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Characterization of classical swine fever virus entry by using pseudotyped viruses: E1 and E2 are sufficient to mediate viral entry

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Abstract

Classical swine fever virus (CSFV) is the causative agent of classical swine fever. Its envelope comprises glycoproteins E^{rns}, E1, and E2. In this study, we showed that the unmodified CSFV glycoproteins could incorporate into the HIV core to generate an infectious CSFV pseudotyped virus. The infection was specific to several porcine cell lines, and could be neutralized by anti-E2 monoclonal antibodies (mAbs) completely and by anti-E^{rns} mAbs partially, indicating that this pseudotyped virus can mimic the early infection steps of parental CSFV. To investigate the specific role of each envelope protein involved in viral entry, a series of pseudotyped viruses were generated bearing CSFV glycoproteins in various combinations. It was found that specific infectivity was also achieved with non-E^{rns} pseudotyped virus carrying E1 and E2 glycoproteins. This indicated that E1 and E2 are sufficient to mediate CSFV entry, and E^{rns} is not indispensable in this process.

Keywords: CSFV; Pseudotyped virus; Glycoprotein; pH-dependent

Introduction

Classical swine fever (CSF) caused by the classical swine fever virus (CSFV) is an important, highly contagious and often fatal pig disease with widespread economic ramifications. CSFV belongs to the *pestivirus* genus within the *Flaviviridae* family (van Regenmortel et al., 2000). It is an enveloped, nonsegmented, positive-strand RNA virus encoding three envelope glycoproteins: E^{rns} (also named E0), E1, and E2 (Rumenapf et al., 1993; Weiland et al., 1990; Wensvoort et al., 1990). E1 and E2 have transmembrane domains. They can be present as an E1–E2 heterodimer, and also as an E2–E2 homodimer, by disulfide linkage (Weiland et al., 1990; Wensvoort et al., 1990). E^{rns} lacks the membrane anchor and is secreted from infected cells (Rumenapf et al., 1993). It can also associate with virions by an as yet unknown mechanism (Weiland et al., 1992).

E2 and E^{rns} have been reported to be involved in CSFV entry (Hulst and Moormann, 1997), but their exact functions in this process are still unclear. E2 is the major neutralizing antigen for CSFV infection (Greiser-Wilke et al., 1990; Rumenapf et al., 1993), while E^{rns} is considered the second glycoprotein that mediates neutralization (Weiland et al., 1992). However, because of the low titer of CSFV in cell cultures and the inherent obstacles in traditional virus entry assays, the details concerning the CSFV entry mechanism remain obscure. Therefore, a specific and sensitive CSFV entry assay is required to separate viral entry and post-entry stages, and to dissect the function of each glycoprotein in this process.

Retroviral pseudotyped viruses have been widely used to study viral entry, because retroviruses can incorporate heterologous virus envelope proteins into their lipid membranes and acquire the host range of the virus from

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which the heterologous glycoprotein is derived (Bartosch et al., 2003b; Dong et al., 1992; Hsu et al., 2003; Landau et al., 1991; Suomalainen and Garoff, 1994). Furthermore, the pseudotyped viruses only undergo a single-cycle infection and carry a reporter gene, thus facilitating quantitative assays. In this study, we incorporated unmodified CSFV glycoproteins into the HIV core to generate infectious CSFV pseudotypes, and showed that their infection could be neutralized by anti-E2 mAbs completely and by anti-E^{rns} mAbs partially. Moreover, specific infectivity could be achieved with non-E^{rns} pseudotypes carrying E1 and E2, indicating that E1 and E2 are sufficient to mediate CSFV entry, and E^{rns} is not indispensable in this process.

Results

Infectious CSFV pseudotyped particles can be assembled in vitro

The CSFV genomic RNA is translated into a polyprotein that is cleaved into a number of proteins by the host cells and virus-encoded proteases. A glycoprotein precursor $E^{rns}12$ is formed, which is processed into E^{rns}, E1, and E2 (Rumenapf et al., 1993). During this process, the C terminus of the C protein serves as a signal peptide for CSFV glycoproteins and is recognized by signalase, which causes a nascent cleavage between the C protein and the trans-located $E^{rns}12$. Therefore, for the expression of CSFV E^{rns}, E1, and E2 glycoproteins, the putative internal signal sequence localized immediately upstream of the E^{rns} N terminus was included. While the exact start of this signal peptide is unknown, we reserved 60aa of the C terminus of the core protein containing the signal peptide for E^{rns}, E1, and E2 (Fig. 1)—a similar strategy was also used for the generation of HCV pseudotyped viruses (Bartosch et al., 2003b).

The $E^{rns}12$ gene was cloned into pcDNA3.1(+) under control of the cytomegalovirus immediate–early promoter ($pE^{rns}12$). The polyprotein was expressed by transient transfection in 293T cells. We first detected the cell surface expression of CSFV glycoproteins, as HIV budding and incorporation of glycoproteins occurred at this place. CSFV E^{rns} was reported to be located on the cell surface, while E2 was located in the endoplasmic reticulum and was not targeted to the cell surface. But when overexpression of E2 occurred, cell surface expression of this glycoprotein could be detected (Konig et al., 1995; Rumenapf et al., 1993; Thiel et al., 1991; Weiland et al., 1999). Our flow cytometric analysis (Fig. 2) demonstrated that both E^{rns} and E2 proteins could be detected on the cell surface. E2 dimmer formation was examined by using a nonreduced immunoblot assay (Rumenapf et al., 1991; Weiland et al., 1990). The apparent band with molecular mass of approximately 75 kDa corresponded to the E1–E2 heterodimers (Fig. 3A), while the E2–E2 homodimers were about 100 kDa and could also be detected (Rumenapf et al., 1991; Wensvoort et al., 1990). HIV p24 as a control could also be detected in the virus pellet (Fig. 3B).

To produce the CSFV pseudotyped virus, 293T cells were co-transfected with the plasmid pNL4.3.Luc. $R^-E^-pro^-$ encoding an envelope-defective HIV-1 proviral genome with a luciferase reporter gene (Connor et al., 1995), and $pE^{rns}12$ encoding CSFV glycoproteins. Controls were generated with the pcDNA3.1 (+) vector or with plasmid encoding VSV G. Pseudotyped particles were purified from supernatants by ultracentrifugation through

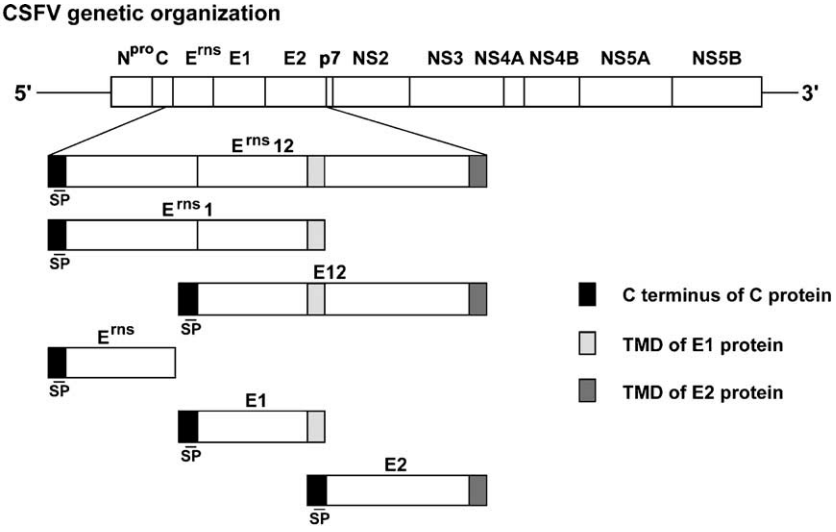

Fig. 1. CSFV E^{rns}E1E2 expression constructs. A cDNA derived from the CSFV polyprotein gene was used to express the E^{rns}, E1, and E2 glycoproteins individually or in different combinations. The C terminus of the C protein, which natively provides the signal peptide (SP) for E^{rns}, was here used to provide the SP for each construct. TMD: transmembrane domain.

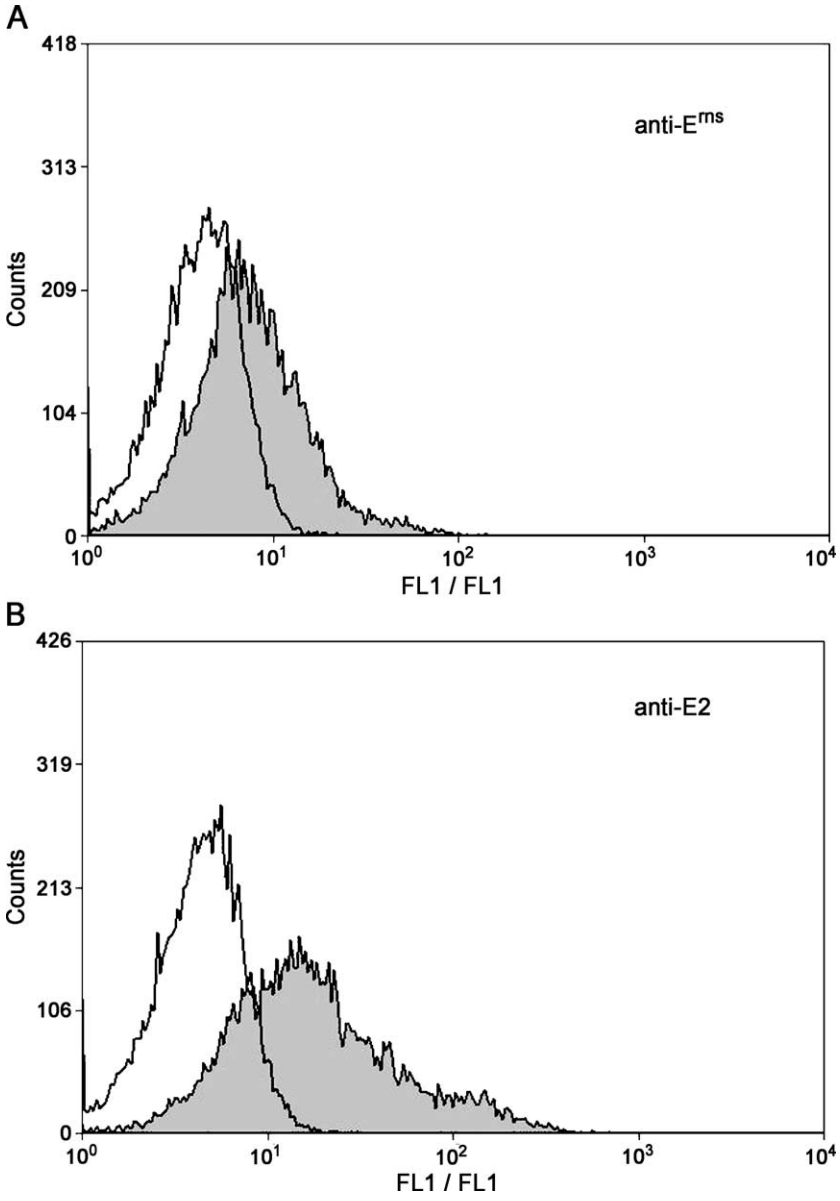

Fig. 2. Detection of CSFV E^{rns} and E2 expression on the cell surface by flow cytometry. 293T cells were transfected with $pE^{rns}12$. Forty-eight hours after transfection, cells were stained with anti-E^{rns} mAbs 140.1 (A) or anti-E2 mAbs V3 (B), respectively (shaded shape). Cells transfected with pcDNA3.1 (+) vector were used as negative controls (open shape).

the 20% density sucrose cushion, and viral lysate underwent the nonreduced immunoblot. As shown in Fig. 3B, a 75-kDa band representing CSFV E1–E2 heterodimers could be detected, indicating they can incorporate into HIV cores.

To test if the pseudotyped virus bearing CSFV $E^{rns}12$ (termed HIV/$E^{rns}12$) was infectious, PK15 cells were incubated with supernatants containing the pseudotyped virus (5 ng per well p24) for 3 h, and the luciferase activity of the cell lysates was tested 48 h later. It was found that HIV/$E^{rns}12$ was infectious to PK15 (Fig. 4A). The tropism of HIV/$E^{rns}12$ was then investigated on a panel of target cell lines derived from swine, mouse, hamster, canine, monkey, and human. We found that only swine cell lines PK15, LLC-PK1, SK6, MPK, and ST could be infected by HIV/$E^{rns}12$, and among these cell lines we detected the highest infectivity occurred on PK15 cells (Fig. 4A). HIV/VSV G was used as a positive control and could infect all cell lines. These data indicated that the infectious CSFV pseudotyped virus can be assembled in vitro, and its tropism restricts to swine cell lines, as previously reported with the wild-type CSFV (Carbrey et al., 1976).

CSFV pseudotyped virus can be neutralized by anti-E2 and anti-E^{rns} mAbs

To further ascertain whether the specific infectivity of CSFV pseudotyped virus was mediated by CSFV glycoproteins, two anti-E^{rns} mAbs (24/16, 140.1) and two anti-E2

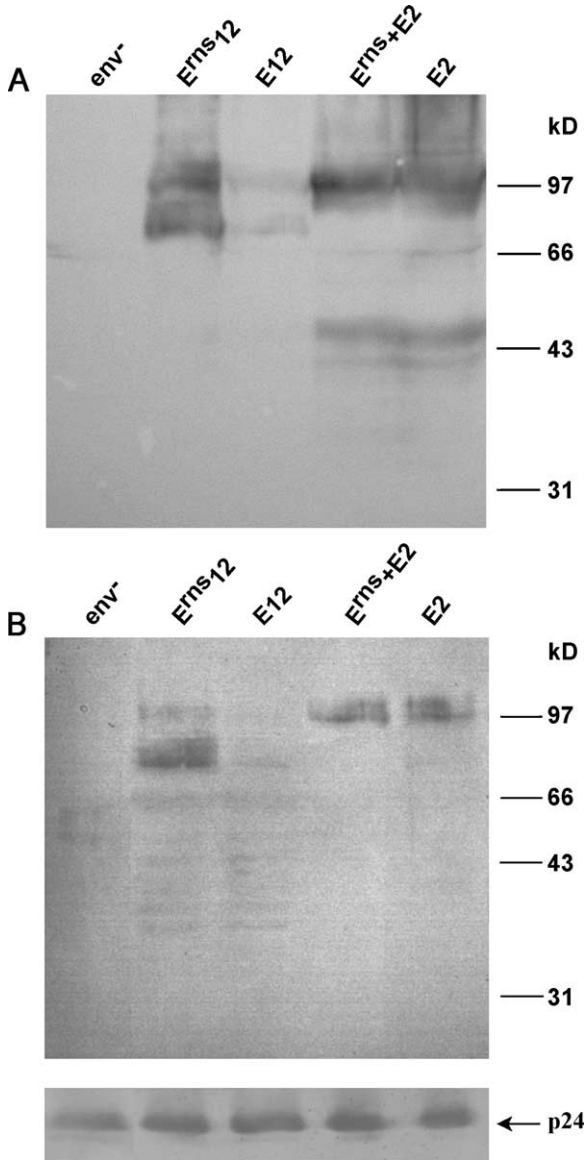

Fig. 3. Western blotting analysis of E2 expression from transfected cells and purified pseudotyped viral particles. (A) 293T cells were transfected with expression constructs (E2, E^{rns} plus E2, E12, $E^{rns}12$). After 48 h, cell lysates were separated under 10% nonreduced SDS-PAGE, and blotted with anti-E2 mAb a18. 293T cells transfected with pcDNA3.1 (+) vector were used as negative controls. (B) Pseudotyped viruses bearing CSFV glycoproteins in various combinations (E2, E^{rns} plus E2, E12, $E^{rns}12$) were purified through 20% sucrose cushions. Pellet samples were submitted to run 10% nonreduced SDS-PAGE and blotted with anti-E2 mAb a18 or HIV p24 antiserum as a control. Empty HIV cores with no glycoprotein provided were used as negative controls.

mAbs (a18, V3) were tested for their ability to neutralize the infection of HIV/$E^{rns}12$ on PK15 cells. The antibody anti-p125 against the nonstructural protein NS3 was used as a negative control. We found both of the two anti-E2 mAbs showed more than 95% neutralizing ability at 5 μg/ml, while both of the two anti-E^{rns} mAbs showed weak neutralizing ability against HIV/$E^{rns}12$ infection at a higher concentration of 20 μg/ml (Fig. 4B).

CSFV pseudotyped virus entry is inhibited by NH_4Cl

To assess whether the CSFV pseudotyped virus entry is pH-dependent (Flores et al., 1996), we treated PK15 cells with well-characterized lysosomotropic agent NH_4Cl. Exposure of cells to NH_4Cl prevents endosomal acidification, thus blocking the pH-dependent fusion between the virus envelope and the intracellular endosomal membrane (Sieczkarski and Whittaker, 2002). HIV bearing VSV G and amphotropic murine leukemia virus (AMLV) glycoprotein were used as controls, which demonstrated pH-dependent and pH-independent routes of entry, respectively. Treatment with 20 mM NH_4Cl caused >90% inhibition of infection by HIV/VSV G or HIV/$E^{rns}12$, while no inhibition could be detected with AMLV pseudotyped virus (Fig. 5). These data indicated the HIV/$E^{rns}12$'s entry may be pH-dependent.

Infectious CSFV pseudotyped virus can be generated without E^{rns}

To study the specific role of each CSFV glycoprotein in pseudotyped virus entry, a series of expression plasmids were constructed, encoding E^{rns}, E1, E2, $E^{rns}1$, and E12, respectively (Fig. 1). These plasmids allowed us to generate various pseudotyped viruses bearing CSFV glycoproteins in various combinations. We checked the expression of glycoprotein E2 in cell lysates, and also in pseudotyped viruses with HIV p24 detection as a control. As shown in Western blots (Figs. 3A,B), when E2 was expressed without E1, it strongly formed a homodimer of about 100 kDa; and when matured from the polyprotein $E^{rns}12$ or E12, the E1–E2 heterodimer, at about 75 kDa, was the major form that could be detected.

We then tested the infectivity of these pseudotyped viruses on PK15 cells. Besides pseudotypes bearing E^{rns}, E1, and E2, we found pseudotyped virus bearing E1 and E2 could also achieve high infectivity, especially when matured from E12 (termed HIV/E12), while other pseudotyped viruses had no infectivity (Fig. 6A). Further experiments were performed to investigate the entry characteristics of HIV/E12. We found its tropism was also restricted to porcine cell lines (Fig. 4A); the infection could be inhibited by NH_4Cl (Fig. 5), and could be completely neutralized by anti-E2 mAbs (a18 and V3) at the concentration of 5 μg/ml (Fig. 6B). All of these characteristics were similar to that of HIV/$E^{rns}12$. These data indicated that CSFV E1 and E2 are sufficient to mediate pseudotyped virus entry, and E^{rns} is not indispensable in this process.

We found that the infectivity of HIV/E12 was lower than HIV/$E^{rns}12$ on all cell lines tested (Fig. 4A). One possible impact factor was the low expression level of CSFV glycoproteins in 293T cells transfected with pE12. As shown in Fig. 3, the expression level of CSFV glycoproteins in 293T cells transfected with pE12 was lower than that of 293T cells transfected with p$E^{rns}12$ (Fig. 3A), and a similar

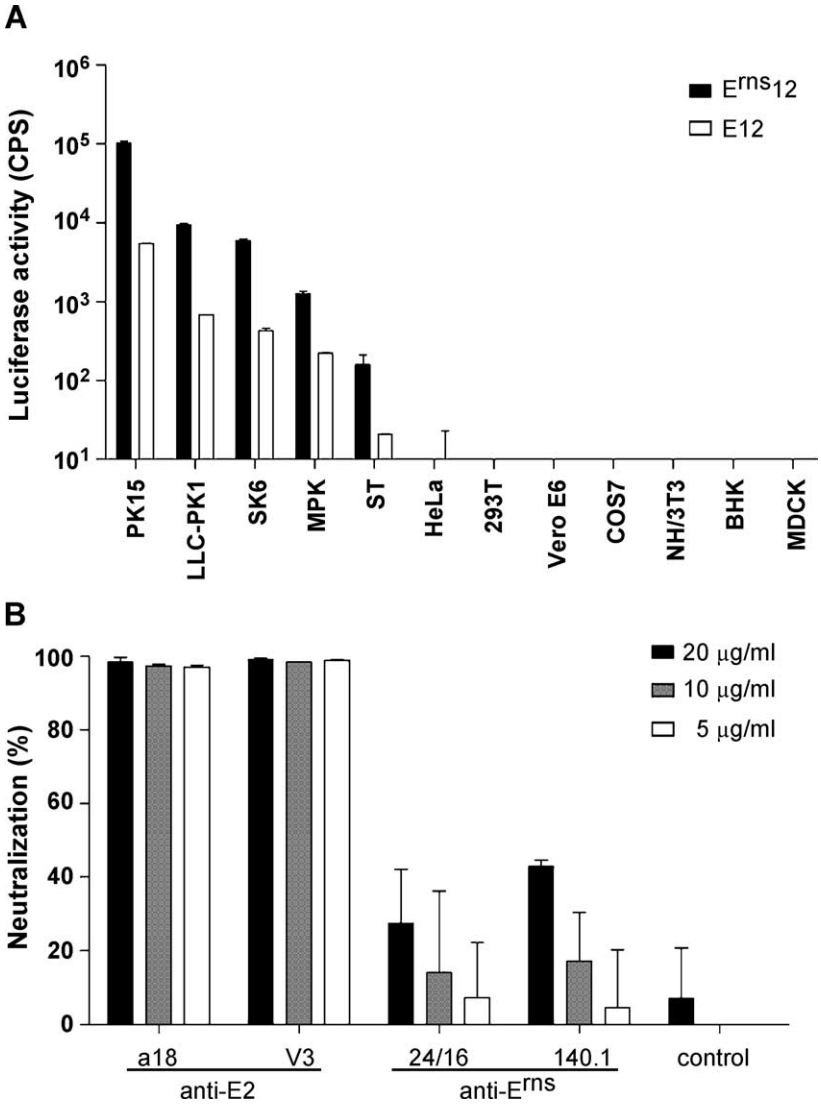

Fig. 4. Specific infection of CSFV pseudotyped viruses. (A) Infectivity of CSFV pseudotypes on different cell types. The results of infection were shown by average of luciferase activity (CPS) of triplicate determinations. (B) Neutralization of CSFV pseudotypes' infectivity. MAbs at concentrations of 20, 10, and 5 μg/ml were used to neutralize CSFV pseudotypes infection on PK15 cells. MAb anti-p125 specific to a nonstructural protein NS3 of CSFV was used as a negative control. Neutralization was determined by inhibition of luciferase activity. Similar results were obtained in two independent experiments.

result was also achieved in corresponding pseudotyped viral particles (Fig. 3B).

Another reason may be the lack of E^{rns} that lead to lower infectivity. However, providing E^{rns} *in trans* with E12 did not assist in improving of the infectivity of the pseudotyped virus, but actually lead to lower infectivity (Fig. 6A). Similarly, pseudotypes bearing E1 and E2 matured from pE1 and pE2 *in trans* also lead to lower infectivity than those matured from pE12 *in cis*. These data suggested the expression and maturation pattern of CSFV glycoproteins might have an impact on protein functions.

CSFV glycoproteins can induce cell–cell fusion

To further ascertain if the CSFV glycoproteins function in the membrane fusion process, we tested their ability by cell–cell fusion assay because the fusion between cells expressing virus glycoproteins and the susceptible cells could mimic the virus–cell fusion process. 293T cells were transiently transfected with $E^{rns}12$, E12, E2 expression constructs, and then mixed and cultured with PK15 cells. As we previously found the CSFV entry might be a pH-dependent process, in this cell–cell fusion experiment, the cocultured cells were treated with PBS at pH 5.5 for 2 min and then cultured normally. Syncytia formation was checked under microscope 6 h post acidification. Cells containing more than five nuclei were considered typical syncytia. As shown in Fig. 7, $E^{rns}12$ could induce syncytia formation. E12 also induced fewer numbers of syncytia formation. 293T cells transfected with E2 alone and pcDNA3.1 (+) vector as a negative control showed no syncytium formation with PK15 cells. We also did this experiment at neutral pH,

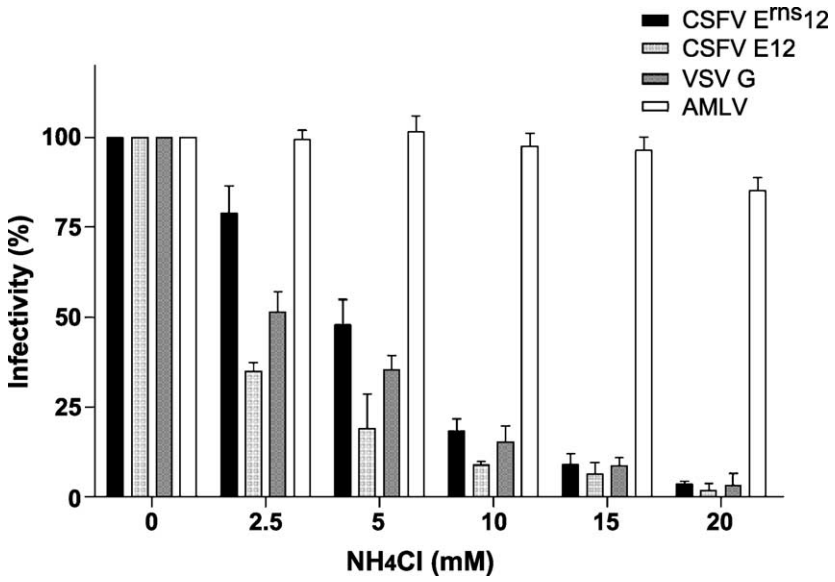

Fig. 5. pH dependency of CSFV pseudotypes infectivity. PK15 cells treated with various concentrations of NH_4Cl were infected with 5 ng (p24) of each pseudotyped virus. The infectivity was determined at 48 h post infection based on the luciferase activity. The luciferase activity from 0 mM NH_4Cl-treated cells was taken as 100%.

but the typical syncytia could hardly be observed (data not shown). We also found that neither PK15 nor 293T expressing CSFV glycoproteins could solely form syncytium, either in a neutral pH or after acidification (data not shown). These results indicated that acidic environment strongly promotes membrane fusion mediated by CSFV glycoproteins, and E1 and E2 are sufficient to mediate this process.

Discussion

In this study, we demonstrated that unmodified CSFV glycoproteins matured from the polyprotein $E^{rns}12$ of CSFV could incorporate into the retroviral core to generate an infectious pseudotyped virus. The infection of pseudotyped virus was specific for several types of porcine cell lines, including PK15, LLC-PK1, SK6, MPK, and ST cells, which was consistent with wild-type virus infection (Carbrey et al., 1976). Because the CSFV glycoproteins we used were derived from cF114 strain, which was propagated on PK15 cells (Nie et al., 2003), it was reasonable to detect that PK15 cells exhibited the highest susceptibility among the tested porcine cell lines. This CSFV pseudotyped virus can be completely neutralized by anti-E2 mAbs, while anti-E^{rns} mAbs also show a partial neutralizing ability. These findings were consistent with previous studies that showed E2 to be the major target for neutralizing antibodies, and E^{rns} is the second envelope protein mediating neutralization (Greiser-Wilke et al., 1990; Weiland et al., 1992). Thus, we established, for the first time, a specific, reliable, and sensitive in vitro pseudotyped virus assay for CSFV research, which may further reveal the entry mechanism of the parental virus. Furthermore, this assay can be used for testing anti-CSFV neutralizing antibodies, evaluating CSFV vaccines, and mapping neutralizing epitopes within envelope proteins (Bartosch et al., 2003a). Within the *Flaviviridae* family, although several putative receptors were identified as virus receptors (Agnello et al., 1999; Maurer et al., 2004; Pileri et al., 1998), expressing these molecules alone does not permit effective infection. Our CSFV pseudotypes thus can facilitate receptor identification.

We demonstrated that, as a representative pestivirus, the infectivity of CSFV pseudotyped virus may be pH-dependent, as it could be inhibited by an endosomal pH acidification inhibitor. This indicated that CSFV enters cells through a receptor-mediated endocytic route, while the fusion function of the virus glycoprotein is activated by acidification of the virion environment (Sieczkarski and Whittaker, 2002). Within the *Flaviviridae* family, flavivirus and hepatitis C virus (HCV) pseudotyped particles were proved to exhibit pH dependency when entering cells (Bartosch et al., 2003c; Gollins and Porterfield, 1985, 1986; Heinz and Allison, 2001; Heinz et al., 1994; Hsu et al., 2003). CSFV as a representative of *pestivirus* shares the same characteristics as them, therefore suggesting that the members within the *Flaviviridae* family may use a similar cell entry mechanism.

Our experiments with pseudotyped viruses bearing various combinations of CSFV glycoproteins showed that, besides the parent-like CSFV pseudotyped virus HIV/$E^{rns}12$, non-E^{rns} pseudotyped virus HIV/E12 bearing only E1 and E2 was also infectious. Furthermore, the tropism of HIV/E12 was identical to that of HIV/$E^{rns}12$. Its infectivity could be inhibited by NH_4Cl, and could be completely neutralized by anti-E2 mAbs. These all indicated E1 and E2 are sufficient to mediate CSFV entry.

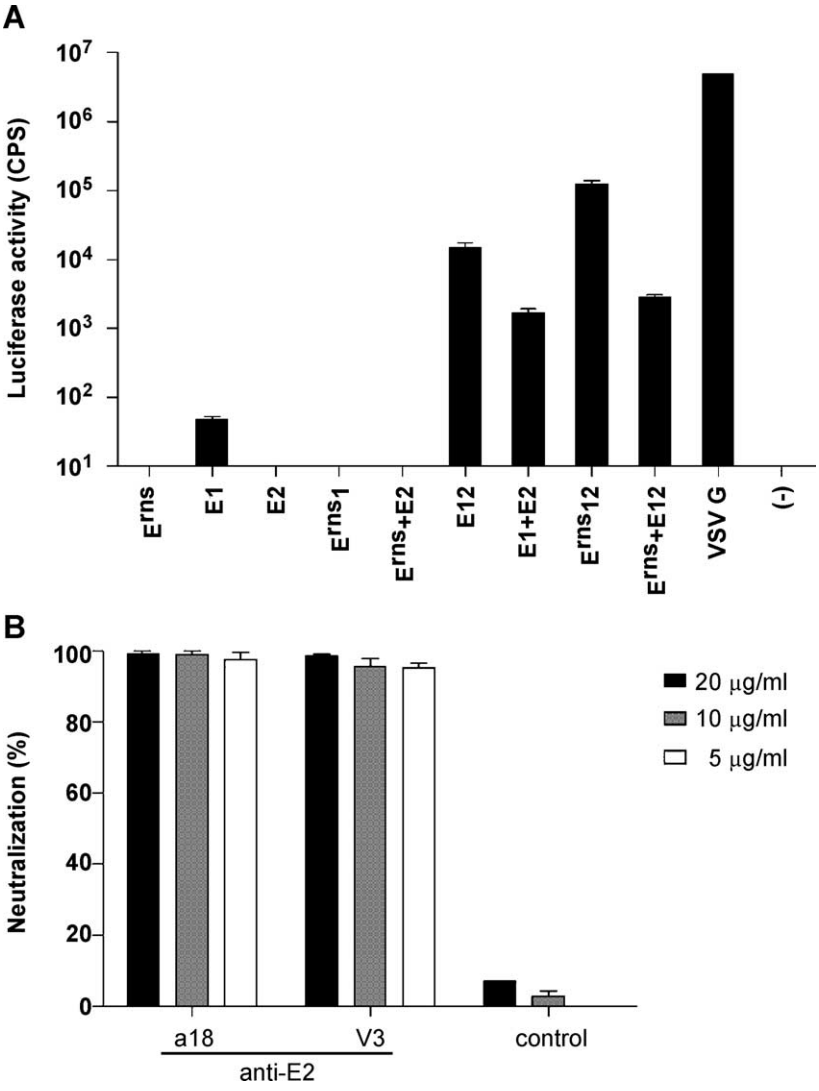

Fig. 6. Infectious CSFV pseudotyped virus can be generated without E^{rns}. (A) Pseudotyped viruses were generated from CSFV glycoproteins in various combinations and their infectivity was test on PK15 cells. Pseudotypes generated with VSV G and with no glycoprotein were used as positive and negative controls, respectively. (B) Neutralization assay of HIV/E12. Anti-E2 mAbs a18 and V3 at concentration of 20, 10, and 5 μg/ml were used to neutralize HIV/E12 infection on PK15 cells. MAb anti-p125 specific to a nonstructural protein NS3 of CSFV was used as a negative control. Neutralization was determined by inhibition of luciferase activity.

The function of E1 and E2 was further affirmed in the cell–cell fusion assay. 293T cells transfected with $pE^{rns}12$ or pE12 could induce syncytia formation between PK15 cells with acid pH treatment, while E2 alone could not, indicating E1 and E2 are sufficient in the membrane fusion process, and an acidic environment can promote this process. As only part of CSFV glycoproteins were leaked to the cell membrane in this overexpression system, it was reasonable that the syncytia we detected were few and small.

It has been reported that E2 can form E1–E2 heterodimers with E1, and E2–E2 homodimers, which are present in both virions and cells infected with CSFV (Thiel et al., 1991; Weiland et al., 1990; Wensvoort et al., 1990). However, it is still unknown which form of E2 is functional during viral entry. In our experiments, we found that in pseudotypes bearing E2, or E^{rns} plus E2, only the E2–E2 homodimers could be detected, and these pseudotypes had no infectivity. This suggested that E2–E2 homodimers may not be the functional form of E2 during viral entry. While in the infectious HIV/E12 and HIV/E^{rns}12, E1–E2 heterodimer was the major form of E2 as we detected. These data suggested that the E1–E2 heterodimer, rather than the E2–E2 homodimer, may be the functional form during viral entry.

Our findings with infectious HIV/E12 also demonstrated that E^{rns} is not indispensable during viral entry. In fact, E^{rns} may take part in the initial attachment process of viral entry, rather than in the specific binding or fusion process. First, the previous report that E^{rns} can also bind to CSFV noninfected cells suggests that E^{rns} interacts with a widely expressed surface molecule (Hulst and Moormann, 1997). Second, E^{rns} can bind to the mannose 6-phosphate receptor

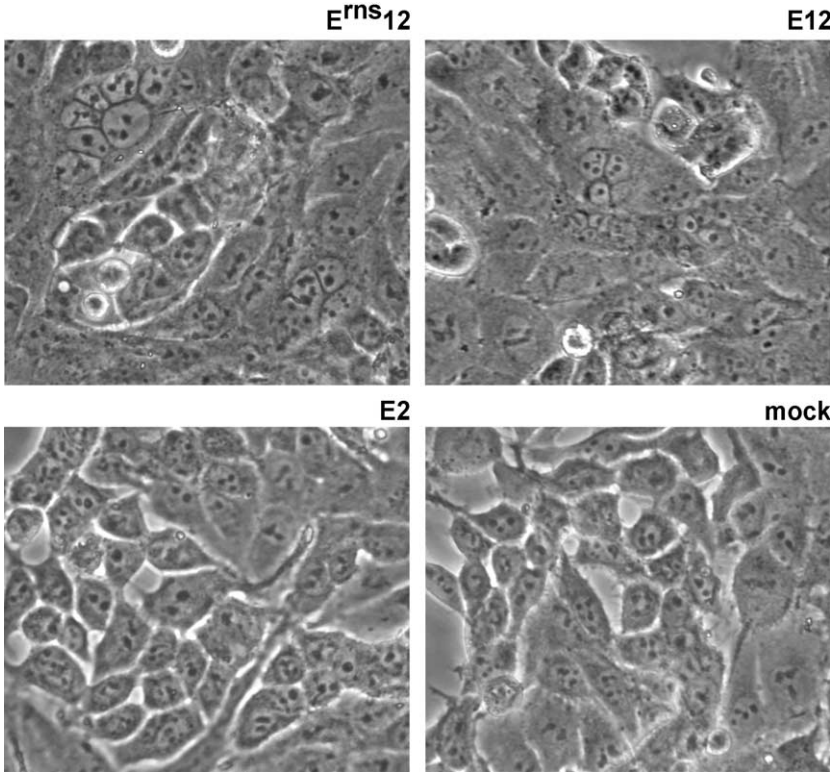

Fig. 7. Syncytium formation between PK15 cells and CSFV glycoproteins transfected 293T cells. 293T cells transfected with pE^{rns}12, pE12, pE2, or pcDNA3.1 (+) vector were trypsinized 36 h later and cocultured with PK15 at the ratio of 1:1 for 12 h. The cocultured cells were bathed in PBS at pH 5.5 for 2 min at 37 °C and then incubated with DMEM containing 10% FBS. The syncytia were observed 6 h later under microscope.

present on the surface of mammalian cells (Kornfeld, 1992). Third, CSFV with a mutant E^{rns} could enter cells through a heparin sulfate-dependent route (Hulst et al., 2000, 2001). Fourth, as we showed, mAbs against E^{rns} could not completely neutralize CSFV pseudotyped virus infection. Based on these facts, we proposed that E^{rns} interacts with a cell surface molecular as an initial attachment, which can facilitate the virus entry but the function is not decisive. Moreover, E^{rns} was found to contain RNase activity (Hulst et al., 1994; Schneider et al., 1993), and can induce cell apoptosis in lymphocytes of several species (Bruschke et al., 1997). These facts suggest that E^{rns} may also play an important role in the post-entry stages, which may be a possible reason causing the CSFV with E^{rns} deletion to be nontransmissible as reported (Widjojoatmodjo et al., 2000).

Materials and methods

Cells

Porcine cell lines PK15, ST, LLC-PK1, and SK6; human cell lines 293T and Hela; monkey cell lines VeroE6 and COS7; mouse cell line NIH/3T3; hamster cell line BHK21; and canine cell line MDCK were grown in DMEM containing 10% FBS. Mini-pig cell line MPK was grown in DMEM containing 20% FBS.

Plasmids

Plasmids pE^{rns}, pE^{rns}1 and pE^{rns}12 (Fig. 1) encoding E^{rns} (polyprotein residues 208–494), E^{rns}1 (polyprotein residues 208–689), and E^{rns}12 (polyprotein residues 208–1065) glycoproteins were constructed by insertion into pcDNA3.1 (+) a DNA fragment encoding the last 60 residues of CSFV C protein and E^{rns}, E^{rns}1, and E^{rns}12 glycoproteins that were amplified by PCR from pMC12297 plasmid (Nie et al., 2003), a construct containing the full-length cDNA of cF114 strain (F114 strain propagated on PK15 cells).

Plasmids pE1, pE12, and pE2 (Fig. 1) encoding E1, E12, and E2 glycoproteins were constructed by fusing the DNA fragment encoding the last 60 residues of CSFV C protein (208–267) and the fragments encoding E1 (polyprotein residues 495–689), E12 (polyprotein residues 495–1065), and E2 (polyprotein residues 690–1065) glycoproteins amplified from pMC12297 by overlapping PCR.

Antibodies

Anti-E2 mAb a18 (Weiland et al., 1990), anti-E^{rns} mAb 24/16 (Weiland et al., 1992), and mAb anti-p125 were generously provided by Dr. R.J.M. Moormann, Institute for Animal Science and Health, the Netherlands. Anti-E2 mAb V3, and anti-E^{rns} mAb 140.1 were purchased from CEDI-Diagnostics, the Netherlands. HIV p24 antiserum

was generously provided by Dr. Yiming Shao at China CDC.

Flow cytometric analysis

293T cells in 10-cm dishes were transfected with pE^{rns}12 (20 μg) and collected 48 h later. The cells were washed with PBS, and then incubated with anti-E^{rns} mAb 140.1 or anti-E2 mAb V3 for 1 h. After washing with PBS, the cells were incubated with FITC conjugated rabbit anti-mouse IgG (Sigma) for 30 min. Finally, the cells were analyzed by a flow cytometer (MoFlo High-Performance Cell Sorter, Dako Cytomation).

Preparation of pseudotyped viruses

The pseudotyped virus was prepared as previously described (Deng et al., 1996). Briefly, 293T cells were transfected with 10 μg plasmids expressing the viral glycoproteins or pcDNA3.1 (+) vector, and 10 μg envelope-defective pNL4.3.Luc.R$^-$E$^-$pro$^-$ (Connor et al., 1995) viral genome using the calcium phosphate transfection method (Deng et al., 1996). The medium was replaced 12 h after transfection. Supernatants were harvested 36 h later and centrifuged at 3000 × *g* for 10 min to remove the cell debris; these were then used in infection assays. HIV p24 antigen content was assessed by using a commercially available EIA (Beckman Coulter).

Western blotting assays

293T cells were transfected with CSFV glycoprotein expression constructs. After 48 h, the cells were collected. Pseudotyped viruses prepared as described above were purified by ultracentrifugation of 10-ml viral supernatants through a 2 ml 20% sucrose cushion in a SW 41 Beckman rotor (50000 × *g*, 1.5 h, 4 °C). Fractions were resuspended in 100 μl PBS, and HIV p24 was measured as described above. Transfected cells, or purified viral pellets, were diluted with 2% SDS without reducing reagents and were heat-denatured. Lysates of 10^5 cells, or 10 ng purified viral particles each lane were subsequently separated by 10% SDS-PAGE (Schagger and von Jagow, 1987). The proteins were transferred onto nitrocellulose membranes and underwent immunostaining. Anti-E2 mAb a18 or HIV p24 antiserum was used as the primary antibody. Goat anti-mice IgG labeled with alkaline phosphatase was used as secondary antibody.

Infection assays

Target cells were distributed to a 24-well plate (6×10^4 cells/well). After 24 h, cells were incubated with supernatant containing 5 ng pseudotyped virus (p24) and 4 μg/ml polybrene per well for 3 h. The supernatants were removed and the cells were incubated in fresh medium at 37 °C for another 48 h, washed in PBS once, and lysed with 100 μl lysis buffer (Promega). Twenty microliters of lysate was tested for luciferase activity by the addition of 50 μl of luciferase substrate, and was measured for 10 s in a Wallac Multilabel 1450 Counter (Perkin Elmer).

For neutralization assays, mAbs specific for E^{rns} (24/16, 140.1) or E2 (a8, V3) at concentrations of 20, 10, and 5 μg/ml were incubated with pseudotyped viruses for 30 min at 37 °C, and the mixtures were tested for infectivity on PK15 cells as described above.

The pH dependency of infection was evaluated by pretreating PK15 cells for 1 h with serum-free DMEM containing ammonium chloride at various concentrations (0–20 mM) at 37 °C; this was followed by incubation with supernatants containing the pseudotyped viruses in the presence of ammonium chloride at the consistent concentration as in the pretreating procedure. After 3 h, the supernatants were replaced with DMEM containing 10% FBS. The luciferase activity was determined 48 h later as described above.

Cell–cell fusion assay

293T cells were transfected with pE^{rns}12, pE12, pE2, or pcDNA3.1 (+) vector. Thirty-six hours after transfection, 293T cells were trypsinized and mixed with PK15 at a ratio of 1:1 for 12 h. The cocultured cells were bathed in PBS at pH 5.5 for 2 min at 37 °C and then incubated with DMEM containing 10% FBS. Six hours later, the syncytia were examined under microscope.

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