preferential formation of E1-E2 heterodimers was observed after infection with VVR that expressed E1 and E2 on a polyprotein (VAC-3.8 and VAC-E1/2). Interestingly, when E2 was expressed without E1, significant homodimerization occurred.

Immunofluorescence studies demonstrated that structural glycoproteins E0 and E2 localize differently inside cells infected with CSFV (34). The proteins expressed by different VVR showed essentially the same distribution (with accumulation of E0 most likely in a post-endoplasmic reticulum compartment), suggesting authentic intracellular transport. In addition, we demonstrated expression of CSFV E0 and E2 at the cell surface. So far, attempts to detect viral glycoproteins on the surfaces of cells infected with pestiviruses were unsuccessful (5). It is tempting to speculate that the infection with VVR led to enhanced expression which enabled the detection of E0 and E2 at the cell surface. However, the cytopathic effect of vaccinia virus may also play a role, even though no apparent lysis had occurred before the staining of the cells.

The protective capacity of recombinant CSFV proteins was determined in animal experiments. The wide host range of vaccinia virus makes vaccination experiments in several animal species feasible (13). However, previous experiments demonstrated a low susceptibility of swine for infection with vaccinia virus (20). High virus doses and a complicated inoculation technique were used to achieve a generalized infection. Successful vaccination of the animals was demonstrated by clinical reactions and a humoral immune response against vaccinia virus. Animals vaccinated with VAC-N^{pro}/C or VAC-E1 were not protected against a lethal challenge infection with CSFV. It is noteworthy that infections of pigs with CSFV gave no indications for the induction of Ab against these three proteins, namely, N^{pro}, core protein C, and E1 (29, 37). Resistance of swine inoculated with VVR expressing glycoprotein E2, i.e., VAC-E1/2, VAC-E2, and VAC-3.8, against the challenge infection confirmed earlier results (7, 20, 32). When protection mediated by VAC-E1/2 and that by VAC-E2 were compared, only a slight difference was noted: one of the animals immunized with VAC-E2 showed a febrile reaction after the challenge infection, whereas no clinical symptoms were observed in swine vaccinated with VAC-E1/2. The influence of intermolecular dimerization upon protein structure and antigenic domains of E2 is unknown. So far, only intramolecular disulfide bonds located in the N-terminal half of E2 have been shown to be important for antigen recognition by MAb (31).

Interestingly, pigs immunized with VAC-E0 were fully protected, even though nAb were not detected. Virus neutralization may still contribute to the protection of animals vaccinated with VAC-E0. In cell culture experiments, MAb and monospecific sera derived against E0 exhibited a weak neutralizing effect toward CSFV (29, 34). The failure to demonstrate nAb in animals immunized with VAC-E0 can be explained by low titers of induced Ab not detectable by standard neutralization assays. The test virus preparation used in serum neutralization tests was obtained from culture supernatant of cells infected with CSFV. It should be noted that cell culture supernatant contains significant amounts of soluble E0 (21); binding of anti-E0 Ab to soluble E0 may decrease the sensitivity of neutralization assays.

Recently, an RNase activity of E0 was reported (6, 24). The role of E0 in CSF pathogenesis remains to be established, but an effect upon distant noninfected cells seems possible, since soluble E0 has been demonstrated in the sera of infected animals (24). Binding of Ab to soluble E0 might interfere with the in vivo RNase activity, thereby influencing CSF pathogenesis.

In addition, an induction of the host's cytotoxic T-cell response by vaccination with recombinant E0 has to be considered. Proteins expressed by VVR are capable of stimulating cytotoxic T cells in vivo (16), and an antiviral effect of cytotoxic T cells has been demonstrated in several virus systems (1, 38). However, in experiments in which VAC-E0-infected MAX cells were used as targets for syngeneic, porcine, CSFV-specific cytotoxic T cells, we were unable to identify a cytotoxic T-cell epitope on E0 (17).

A basic requirement of vaccines against CSF is the induction of protective immunity against a broad range of virus strains. Challenge infections in previous protection experiments with recombinant E2 were carried out exclusively with homologous CSFV strains (7, 20, 32). It remains to be established whether such immunizations will protect against heterologous CSFV strains. Animal experiments with the highly virulent CSFV strain Eystrup revealed partial protection after vaccination with either VAC-E0 or VAC-E1/2 (9). With respect to their reactivity patterns with MAb against E0 and E2, CSFV Eystrup and CSFV Alfort Tübingen are clearly different (10). The incomplete protection observed underlines the necessity for further experiments. The activation of T-helper cells is needed for an effective humoral immune response, including long-term memory. Kimman et al. failed to identify the E2 protein of CSFV as a major target for the porcine T-cell response (8). This finding suggests the absence of immunodominant T-helper-cell epitopes. It will be interesting to investigate E0 in this regard. A future vaccine against CSF may well refer to both envelope proteins.

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