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Involvement of a joker mutation in a polymerase-independent lethal mutagenesis escape mechanism

Rubén Agudo [a], Ignacio de la Higuera [a], Armando Arias [a], Ana Grande-Pérez [b], Esteban Domingo [a,c,*]

[a] Centro de Biologia Molecular "Severo Ochoa" (CSIC-UAM), Cantoblanco, E-28049 Madrid, Spain
[b] Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", Universidad de Málaga – Consejo Superior de Investigaciones Científicas, (IHSM-UMA-CSIC) Área de Genética, Campus de Teatinos, 29071 Málaga, Spain
[c] Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Barcelona, Spain

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ABSTRACT

We previously characterized a foot-and-mouth disease virus (FMDV) with three amino acid replacements in its polymerase (3D) that conferred resistance to the mutagenic nucleoside analogue ribavirin. Here we show that passage of this mutant in the presence of high ribavirin concentrations resulted in selection of viruses with the additional replacement I248T in 2C. This 2C substitution alone (even in the absence of replacements in 3D) increased FMDV fitness mainly in the presence of ribavirin, prevented an incorporation bias in favor of A and U associated with ribavirin mutagenesis, and conferred the ATPase activity of 2C decreased sensitivity to ribavirin-triphosphate. Since in previous studies we described that 2C with I248T was selected under different selective pressures, this replacement qualifies as a joker substitution in FMDV evolution. The results have identified a role of 2C in nucleotide incorporation, and have unveiled a new polymerase-independent mechanism of virus escape to lethal mutagenesis.

1. Introduction

High mutation rates and quasispecies dynamics endow viruses with phenotypic flexibility and capacity to respond to selective constraints, including antiviral agents [as relevant articles and reviews, see Domingo et al. (2012), Figlerowicz et al. (2003), Lauring and Andino (2010), Perales et al. (2010), and Vignuzzi et al. (2006)]. RNA virus escape to inhibitors has led to a number of proposals to control viral infections that aim at creating simultaneous selective pressures that force selected or resistant virus to reduce its fitness (Bonhoeffer et al., 1997; Domingo, 1989,1992). One of the approaches consists in the use of mutagenic agents to increase the virus mutational load to provoke the deterioration of viral functions and virus extinction. It has been termed entry into error catastrophe or lethal mutagenesis of viruses (Loeb et al., 1999), and has found support in studies in cell culture and *in vivo* (Chung et al., 2013; Dapp et al., 2013; Dietz et al., 2013; Graci et al., 2008; Holland et al., 1990; Loeb et al., 1999; Ortega-Prieto et al., 2013; Pariente et al., 2003; Ruiz-Jarabo et al., 2003; Severson et al., 2003).

The adaptive potential of viral quasispecies towards a mutagenic nucleotide analogue was evidenced by selection of viral mutants that display decreased sensitivity to ribavirin (1-β-D-ribofuranosyl-1-*H*-1,2,4-triazole-3-carboxamide) (Bordería et al., 2016; Pfeiffer and Kirkegaard, 2003; Sierra et al., 2007; Vignuzzi and Andino, 2010; Vignuzzi et al., 2006). Replication of foot-and-mouth disease virus (FMDV) in the presence of increasing concentrations of ribavirin (from 200 μM to 5 mM added to the culture medium) resulted in the selection of virus populations that incorporated sequentially M296I, P44S and P169S in their RNA-dependent RNA polymerase (RdRP, termed 3D) (Agudo et al., 2010; Arias et al., 2008; Sierra et al., 2007). These three substitutions conferred a selective advantage to the virus when it replicated in the presence of ribavirin but they reduced viral fitness in its absence, suggesting that there is a fitness cost in the acquisition of ribavirin resistance. The polymerase that included only M296I showed decreased capacity to use ribavirin-5′-triphosphate (RTP) as substrate, without a significant increase of template copying fidelity (Arias et al., 2008; Sierra et al., 2007). The major effect of substitutions P44S and P169S in 3D was to avoid a significant fitness loss while maintaining a balance among the four transition types in progeny genomes; mutant spectrum complexity, which is important for virus adaptability, was ensured despite exposure to high ribavirin concentrations. Biochemical and structural studies with 3D containing P44S, P169S and M296I [termed 3D(SSI)]

* Corresponding author at: Centro de Biologia Molecular "Severo Ochoa" (CSIC-UAM), Cantoblanco, E-28049 Madrid, Spain.
E-mail address: edomingo@cbm.csic.es (E. Domingo).

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documented that the avoidance of ribavirin-mediated G→A and C→U transitions was associated with a conformation modification of the N-terminal region of the polymerase that results in reorientation of template residues and altered nucleotide incorporation (Agudo et al., 2010).

The virus engineered to include the three substitutions P44S, P169S and M296I in 3D [termed FMDV 3D(SSI)] was still partially sensitive to 5 mM ribavirin. Therefore, it was possible to subject FMDV 3D(SSI) to additional passages in the presence of ribavirin to study if the virus could still evolve towards a higher level of ribavirin resistance. Here we show that, unexpectedly, FMDV 3D (SSI) survival in the presence of continued high ribavirin concentrations was achieved through a single amino acid substitution (I248T) in non-structural protein 2C, without additional substitutions in 3D. Picornaviral 2C is a multifunctional protein involved in virus uncoating, genome replication, encapsidation, and production and organization of membrane structures where viral replication takes place (Aldabe and Carrasco, 1995; Bienz et al., 1987; Bienz et al., 1992; Cho et al., 1994; Gladue et al., 2012; Gromeier et al., 1999; Li and Baltimore, 1990; Liu et al., 2010; Palmenberg et al., 2010; Pfister and Wimmer, 1999b; Rodriguez and Carrasco, 1993; Teterina et al., 1997a, 1997b; Tolskaya et al., 1994; van Kuppeveld et al., 2010). Replacements in rhinovirus 2C affected rhinovirus host range (Harris and Racaniello, 2003). 2C substitution I248T is involved in guanidine resistance of FMDV (Pariente et al., 2003), and it became dominant also upon adaptation of the virus to the guinea pig (Núñez et al., 2001) or mouse (Sanz-Ramos et al., 2008). Thus, I248T belongs to the class of joker substitutions defined as those that serve to increase fitness of a virus in different environments (Domingo, 2016). By engineering a clone of FMDV with I248T in 2C as the only replacement relative to the wild type virus, we show that this substitution can also modulate the types of mutations introduced by 3D, without participation of any 3D substitution. 2C substitution I248T allows FMDV to survive under ribavirin mutagenesis despite the mutant spectrum achieving elevated average mutation frequencies. These observations provide evidence of the association of protein 2C with picornaviral replication fidelity, and define a two-layer mutagenesis response mechanism, which is redundantly associated with two essential viral proteins.

2. Results

2.1. Selection of substitution I248T in 2C in FMDV 3D(SSI) passaged in the presence of ribavirin

Passage of FMDV 3D(SSI) in the presence of ribavirin resulted in a population that gained replicative capacity in the presence of the drug. The consensus sequence of the 2C-coding region of the FMDV 3D(SSI) populations at passages 2, 4, 6, 8 and 10 with ribavirin documented a gradual increase in the proportion of mutation U5087C (corresponding to amino acid subsitution I248T) that paralleled an increase of virus titer (Fig. 1). This mutation was the only one detected in the consensus sequence of the entire genome at passage 10, while the three 3D replacements in the parental FMDV 3D(SSI) were maintained. Parallel passages of FMDV 3D(SSI) in the absence of ribavirin did not result in selection of mutation U5087C. However U5087A (corresponding to amino acid substitution I248N) represented about 60% of the population at passage 10 (p10). When the virus passaged 10 times with ribavirin [termed population p10(Rib) (Fig. 1)] was subjected to 5 additional passages in the absence of ribavirin, pseudoreversion C5087A was observed. No other substitutions were detected in the 2C-or P3-coding regions. These results suggest that 2C replacement I248T was selected upon passage of FMDV 3D(SSI) in the presence of ribavirin.

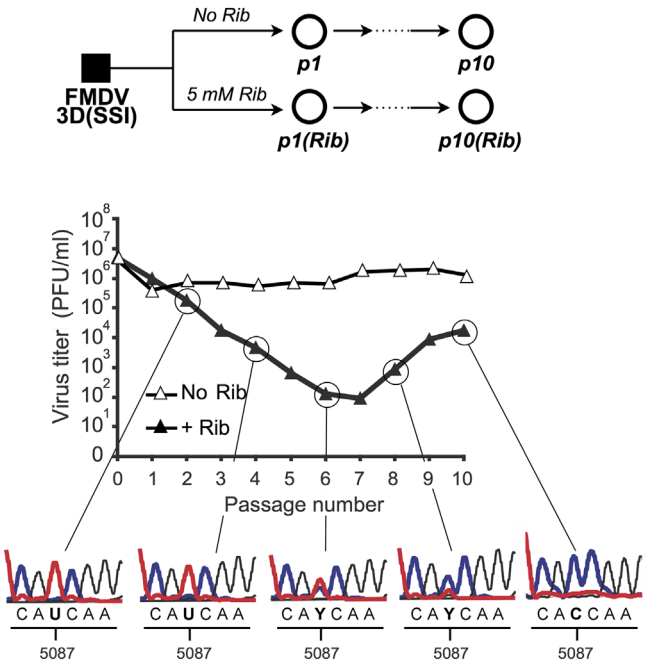

Fig. 1. Passage of FMDV 3D(SSI) in the absence or presence of ribavirin. Top: scheme of virus passages. The initial biological clone is depicted as a filled square and the uncloned, passage populations as empty circles. Bottom: Evolution of virus titer in the absence or presence of ribavirin. For the indicated passages the consensus sequences at genomic residues 4345–5298 (2C-coding region) were determined. The peak pattern for nucleotides 5085–5090 is shown, indicating the gradual dominance of mutation U5087C (amino acid substitution I248T in 2C); Y indicates a mixture of U and C. Residue numbering is according to Escarmís et al. (1996) and proteins are numbered individually beginning at the amino-terminal residue. Procedures for virus passage, titration of infectivity and nucleotide sequencing are described in Section 4.

2.2. 2C substitution I248T in the response of FMDV to ribavirin

To investigate the effect of 2C replacement I248T on the response of FMDV to ribavirin in the context of the triple 3D substitution SSI, pMT28-(TSSI) (infectious clone encoding I248T in 2C, and P44S, P169S and M296I in 3D) was constructed as detailed in Section 4; the corresponding progeny virus is termed FMDV(TSSI). The fitness of FMDV(TSSI) relative to FMDV 3D(SSI) was 3.5 ± 2.0 in the absence of ribavirin and 6.9 ± 1.7 in the presence of 5 mM ribavirin (Fig. 2A). Thus, amino acid substitution I248T in 2C had a positive effect on the replicative fitness of FMDV 3D(SSI) mainly in the presence of ribavirin.

To investigate if replacement I248T could play a role in the adaptation to ribavirin in the absence of substitutions in 3D, virus FMDV 2C(I248T) (with I248T in 2C as the only substitution in the genetic context of pMT28) was constructed and its fitness measured in growth-competition experiments with the wild type (FMDV Wt). The fitness of FMDV 2 C(I248T) relative to FMDV Wt was 2.0 ± 2.2 in the absence of ribavirin and 4.8 ± 1.2 in the presence of 5 mM ribavirin, indicating a selective advantage of FMDV harboring 2C(I248T) in the sequence context of the wild type virus (Fig. 2B). For both FMDV(TSSI) and FMDV 2C(I248T) the fitness increase conferred by I248T was statistically significant (Fig. 2).

To explore if substitution I248T in 2C could affect FMDV extinction by ribavirin, FMDV Wt and FMDV 2C(I248T) were subjected to serial passages in the presence or absence of 5 mM ribavirin (Fig. 3). In the presence of ribavirin, FMDV 2C(I248T) showed a decrease of infectious progeny production that was less pronounced than the decrease observed with FMDV Wt. FMDV Wt