Virology 452-453 (2014) 202–211

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Improved genetic stability of recombinant yellow fever 17D virus expressing a lentiviral Gag gene fragment

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ARTICLE INFO

Article history:
Received 14 October 2013
Returned to author for revisions
11 November 2013
Accepted 20 January 2014
Available online 8 February 2014

Keywords:
Yellow Fever 17D virus
Viral vector
Genetic stability
SIV *gag* IRES
SIV Gag
Recombinant virus

ABSTRACT

We have previously designed a method to construct viable recombinant Yellow Fever (YF) 17D viruses expressing heterologous polypeptides including part of the Simian Immunodeficiency Virus (SIV) Gag protein. However, the expressed region, encompassing amino acid residues from 45 to 269, was genetically unstable. In this study, we improved the genetic stability of this recombinant YF 17D virus by introducing mutations in the IRES element localized at the 5′ end of the SIV gag gene. The new stable recombinant virus elicited adaptive immune responses similar to those induced by the original recombinant virus. It is, therefore, possible to increase recombinant stability by removing functional motifs from the insert that may have deleterious effects on recombinant YF viral fitness.

Introduction

Yellow Fever (YF) 17D is one of the most effective vaccines currently available. It was developed by Max Theiler and associates, who attenuated wild type strain Asibi by serial passages in animal tissues. This vaccine has been used for 75 years in more than 540 million people with an outstanding record of efficacy and safety. A single subcutaneous injection confers protection for at least 10 years (Ciczora et al., 2010; Monath, 2004). The basis of this strong and durable immune response is currently being investigated. It is known that this vaccine virus activates different pathways of the innate immune response, which lead to a polyvalent adaptive response. Vaccination induces cytotoxic CD8+ T memory cells, neutralizing antibody production and a mixed T_H1/T_H2 response (Barba-Spaeth et al., 2005; Miller et al., 2005; Querec et al., 2006; Santos et al., 2008).

These exceptional properties of the attenuated YF 17D vaccine have led to the idea that this virus could be used as a vector for the generation of new human vaccines (Bonaldo et al., 2000; Pugachev et al., 2005). The YF virus is the prototype member of the genus Flavivirus, which contains a positive strand RNA genome of about 11 kb (Chambers et al., 1990). Recombinant virus recovery is possible by modifying the complete cDNA infectious clone of the YF 17D vaccine virus, in vitro transcription and transfection of infectious RNA molecules.

So far, several strategies have been developed to insert gene sequences encoding microbial antigens in the YF genome. In some of these approaches, short sequences that encode epitopes were inserted into different genomic regions (Barba-Spaeth et al., 2005; Bonaldo et al., 2002, 2005; McAllister et al., 2000; Tao et al., 2005). However, a major concern in the development of recombinant YF 17D vaccines relates to the limited size of the insert, because, unfortunately, inserts longer than 40 codons do not generally produce genetically stable viruses. The expression of larger fragments would be desirable to promote a broader immune response. Hence, we have developed a methodology to construct viable and immunogenic recombinant YF 17D viruses that used the presence of functional motifs and amino acid sequence conservation flanking the E and NS1 intergenic region. Duplication of these sequences and fusion to the exogenous gene facilitated the correct processing of the viral

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0042-6822/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2014.01.017

polyprotein precursor (Bonaldo et al., 2007). Using this strategy, we recovered a viable and immunogenic recombinant YF 17D virus expressing a fragment of the SIV Gag protein (residues 45–269), which elicited SIV-specific CD8+ T cell responses after immunization of rhesus macaques (Bonaldo et al., 2010). However, this recombinant virus was not stable, resulting in insert loss after serial passages in Vero cell culture. Interestingly, this *gag* 45–269 minigene contains part of a lentiviral IRES motif (Weill et al., 2010). We hypothesized that the IRES motif might cause a substantial reduction in viral fitness leading to the positive selection of recombinant viruses in which the *gag* gene insert has been partially deleted. To explore this hypothesis and create new approaches to overcome this limitation, we constructed a variant recombinant YF 17D virus in which the IRES element was knocked out. The resulting mutant virus retained the foreign cassette for higher numbers of passages when compared to the original recombinant YF17D/SIV Gag_{45-269} virus. It also retained its biological and immunological properties, providing the basis for further development of this platform for expressing relevant SIV/HIV antigens and the development of new HIV vaccine candidates.

Results and discussion

Design of SIV gag minigene expression cassettes and recombinant YF 17D virus recovery

We have previously described a strategy for the insertion and expression of the heterologous sequences in the YF genomic E/NS1 intergenic region (Bonaldo et al., 2007). The rationale for this approach was based on the fact that this insertion site represents a functional shift from the structural to non-structural flavivirus genes accommodating larger inserts better than any other site in the YF 17D virus genome. In our first attempt to establish the use of the YF 17D virus as a vector to express lentiviral antigens, we inserted the SIV *gag* 45–269 gene region at the YF 17D E-NS1 site (SIVmac239 *gag* gene position: 133–807; GenBank, AY588945). This SIV gene region corresponds to the last 273 nucleotides of the p17 Matrix protein gene (coding for 91 amino acid residues of the carboxyl-terminus of the Matrix protein) and 402 nucleotides of the 5′ end of the Capsid gene (p24) encoding its first 134 amino acids.

The choice of this SIV antigen was based on the presence of a well-characterized SIVmac239 CD8+ T cell epitope recognized by rhesus monkey T cells (Allen et al., 2001). Recombinant YF17D/SIV viruses could therefore be evaluated for immunogenicity and safety in the rhesus monkey model. Accordingly, we demonstrated that the recombinant YF17D/SIV Gag_{45-269} virus elicited SIV-specific CD8+ T cell responses in the Indian rhesus macaques after immunization (Bonaldo et al., 2010).

The second criterion for the rational design of the recombinant virus was the physical chemical properties of this SIV Gag_{45-269} antigen. We also investigated whether the insert encoded any transmembrane domains and whether it was hydrophobic. Both characteristics might be deleterious to the correct topology and processing of the downstream YF polyprotein precursor. The selected SIV Gag_{45-269} expression cassette encoded 308 amino acids, which included 10 residues from the N-terminus of the YF 17D virus NS1 protein (YF genome position from 2453 to 2482) fused at its 5′ terminal (Fig. 1A). At the C-terminal, the Gag_{45-269} cassette contains the motif KESSIG (YF genomic position from 2145 to 2164) followed by the truncated stem anchor domain of dengue 4 (DENV4) virus E protein containing two transmembrane helix motifs called TM1 and TM2 (DENV4 genome positions 2226–2423; GenBank GU289913) (Fig. 1A). Both transmembrane domains could be predicted by the TMHMM software, specific for transmembrane helices in proteins (Fig. 1B). The presence of these

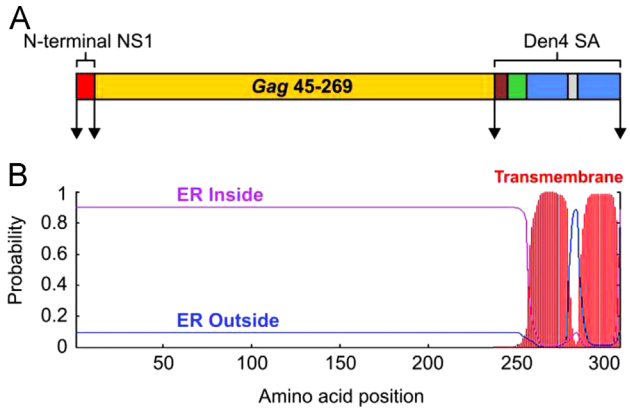

Fig. 1. Schematic diagram of the YF17D SIVmac239 Gag_{45-269} expression cassette. (A) Gag_{45-269} recombinant cassette fused at its 5′ and 3′ termini to YF motifs. The N-terminus of NS1 is shown in red. The C-terminus is made up of different components including the motif KESSIG (brown) and the truncated stem anchor domains of Dengue 4 virus E protein, the putative stem H2 motif (green) followed by the transmembrane domains T1 and T2 (blue). (B) Prediction plot of transmembrane helices in the Gag_{45-269} cassette using the TMHMM Programm. The sequence was positioned inside or outside of endoplasmic reticulum (ER) based on the absence of transmembrane helices and the expected viral polyprotein topology.

transmembrane motifs in the recombinant protein promotes its anchoring in the ER membrane, facing the ER lumen (Bonaldo et al., 2007). Additionally with the ProtParam Tool, we also determined various physicochemical properties, such as the expected molecular weight of 33.7 kDa and its hydrophilic character based on its Grand average of hydropathicity (GRAVY's index) of – 0.138 (Supplementary Table 1). The hydrophilicity of the foreign protein is probably an important property of the recombinant protein, since it should be soluble and exposed in the lumen of ER thereby not disturbing the arrangement of the flanking viral E and NS1 proteins.

Finally, the selected sequence was codon-optimized, based on YF virus codon usage frequency. The synthetic gene was cloned into YF genomic cDNA, which in turn was submitted to in vitro transcription. Following Vero cell transfection with RNA, we recovered the recombinant YF virus, identified as YF17D/SIV Gag_{45-269} virus and re-infected Vero cell monolayers generating a second passage viral stock, called P2, with a viral titer of 6.2 $\log_{10}$ PFU/ml.

The recombinant YF17D/SIV Gag_{45-269} virus is genetically unstable

Genetic stability is one of the most important properties of a recombinant live attenuated virus for a vaccine candidate. We, therefore, genetically characterized the recombinant YF17D/SIV Gag_{45-269} virus by sequencing the whole genome to confirm the expected nucleotide sequence of this virus as well as the integrity of the heterologous cassette under successive passages in Vero cells. We did not detect any point mutations in the second passage virus samples (P2). However, by analyzing the size of the RT-PCR amplicons containing the heterologous insertion, we observed a reduction of the cassette size after the fifth Vero cell serial passage (Supplementary Fig. 1B). At the twentieth continuous cell passage, most of the amplicons contained only recombinant Gag sequence of the first 27 nucleotides at the 5′end and the last 178 nucleotides of the gag_{45-269} gene. We reasoned that the particular region from nucleotide 57 through 528 of the expression cassette might be deleterious to the virus, and therefore, recombinant YF 17D viruses with smaller foreign inserts were positively selected.

With the aim of understanding the nature of this constraint, we compared the recombinant YF17D/SIV Gag_{45-269} virus to six other