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The CD2v protein enhances African swine fever virus replication in the tick vector, *Ornithodoros erraticus*

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ABSTRACT

The NH/P68 non-haemadsorbing (non-HAD) African swine fever virus (ASFV) isolate contains frameshift mutations in the EP402R and adjacent EP153R genes. These encode, respectively, the protein (CD2v) that is required for the haemadsorption (HAD) of swine erythrocytes to ASFV-infected cells and a C-type lectin protein. Two recombinant HAD viruses were constructed in this parental strain. In one of these the intact EP153R gene sequence was restored. Although restoration of the HAD phenotype did not increase virus virulence in pigs, a significant increase was observed in the number of pigs which developed viraemia. These HAD recombinant viruses replicated to titres approximately 1000-fold higher than the parental non-HAD isolate when membrane fed to *Ornithodoros erraticus* ticks. Inoculation of the non-HAD isolate across the gut wall increased viral replication to levels comparable to that of the HAD recombinant viruses. These results demonstrate a novel role for the CD2v protein in virus replication in ticks.

Introduction

African swine fever (ASF) is a severe haemorrhagic fever of domestic pigs, which is caused by a large, cytoplasmic, enveloped, double-stranded DNA virus called African swine fever virus (ASFV). ASFV is the only member the *Asfarviridae* (Dixon et al., 2000). Virulent isolates of ASFV can cause up to 100% mortality in domestic swine (*Sus scrofa*) and result in major economic losses. In Africa, ASF is maintained in a transmission cycle between its natural hosts: warthogs (*Phacochoerus* spp.), bushpigs (*Potomochorus porcus*) and soft ticks of the species *Ornithodoros*, which act as a virus reservoir (Plowright et al., 1969). The virus is highly adapted to these hosts and causes a subclinical persistent infection. The tick vector is thought to play an essential role for virus transmission between warthogs, although direct transmission can occur between domestic pigs without a tick vector (Wilkinson, 1989). ASFV is therefore a risk to pig populations, even in countries that do not contain the arthropod vector (Wilkinson et al., 1988).

Following ingestion of an infectious blood meal, ASFV replicates within the tick midgut epithelium (Kleiboeker et al., 1999) and gains entry into the haemocoel, where it can disseminate to the salivary glands and remaining organs. ASFV infection of *Ornithodoros* ticks is characterised by the establishment of long term, persistent infections. This involves replication in several tissues and is associated with minimal cytopathology (Greig, 1972; Hess et al., 1989; Kleiboeker et al., 1998; Kleiboeker and Scoles, 2001; Kleiboeker et al., 1999; Plowright et al., 1970; Rennie et al., 2000). In *Ornithodoros moubata*, the vector for ASFV in the sylvatic cycle in East and South Africa, transovarial, transtadial and transsexual transmission of virus occurs, demonstrating that the virus is very well adapted to replicate in this tick vector. It is therefore expected that the virus should encode genes which facilitate virus replication in the arthropod host. Given the importance of the tick vector in maintaining a virus reservoir and in transmission, there should be a strong selection for these genes. As yet few studies have been directed to identify these. One study demonstrated a role for members of multigene family (MGF) 360 in increasing ASFV replication in ticks (Burrage et al., 2004). ASFV also replicates in other *Ornithodoros* species and Ornithodoros erraticus acted as a virus reservoir in southern Spain and Portugal. In *Ornithodoros erraticus*, sexual transmission (Boinas, 1994; Endris and Hess, 1994) and transstadial transmission (Endris and Hess, 1992) have been demonstrated, but transovarial transmission has not (Endris and Hess, 1994) suggesting the virus may be less well adapted to replicate in this species. Experimental ASFV infection and transmission have also been demonstrated for *O. savigni* (Mellor and Wilkinson, 1985), *Ornithodoros coriaceus* and *Ornithodoros turicata* (Groocock et al., 1980; Hess et al., 1987), ticks indigenous to America and *Ornithodoros puertoricensis*, indigenous to the Caribbean (Endris et al., 1991; Hess et al., 1987).

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The majority of ASFV isolates cause the haemadsorption (HAD) of red blood cells to infected cells and extracellular virus particles. Less frequently, non-HAD isolates have been described (Pini and Wagenaar, 1974; Thomson et al., 1979). A viral transmembrane protein, which resembles the host CD2 protein, is required for HAD (Borca et al., 1994; Rodriguez et al., 1993). In pigs infected with HAD isolates of ASFV viraemia is primarily erythrocyte associated (Borca et al., 1998; Quintero et al., 1986). The CD2-like protein, designated CD2v (Kay-Jackson et al., 2004), is encoded by the open reading frame (ORF) EP402R. Deletion of this gene from virulent isolates did not reduce virus replication in cells and did not reduce the mortality rate in pigs (Borca et al., 1998). However, the progress of infection differed since the onset of viraemia and dissemination of virus to tissues was delayed compared to infection with the wild-type virus (Borca et al., 1998; Rodriguez et al., 1993; Thomson et al., 1979).

The similarity in structure and function of the extracellular domain of CD2v to the T-cell adhesion molecule CD2 and the observation that CD2v expression is required to suppress the proliferation of lymphocytes in response to mitogens *in vitro* suggest that CD2v plays an important role in evading the immune system of the host (Borca et al., 1998).

The interaction between CD2v and its ligand on red blood cells may be stabilised by expression of an ASFV member of the C-type lectin family of adhesion proteins encoded by the ORF EP153R (Galindo et al., 2000; Haynes et al., 1989; Neilan et al., 1999), since deletion of the EP153R gene has been reported to reduce HAD around ASFV-infected cells (Galindo et al., 2000). EP153R protein has also been shown to inhibit apoptosis (Hurtado et al., 2004).

In this report, we demonstrate that the EP153R and EP402R genes are disrupted in the genome of a natural non-HAD, low virulence ASFV isolate, NH/P68 (Leitao et al., 2001). We demonstrate that the NH/P68 isolate is less efficient in establishing a persistent infection in *O. erraticus* ticks than two recombinant viruses with the HAD phenotype restored by reinsertion of the extracellular domain of the EP402R gene. In one of these HAD recombinants the intact EP153R gene is restored (Rec 4) but not in the other (Rec 34). Furthermore, inoculation of the non-HAD NH/P68 virus across the tick gut wall directly into the haemocoel was shown to increase the titres to which this virus replicates to levels comparable to those observed with the recombinant HAD viruses. These results demonstrate that expression of the CD2v protein significantly increases virus replication in the tick vector at the step of virus uptake or replication in the tick gut. Pigs infected with this recombinant HAD virus did not show any increase in clinical scores compared to those inoculated with the parental NH/P68 virus, although the number of pigs which developed low viraemia was significantly higher. This is one of the first studies to have identified an ASFV protein which has an important role in replication in ticks.

Results

Sequence analysis of the EP153R and EP402R ORFs from the non-HAD NH/P68 isolate

The EP153R and EP402R ORFs are adjacent to each other in the centre of the ASFV genome (positions 56166–55705 and 56242–57450, respectively, on the BA71V genome, accession number U18466). The nucleotide sequence of the fragment of the NH/P68 genome encoding these ORFs was determined and compared with the sequence in HAD isolates to establish the basis of the non-HAD phenotype of NH/P68. This showed that in the NH/P68 isolate genome both of these ORFs were interrupted by frameshift mutations. A single base pair (bp) deletion at position 49 downstream from the ATG codon of the NH/P68 EP153R ORF brings in frame a stop codon at position 58 downstream from the ATG. A second ATG, located 120 bp downstream from the first, is in frame with the remainder of the EP153R ORF and terminates at the same position (Fig. 1). The polypeptide encoded is 113-amino acid long and is truncated at the amino terminus compared to other EP153R predicted proteins. This ORF is unlikely to be efficiently translated as it is downstream of the short ORF that terminates at position 58. In the EP402R ORF of the NH/P68 isolate a single bp deletion at position 85 downstream from the first ATG causes a frameshift shortening this ORF to 90 bp (Fig. 1A). An ATG codon, located 25 bp upstream from this nucleotide deletion, is in frame with the remainder of the coding region of the EP402R protein. This ORF is truncated by 59 bp at the 5′ end compared to the ORFs in the BA71 V and Lisbon 60 isolates. Another two single-bp deletions, located 798 and 963 bp downstream from the first ATG codon, cause frame shifts. The latter deletion brings a stop codon in frame 13 bp downstream. This ORF encodes a polypeptide of 304 amino acids, which lacks the N-terminal signal sequence, has an altered sequence of 55 amino acids within the cytoplasmic domain and is also truncated by 67 amino acids at the C-terminus (Fig. 1A and B). The reason for the loss of HAD phenotype of the NH/P68 parent is most likely because just a short truncated polypeptide is expressed from the first ATG codon of the EP402R gene. The downstream larger ORF is unlikely to be translated into protein efficiently due to the upstream ATG codon and would lack the signal sequences.

Construction of recombinant HAD viruses

Recombinant ASF viruses were generated to examine the effect of restoring the HAD phenotype to the NH/P68 virus on replication in ticks and pathogenesis in pigs. Plasmids containing the full-length intact EP153R and EP402R genes and flanking regions contained on the EcoRI E′ fragment of the HAD viruses Lisbon 60 and Ba71V were transfected into swine macrophages that had been infected with the NH/P68 virus. DNA fragments from these viruses were selected because they are closely related to the NH/P68 sequences and therefore homologous recombination between the transfected plasmids and genome of the NH/P68 virus would be facilitated. Two recombinant viruses were identified by the restoration of their ability to induce HAD and were purified by 10–12 rounds of limiting dilution. Red blood cell rosettes formed in swine macrophages infected with both purified recombinants Rec 34 and Rec 4 were indistinguishable from those formed following infection with the wild-type HAD viruses (data not shown).

Comparison of the nucleotide sequences of EP153R and EP402R ORFs of recombinant HAD viruses Rec 4 and Rec 34

The genome regions containing the EP153R and EP402R genes from Rec 34 and Rec 4 viruses were amplified by PCR and cloned, and the nucleotide sequences were determined (Rec 34: accession number AY463915; Rec 4: accession number AY463916). As shown in Fig. 1, one of the recombinants, Rec 34, had the same N-terminal mutation observed in the EP153R gene of NH/P68 isolate, whereas the other recombinant, Rec 4, had an intact EP153R gene restored (Fig. 1). Thus, the recombination event at the 5′ end of the inserted sequences must have occurred downstream from the EP153R frameshift mutation in Rec 34 virus but upstream from this in Rec 4 virus. The EP402R genes in both recombinant viruses encoded CD2v proteins with the same N-terminal signal peptide, extracellular domain and transmembrane domain as the parental HAD sequences. However, the cytoplasmic domain encoded by both Rec 34 and Rec 4 viruses was the same as in the parental NH/P68 virus and was thus truncated and differing in sequence compared to the proteins encoded by the parental HAD proteins (Fig. 1B). Thus in both Rec 34 and Rec 4 viruses the recombination event at the 3′ end of the inserted sequence must have occurred upstream from the frameshift mutation in the cytoplasmic domain in the NH/P68 EP402R gene. It is not possible to determine the precise site of recombination due to the very close similarity of the sequences in the parental NH/P68 virus and the sequences in the plasmids used to generate recombinants.