Design and construction of African swine fever virus chimeras incorporating foreign viral epitopes

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Accepted March 12, 1999

Summary. In the present work we have studied the feasibility of introducing foreign epitopes into the African swine fever virus (ASFV) particle by genetic manipulation of the virus. For this purpose, we developed specific transfer vectors containing the gene encoding for the highly antigenic structural ASFV protein p54 in which foreign sequences were introduced. DNA sequences encoding continuous linear epitopes, the antigenic site A from foot-and-mouth disease virus (FMDV) VP1 protein and the DA3 antigenic determinant from transmissible gastroenteritis coronavirus (TGEV) nucleoprotein N, were separately cloned into the p54 gene, in a region encoding a non-essential domain of the protein. Chimeric p54 genes were inserted by homologous recombination into the thymidine kinase (TK) locus of ASFV genome. The resulting recombinant viruses efficiently expressed both chimeric proteins under transcriptional control of the p54 promoter, and the chimeric gene products were recognized by antibodies to both p54 and foreign epitopes. The modified p54 proteins were also found in the viral particles and complemented the function of the wild-type p54, since deletion of the p54 gene from recombinant viruses did not affect virus replication in Vero cells. This work demonstrates for the first time the feasibility of incorporating foreign amino acid sequences (up to 18 residues) into a protein component of the ASFV particle without affecting virus viability.

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Introduction

African swine fever virus (ASFV), the sole member of the family Asfarviridae (L. Dixon, pers. comm.), is a large DNA virus which infects species of the Suidae family and soft ticks of the Ornithodoros genus [22, 24]. It was previously shown that the genome of ASFV, a double-stranded DNA molecule, can accept foreign sequences by homologous recombination [17]. This fact was further extended with the construction of plasmid vectors for the generation of deletion mutant viruses or viruses expressing foreign reporter genes [2, 6, 7, 13, 19]. Recently, it was possible to attenuate a virulent strain of ASFV by specific elimination of a viral gene. Animals immunized with the attenuated virus survived the lethal challenge with the homologous virulent virus [26]. The feasibility of manipulating the ASFV genome and its restricted host range makes reasonable to explore the use of ASFV as a swine vector when the incoming information about the genes involved in virulence and pathogenesis allows the stable attenuation of the virus and, consequently, a safer use of such vector.

Previous studies in our laboratory have shown that the ASFV structural protein p54 is an essential protein [19], that localizes in the surface of the virion [3, 19]. Accordingly to its highly immunogenic character, p54 induces neutralizing antibodies during virus infection [8], in agreement with more recent data regarding the functionality of this polypeptide as one of the virus attachment proteins [9]. Furthermore, it has been shown that the p54 gene suffers rearrangements within the coding region without affecting virus viability [18, 21]. These rearrangements produce alterations in the number of copies of the P A A A tetrapeptide present in p54, thus resulting in stepwise modifications in the molecular weight of this protein which are dispensable for virus replication in cell culture.

The above data has favoured the design of a rational strategy to generate ASFV chimeras intended to explore the use of ASFV as a putative virus vector. Here, we have built several recombinant ASF viruses expressing in the surface of the viral particle chimerical p54 proteins containing linear epitopes from two different pathogens: a 18 amino acid epitope corresponding to the immunodominant antigenic site A of foot-and-mouth disease virus (FMDV) VP1 protein [12] and the 12 amino acid DA3 epitope [10] from transmissible gastroenteritis coronavirus (TGEV) nucleoprotein N. Our results indicate that at least 18 amino acid residues can be efficiently inserted in the ASFV particle in a stable form, without affecting virus viability.

Materials and methods

Cells and viruses

Vero cell cultures were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The highly cell culture adapted ASFV isolate BA71V, apathogenic for swine, and the low cell culture passaged attenuated viruses 608V and 1207V, were used in experiments of genetic manipulation.
**Transfection-infection of cell cultures**

Semiconfluent Vero cell monolayers seeded onto six well culture plates were transfected with 5–10 μg of plasmid DNA per well, using a liposome-mediated transfection procedure [17]. Eight to sixteen hours after transfection cells were washed in DMEM and infected with ASF virus at a multiplicity of infection (m.o.i.) of 1. When the cytopathic effect (c.p.e.) was complete the cultures were harvested, frozen-thawed, and sonicated in ice bath for 1 min. at 80 watts power setting.

**Selection and purification of recombinant viruses**

Transfection-infection mixtures were used to infect preconfluent Vero cell monolayers seeded onto 100 mm Petri dishes at different dilutions. Two hours post infection the inoculum was removed and cells were covered with 0.4% agarose (Sigma) in DMEM and incubated at 37°C until viral plaques were observed. X-gal or X-gluc (Biomol) was added to the cultures at a final concentration of 300 μg/ml. Blue plaques were harvested, suspended in DMEM containing 10% FBS and sonicated to disperse the virus from the agarose plugs. The selected viruses were then plaqued for three consecutive rounds of purification in six well dishes.

**Plasmid construction**

Oligonucleotides 5′GGCCGCCGATGCACGCGGGGATTTGGCTCACCTAACGACGACGATGCTCGGATTTGCGCCG 3′ and 5′GGCCGCCGCCGCAAATGCCGAGCATGCTCGGCATTGCCGGC 3′ were annealed to generate the nucleotide sequence of the site A from FMDV VP1 protein. Similarly, oligonucleotides 5′GGCCGCCGATCGAAAATTATACAGATGTGTTTGATGACGCGCGGCCTGC 3′ and 5′GGCCGCCAGGCTGTGTGTCATCGGGCGCGTCTGTATAATTTTCGATCGC 3′ were annealed to generate the nucleotide sequence corresponding to the DA3 epitope from TGEV nucleoprotein N. The annealed oligomers were then ligated to NotI digested plasmid pINS β-gus p54 [19]. The resulting plasmids were named pINS β-gus p54/siteA and pINS β-gus p54/DA3 respectively.

The construction of plasmid pΔp54, designed to inactivate the wild type copy of the p54 gene, has been previously described [19]. Briefly, a 862 bp PCR product containing the ORF and flanking sequences from p54 gene was cloned into pUC19 vector. The resulting plasmid, pUCp54, was then digested with Accl and NotI in order to eliminate 322 bp from the p54 ORF, blunt ended with Klenow enzyme, dephosphorylated and ligated to a 3.3 kb fragment, containing a 200bp late ASFV p72 protein promoter region sequence immediately upstream the β-gal gene, that was obtained by blunt ending the XbaI digested plasmid pINS β-gal.

**Western blot and immunoblotting**

Infected Vero cell extracts or purified virus were subjected to electrophoresis in 15% polyacrylamide gels and electrotransferred to nitrocellulose sheets. Nitrocellulose filter strips were blocked with 2% non-fat dried milk in phosphate buffered saline (PBS) for 30 min. at room temperature and incubated either with anti-p54 polyclonal serum raised against a baculovirus-expressed p54 [8], monoclonal antibody SD6, which recognizes VP1 site A in type C1 FMDV [11] or monoclonal antibody DA3, which recognizes TGEV nucleoprotein N [10]. Strips were then washed extensively and incubated with a rabbit anti-mouse IgG conjugated with peroxidase. Immunocomplexes were detected either by chromogenic or chemiluminescent reactions.
Purification of viral particles and analysis of recombinant virus DNA

Viral particles from infected Vero cell culture supernatants were purified in Percoll gradients [4]. For the isolation of viral DNA, ASFV infected Vero cells were incubated with 500 μg/ml proteinase K in 50 mM Tris-Cl pH 7.8, 0.5% SDS, 6% (w/v) sucrose. DNA was phenol extracted, ethanol precipitated and digested with Eco RI restriction endonuclease. The resulting DNA fragments were resolved by agarose gel electrophoresis, and then blotted onto uncharged Nylon membranes (Amersham). The membranes were probed with a digoxigenin-11-dUTP labeled 322 bp DNA fragment corresponding to the region replaced by insertional inactivation of wild-type p54 gene [19].

Growth curve of recombinant viruses

Approximately 3.5 × 10⁴ cells were infected at a moi of 1 with either parental viruses BA71V, 608V and 1207V, with double recombinant viruses BA71V and 608V (GUS⁺/GAL⁺) or with single recombinant 1207V (GUS⁺) virus. Viral growth was then monitored by plaque assay of viral yields at 0, 8, 16, 24, 48 and 72 h post infection (hpi). Viral inocula were seeded onto Vero cells in 96 well plates and layered with 3% carboximethylcellulose (Sigma) in DMEM supplemented with 5% FBS. The layer was removed by vacuum aspiration and then viral plaques were immunostained [25] using monoclonal antibody 3F8, which recognizes ASFV protein p30. Input virus, defined as number of plaque forming units adsorbed to the cells one hour after infection, was considered as viral titer at 0 hpi.

Results

Construction of ASFV/foreign epitope chimeras

The study of p54 heterogeneity proved that differences in size displayed after adaptation of ASFV to growth in cell culture [1] consisted of changes in the number of copies of an imperfect direct repeat formed by 12 nucleotides coding for a PAAA tetrapeptide [18, 21]. Wild type and low passage viruses contained only two repeats while one to more than four copies were found in highly passaged viruses. These alterations in the number of PAAA blocks produced stepwise changes in the electrophoretic mobility of p54. Nevertheless, all p54 species were functional since they were specified by single virus clones and incorporated to the viral particle without affecting virus morphogenesis [18]. We took advantage of the occurrence of these dispensable repetitions to introduce foreign sequences between two consecutive PAAA motifs. The coding sequence for the three alanine residues provided for a NotI restriction endonuclease recognition site, thus facilitating the insertion of foreign sequences into the p54 coding region (Fig. 1).

Following this approach we generated plasmids containing modified p54 genes including either the site A epitope from FMDV VP1 or the DA3 epitope from TGEV nucleoprotein N. Subsequent generation of recombinant viruses with modified p54 proteins was achieved in a two-step process (Fig. 2) as reported [19]. The first step involved the insertion of the chimeric p54 gene together with a reporter gene, β-glucuronidase (β-gus), into the thymidine kinase locus of the ASFV genome. In the second step, the wild-type copy of the p54 gene was disrupted by the insertion of a second reporter gene, β-galactosidase (β-gal), into the genome of β-gus expressing viruses, generated after the first homologous recombination.
Fig. 1. Strategy of sequence insertion in the p54 gene. Foreign nucleotide sequences are represented in boldface type and the corresponding amino acid sequences have been underlined. Italic letters show the specific amino acid sequences for the introduced viral epitopes.
**Fig. 2.** General outline for the generation of chimeric ASF viruses
The method generates two types of recombinant viruses, either with double or single copies of p54 and with one or two reporter proteins, as a consequence of single or double recombination events, respectively. By this methodology, we obtained single and double recombinant Ba71V and 608V viruses, containing the epitope from FMDV virus defined by monoclonal antibody SD6, a single and double recombinant Ba71V virus containing DA3 epitope, and a single recombinant 1207V virus containing DA3 epitope.

Expression of chimeric p54/foreign epitope proteins

In order to detect the expression of p54 chimeric proteins in Vero cells infected with recombinant viruses, we carried out Western blot assays using infected cell extracts and specific antibodies to both p54 and the viral epitopes inserted in the p54 locus. An immunoreactive band of approximately 26 kDa corresponding to chimeric protein p54/site A was readily detected using both specific anti-p54 serum and monoclonal antibody SD6 (Fig. 3B). Thus, incorporation of the 18 amino acid site A epitope generated a p54 chimeric protein that increased in 1 kDa the relative molecular mass observed in wild-type ASFV-infected cells. In contrast, incorporation of the 12 amino acid DA3 epitope from nucleoprotein N into p54 increased the relative molecular mass of the wild-type protein in about

![Western blot analysis of chimeric p54 proteins. A Detection of p54/DA3 with monospecific polyclonal anti-p54 serum or with monoclonal antibody DA3 directed to N protein of TGEV. WT 1207V wild-type infected Vero cell extracts; SR 1207V/DA3 single recombinant virus; TGEV TGEV infected ST cell extracts. B Detection of p54/Site A with monospecific polyclonal anti-p54 serum or with monoclonal antibody SD6 directed to FMDV antigenic site A (26 kDa). WT BA71V wild-type infected Vero cell extracts; SR BA71V/Site A single recombinant virus; FMDV purified FMDV. The arrows show the position of the chimerical p54 proteins.](image-url)
Fig. 4. Analysis of the replicative phenotype of genetically manipulated viruses in Vero cells. A Parental (WT) and double recombinant (DR) BA71V/Site A viruses. B Parental (WT) and single recombinant (SR) 1207V/DA3 viruses. C Parental (WT) and double recombinant (DR) 608V/Site A viruses.

6 kDa, generating a chimerical p54/DA3 polypeptide of approximately 31 kDa (Fig. 3A). These differences in molecular mass of p54 after incorporation of foreign epitopes were not due to incorporation of repetitive copies of DA3 epitope into the p54. Sequence analysis of the plasmid constructs used for homologous recombination with the parental viruses demonstrated that only one copy of the sequence encoding for each epitope was inserted into the p54 gene. Posttranslational modifications of chimeric p54 proteins incorporated to the ASFV genomes, that may account for the altered molecular mass observed with DA3 epitope, were not analyzed. Importantly, the antigenicity of p54 after incorporation of the foreign epitopes was not substantially altered since a monospecific antiserum reacted with chimeric proteins similarly as it did with wild-type p54.

To assess whether the growth rate of the recombinant viruses was altered by the manipulation of the original p54 gene, a growth curve analysis was performed both with single (1207V/DA3) and double recombinant viruses (Ba71V/Site A and 608/Site A) and referred to their parental viruses. Viral growth was not substantially affected by the insertion of neither the TGEV nor the FMDV epitopes (Fig. 4).

**Genomic analysis of recombinant viruses**

To establish whether the genomic structure of the recombinant viruses was as predicted, DNA was isolated from Vero cell cultures infected either with recombinant or parental viruses. Total DNA was digested with EcoRI and analyzed by Southern blotting. Membranes containing transferred DNA were probed with
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Fig. 5. Genomic analysis of single and double recombinant BA71V/Site A and 608V/Site A viruses. A Schematic representation of the wild-type ASFV genome indicating the position of the EcoRI K and E fragments and the predicted genomic structure of the single (SR) and double (DR) recombinant viruses. B Southern blot analysis of parental (WT) single (SR) and double (DR) recombinant BA71V/Site A and 608V/Site A virus genomes using a probe consisting on a digoxigenin-11-dUTP labeled 322 bp DNA fragment corresponding to the region replaced by insertional inactivation of wild-type p54 gene

a digoxigenin-11-dUTP-labeled internal Acc I/Not I p54 gene fragment, the same gene region deleted by the insertion of β-gal gene. The results obtained were consistent with the predicted genomic structure for the recombinant viruses (Fig. 5). As expected, the p54 probe hybridized, both in parental and single recombinant viruses, to a 8.8 kb fragment corresponding to the ASFV DNA EcoRI E restriction fragment, and to a 7.5 kb fragment, corresponding to an altered EcoRI K fragment, in single and double recombinant viruses.

Detection of inserted epitopes in the viral particle

Since p54 has been found as a component of the virion structure [3, 8, 18, 21], we performed experiments to check whether the chimeric p54 proteins effectively incorporated to the viral particle. For this purpose Percoll purified preparations of single and double recombinant BA71V/DA3 viruses, containing both the chimeric and wild-type copies of the p54 protein or only the chimeric p54 respectively, were subjected to SDS-PAGE and analyzed by Western blotting using a monospecific anti-p54 serum (Fig. 6). Antibodies were able to detect the wild-type (25 kDa) and chimeric (31 kDa) polypeptide incorporated to viral particles, indicating that both forms of p54 were efficiently incorporated to the virions.
Discussion

Infection with attenuated ASFV strains produces a strong cellular and humoral immune responses in pigs [23], conferring in some cases a prolonged immunity protecting for years against homologous virulent infection [14, 20]. Recent studies about ASFV genes involved in virulence and/or pathogenesis allowed for the attenuation of a virulent ASFV or modifications in pathogenesis by obtention of recombinant deletion mutants [2, 26]. These genetically modified attenuated viruses may serve not only as ASFV vaccines but as a potential swine vectors to trigger potent immune responses against foreign proteins or epitopes expressed during ASFV infection, producing high titer systemic and secretory antibodies. In addition ASFV may have practical uses for in vitro studies of T-cell epitope mapping of foreign antigens since this virus infects monocyte/macrophages and other antigen presenting cells [5, 15, 16].

Genetic manipulation of ASFV can be achieved by homologous recombination with a similar efficiency to that routinely obtained for vaccinia virus [17]. Therefore, and as a first step in the development of an ASFV derived vector, we have designed a method to incorporate foreign sequences into the ASFV particle. Three ASFV strains were genetically modified to produce chimeric p54 proteins carrying two different foreign viral epitopes. These chimeric proteins were expressed in cells infected by recombinant viruses at lower level than wild-type p54. This difference in protein expression could be due to the localization of the gene copy in the virus genome, since chimeric p54 was expressed under the control of the same promoter than original p54. Chimeric p54 proteins were efficiently incorporated to the virus particle either when the virus expressed both wild-type and chimerical p54 protein simultaneously or the modified version of the protein alone. The proportion of p54 form incorporated to the virus particle was proportional to their relative abundance in infected cells. The generated recombinant viruses were stable at least after 10 consecutive passages in cell culture and did not present significant alterations in virus yields with respect to parental viruses.

In this study we have inserted an epitope of 18 amino acid residues (antigenic site A) into the p54 protein without affecting significatively the virus morphogenesis and the replication phenotype of the chimeric virus. Since subpopulations with p54 proteins of more than 35 kDa have been described [1], corresponding approximately to a 40 amino acids repetition [18], the possibility of introducing
Construction of African swine fever virus chimeras remains opened and should be further studied. In addition, several copies of chimeric p54 proteins containing foreign epitopes from different swine pathogens could be probably incorporated into the same viral particle, since wild-type and chimeric p54 proteins were incorporated to the virions in single recombinant viruses. Finally, since p54 is one of the most antigenic ASFV proteins, it is reasonable to speculate about the possibility that cloned epitopes could enhance its own antigenicity when they are associated with p54.

Acknowledgements
This work was supported by Grant PB96-0105 from Programa Sectorial de Promoción General del Conocimiento and by Grant Bio 98-0307 from Programa Nacional de Biotecnología.

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Received January 25, 1999