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E2 and E^{rns} of classical swine fever virus C-strain play central roles in its adaptation to rabbits

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Abstract

The classical swine fever virus (CSFV) C-strain has been used as a vaccine strain for over 60 years in China. A recent study has demonstrated that the E2 protein of C-strain plays a major role in its adaptation to rabbits. E2 protein in combination with either E^{rns} or E1 confers rabbit adaptation for the C-strain, and the residues P108 and T109 in domain I of E2 are critical for rabbit adaptation. To further identify the contributions of the glycoproteins to rabbit adaptation, a series of C-strain-based chimeric viruses containing single or double glycoprotein substitutions of the Shimen strain were generated and inoculated into rabbits. Profiles of rectal temperature, viral RNA, E2 protein expression, and antibody responses were compared among the chimeric viruses. Replacement of E^{rns}, E2, E^{rns}–E2, or E1–E2 of the C-strain with the counterpart(s) of the Shimen strain led to decreased fever response, reduction of viral RNA and antibody responses in rabbits, as compared with their parental C-strain. The C-strain-based chimeric virus expressing the Shimen strain E1 exhibited typical fever response and viral RNA level similar to the C-strain. However, substitution of both E^{rns} and E2 in the C-strain backbone abolished fever response, and the chimeric virus did not show adaptation in rabbits as demonstrated by lack of viral RNA and E2 protein expression in the spleen and weak antibody responses. These results indicate that E^{rns} has partial contribution to adaptation of the C-strain in rabbits, and combination of E2 and E^{rns} is essential for the C-strain to have adaptive replication in rabbits.

Keywords Classical swine fever virus · C-strain · Glycoproteins · Rabbit adaptation

Introduction

Classical swine fever (CSF) is a highly contagious disease that threatens the pig industry worldwide. The causative agent, classical swine fever virus (CSFV), is a single-stranded, positive sense RNA virus with an approximate genome size of 12,300 nt [1, 2]. The genome contains a single open reading frame flanked by a 5′-untranslated region (UTR) and a 3′-UTR, and encodes a polyprotein of 3898 amino acids. The polyprotein is processed by cellular and viral proteases to yield four structural proteins (Core, E^{rns}, E1, and E2) and eight non-structural proteins (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [3, 4].

The CSFV C-strain, also known as hog cholera lapinized virus (HCLV), was developed in the mid-1950s in China via serial passages of a virulent strain in rabbits [5, 6]. Domestic pigs and wild boar are the natural hosts of CSFV. Unlike other CSFV field strains, C-strain is characterized by adapted infection in rabbits with typical fever response. The highly pathogenic CSFV Shimen strain does not infect rabbits irrespective of high genomic similarity with C-strain (97.3% similarity at amino acid level). By reverse genetic approach, Li et al. found that the translated region of C-strain, rather than the UTR regions, determined its adaptation in rabbits [7].

E^{rns}, E1, and E2, the glycoproteins of CSFV, are involved in recognizing receptors or ligands for attachment and

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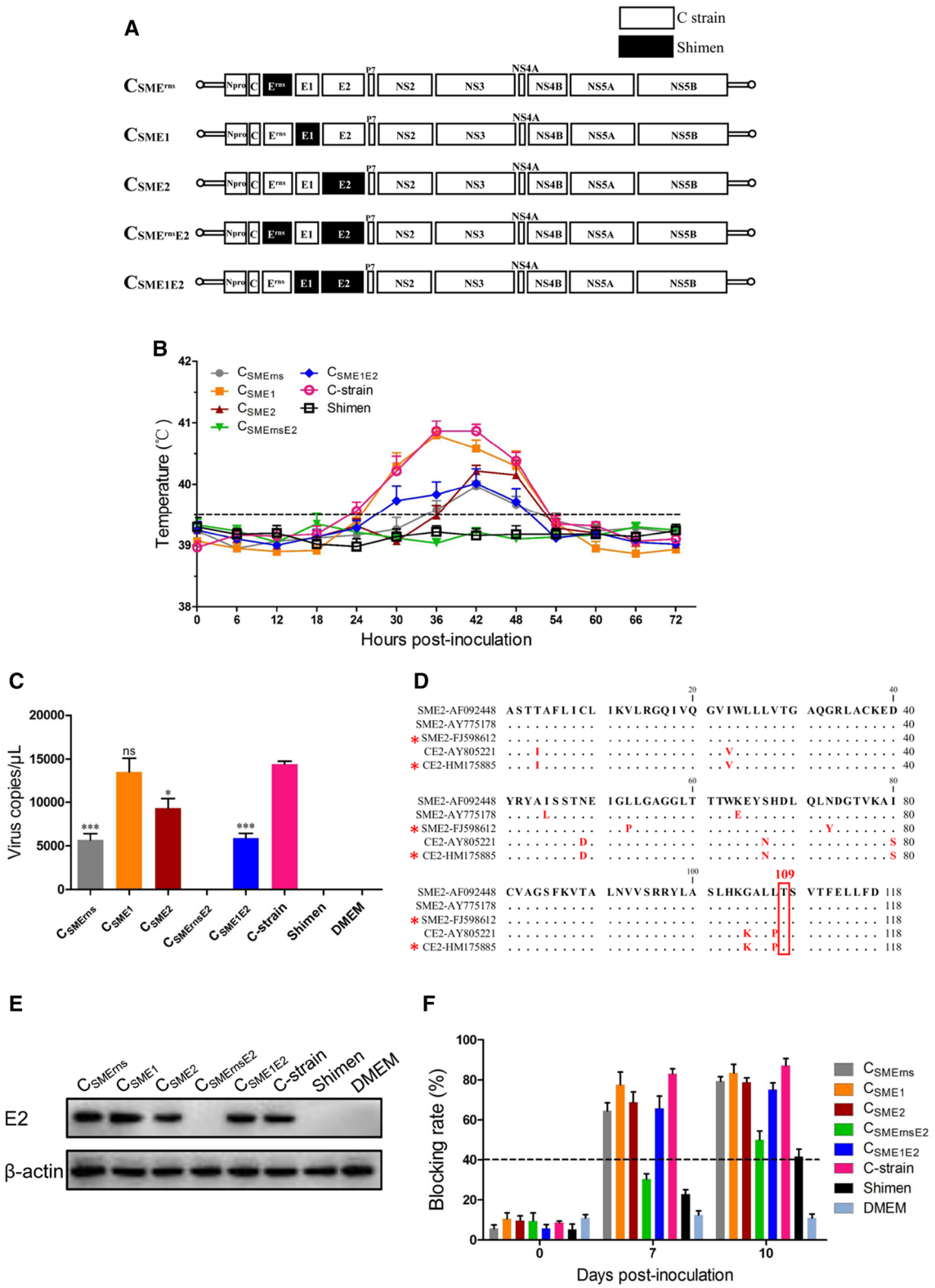

invasion into target cells [8–13]. They appear to determine cell tropism or host adaptation of CSFV. A recent report [14] has demonstrated that the E2 protein, together with E^{rns} or E1, plays a major role in adaptation of C-strain to rabbits when chimerized on the Shimen strain backbone. Moreover, the amino acids P108 and T109 in domain I of E2 are found important in mediating such adaptation. However, most of the viruses in that study were Shimen strain-based

◂**Fig. 1** E^{rns} and E2 confer adaptation of the C-strain in rabbits. **a** Schematic representation of the strategy used for construction of C-strain-based chimeric cDNA clones. **b** Rectal temperature of rabbits inoculated with different chimeric viruses. Rabbits were inoculated with the corresponding viruses (10^4 $TCID_{50}$/rabbit, $n=6$ per virus) via marginal ear vain. Rectal temperature of the rabbits was recorded for three days. The dashed line represents the upper limit of normal rectal temperature of the rabbits. **c** Viral RNA in spleen samples of the rabbits inoculated with the chimeric viruses. Spleen samples of the rabbits were homogenized and viral RNA were extracted for quantification of genome copies. **d** Amino acid sequence alignment of domain I of E2 between Shimen strain and C-strain. The sequences were obtained from the NCBI database with Shimen strain and with C-strain. The asterisks designate the viruses used in this study. The key amino acid residue T109 identified in the recent study was indicated as solid boxes. **e** Western blotting analysis of the E2 protein in spleen samples of rabbits inoculated with chimeric viruses. **f** Antibody levels of rabbits inoculated with chimeric viruses. Sera of the rabbits in each group were collected on 0, 7, and 10 dpi and tested by the IDEXX CSFV antibody test kit. The cutoff value was indicated as a dashed line

chimeras, but limited with the C-strain backbone. For better understanding of the critical components of the C-strain in rabbit adaptation, C-strain-based chimeric viruses should be evaluated.

In this study, five C-strain-based chimeric viruses containing single or double glycoprotein genes of the Shimen strain were generated to further identify the contributions of the glycoproteins E^{rns} and E1 of the C-strain to rabbit adaptation.

Results and discussion

With reverse genetic method [15], five C-strain-based chimeric viruses containing E^{rns}, E1, E2, E^{rns} and E2, or E1 and E2 of Shimen strain (namely C_{SMErns}, C_{SME1}, C_{SME2}, $C_{SMErnsE2}$, and C_{SME1E2}, respectively) were rescued from the corresponding cDNA clones (Fig. 1a). PK-15 cells inoculated with the supernatant samples of the rescued viruses were positive by IFA using anti-E2 monoclonal antibody 6B8 [16], indicating that mature virus particles were obtained. The UTRs and genes covering the region of structural proteins of the rescued viruses were sequenced. There was a single mutation located at E2 (T3310A) in the C_{SMErns} virus genome while all other chimeric viruses remained genetically stable in PK-15 cells as demonstrated by sequencing of the 5th passage viruses.

Forty-eight New Zealand white rabbits (2.0–2.5 Kg) were randomly divided into eight groups ($n=6$). Group A to E were inoculated with the five rescued chimeric viruses (10^4 $TCID_{50}$/ rabbit) via the marginal ear vein. Group F and G were injected with C-strain and Shimen strain (10^4 $TCID_{50}$/ rabbit), respectively. Group H were inoculated with DMEM as uninoculated control. Rectal temperature was recorded every 6 h to detect fever responses. A typical fever is characterized by ≥ 1 °C increase in rectal temperature for a minimum of 18 h starting from 18 to 24 h post inoculation (hpi) [17]. Three rabbits of each group were randomly selected and euthanized at 72 hpi. Spleen samples of the rabbits were collected and used for viral RNA quantification, western blotting of viral E2 protein, and re-sequencing. Blood samples were collected at 0, 7, and 10 days post inoculation (dpi) to examine the antibody responses.

All rabbits inoculated with C-strain or C_{SME1} displayed typical fever response patterns lasting for 18–24 h at 24–30 hpi, while C_{SMErns}, C_{SME2}, and C_{SME1E2} strains induced mild fever responses with lower rectal temperature and short duration. However, rabbits inoculated with $C_{SMErnsE2}$ did not exhibit fever throughout the experiment, suggesting that E^{rns}–E2 combination of C-strain contributed to the rabbit adaptation with fever response (Fig. 1b).

The viral load in the rabbit spleen can reflect the adaptability of different chimeric CSFV strains. The spleen samples collected at 72 hpi were homogenized in a mechanical tissue homogenizer (Bertin Technologies, USA) in PBS followed by centrifugation. The supernatant was retrieved and used for genomic RNA extraction (Tiangen, China), sequencing, western blotting and quantitative RT-PCR as previously described [18]. C_{SMErns} and C_{SME2} groups showed lower virus levels (mean genome copies at 5.68×10^3/μL and 9.32×10^3/μL, respectively) than that of C-strain group (1.44×10^4/μL) ($P<0.05$). The C_{SME1} group had lower viral RNA level (1.35×10^4/μL) than C-strain group, but with no significant difference ($P>0.05$) (Fig. 1c). These results indicate that E^{rns} or E2 plays a more important role than E1 in rabbit adaptation.

Li et al. [14] reported that the chimeric virus vHCLV-SME2 (corresponding to C_{SME2} in this study) did not replicate in rabbits, mainly due to changes of the two amino acids P108 and T109 in the domain I of E2. This seems to be inconsistent with our data because all six rabbits inoculated with C_{SME2} in our study could induce mild fever response and replicate in rabbits, though at a lower level than its parental C-strain. We further compared all three sequences of the Shimen strain in the NCBI database, two uploaded by two Chinese institutions (GenBank accession no. AY775178 and AF092448) and one from this study (GenBank Accession No. FJ598612). Figure 1d shows that aa109 of E2 is threonine, but not isoleucine. This could help explain why C_{SME2} in this study could replicate in rabbits and further support the role of T109 in rabbit adaptation.

Recent findings suggest that Shimen strain-based chimeric viruses with single substitution of E^{rns}, E1, or E2 of C-strain do not improve adaptation in rabbits [14]. In our study with the C-strain backbone chimerized with these glycoprotein genes, we found that single substitution with E2 or E^{rns}, but not E1, from Shimen strain exhibited attenuation

of viral adaptation in rabbits. For chimeric viruses containing double substitutions of the glycoproteins, C_{SME1E2} group exhibited a decreased viral RNA level (5.88×10^3/μL) as compared with C_{SME2} group. However, there was no viral RNA detected in rabbits inoculated with $C_{SMErnsE2}$. Western blotting also showed that E2 protein could be detected in the spleen samples of C_{SMErns}, C_{SME1}, C_{SME2}, C_{SME1E2}, and C-strain groups, but not in $C_{SMErnsE2}$ and Shimen groups (Fig. 1e). All these results indicate that E^{rns} of the C-strain exerts a significant effect on viral adaptation in rabbits particularly when combined with E2.

To further verify if there were mutations of the chimeric viruses in the rabbits that might affect viral adaptation, the regions covering the UTRs and structural proteins of strains C_{SMErns}, C_{SME1}, C_{SME2}, and C_{SME1E2} from the spleen samples were re-sequenced. There were no mutations found in these viruses compared with the initially rescued strains. Besides, the T3310A mutation site in the C_{SMErns} virus remained unchanged.

E2 is an immune dominant glycoprotein of CSFV that induces neutralizing antibodies [19, 20]. On 0, 7, and 10 dpi, sera of the rabbits were collected and anti-E2 antibodies were detected (IDEXX, USA). On 7 dpi, rabbits inoculated with strains C_{SMErns}, C_{SME1}, C_{SME2}, and C_{SME1E2} as well as C-strain had obvious sero-conversion with the C-strain group showing the highest blocking rate (83.1%), while the sera of $C_{SMErnsE2}$ and Shimen groups showed blocking rates (30.3% and 22.9%, respectively) considered as negative according to the manufacture's instruction. On 10 dpi, the $C_{SMErnsE2}$ and Shimen groups showed increased antibody level close to or slightly above the threshold level (50.1% and 41.7%, respectively) (Fig. 1f). The lower antibody levels also suggest that strain $C_{SMErnsE2}$, like Shimen strain, does not have active replication in rabbits.

Our findings with chimeric viruses on the C-strain backbone were consistent with those on the Shimen strain backbone by Li et al. [14] that combination of E2 and E^{rns} plays a key role in adaptation of the C-strain to rabbits. Li et al. reported that Shimen-strain-based chimeric virus containing C-strain E1 and E2 could replicate in rabbits, suggesting that C-strain E1–E2 combination might confer adaptation in rabbit. We found that the C-strain-based chimeric virus containing both E1 and E2 of the Shimen strain (C_{SME1E2}) shows reduced level of fever response and viral genome copies in the spleen. There seems to be some degree of agreement between the studies. However, in this study, Shimen E1 substitution alone on the C-strain backbone (C_{SME1}) did not show significant effect on rabbit adaptation (with similar temperature profile to its parental C-strain), while E2 substitution (C_{SME2}) alone resulted in reduced rabbit adaptation. This clearly indicates that C-strain E2 could be the major factor for rabbit adaptation although there may be other factors yet to be identified. Wu et al. found that substitution of both E2 and 3′-UTR from C-strain contributed to reduced virulence of the Shimen strain to pigs as compared with single substitution alone [21], indicating the synergistic roles of E2 and 3′-UTR from different strains in altering the character of viruses. So the differences in the backbones used for construction of chimeric viruses are likely to have resulted in this variation.

In summary, we demonstrate by rabbit fever response, viral replication in spleen and antibody response that E^{rns} has partial contribution to adaptation of the C-strain in rabbits, and combination of E2 and E^{rns} is essential for the C-strain to have adaptive replication in rabbits. Further study could focus on the key residues of E^{rns} that help adaption of the C-strain in rabbits.

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Compliance with ethical standards

Conflict of interest All the authors declare no conflict of interest.

Ethical approval Animal experiments were conducted following the guidelines and approved protocols of the Laboratory Animal Management Committee of Zhejiang University, China (Approval number: ZJU20180766).

Informed consent All authors read and approved the manuscript.

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