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

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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)]

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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.

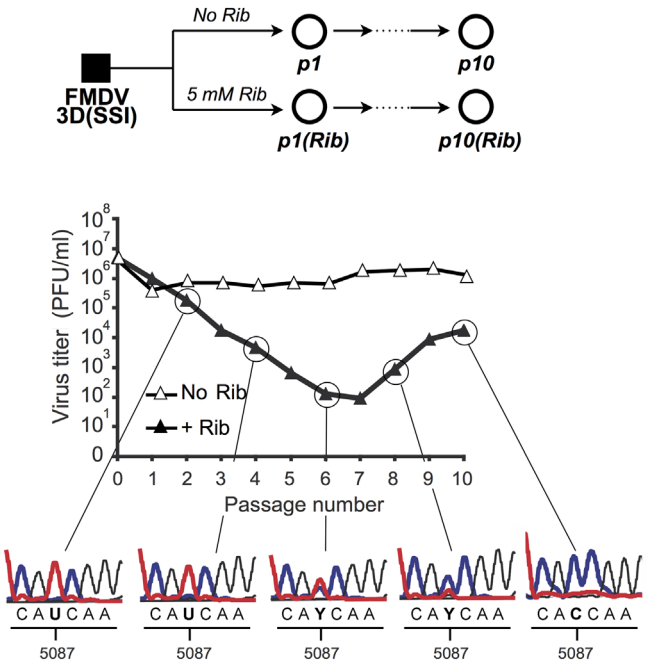

Fig. 1. Passage of FMDV 3D(SSI) in the absence or presence of ribavirin. Top: scheme of virus passages. The initial biological clone is depicted as a filled square and the uncloned, passage populations as empty circles. Bottom: Evolution of virus titer in the absence or presence of ribavirin. For the 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

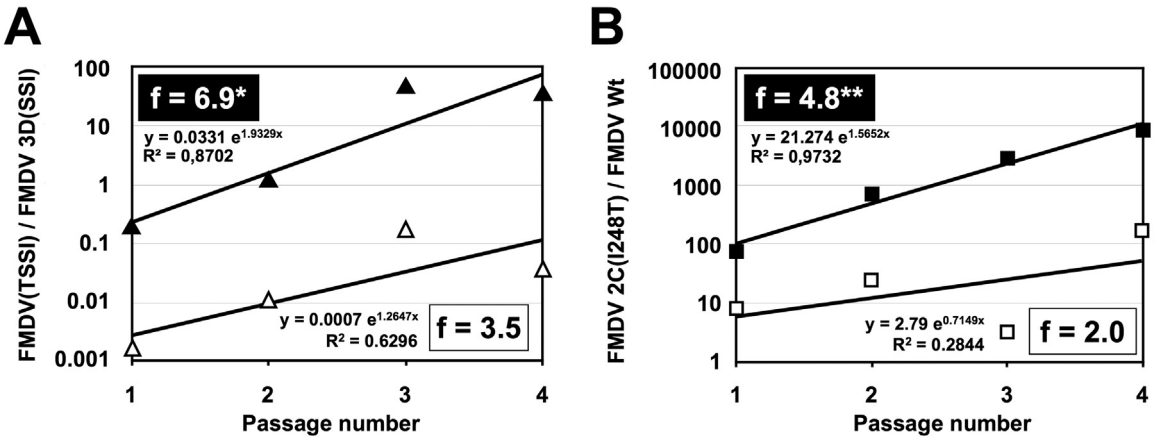

Fig. 2. Fitness of FMDV containing substitution I248T in 2C relative to FMDV Wt in the absence or presence of ribavirin. Competitions were established by infecting BHK-21 cells with a mixture of two viruses [either FMDV(TSSI) and FMDV 3D(SSI) (panel A) or FMDV 2C(I248T) and FMDV Wt (panel B), as indicated in the ordinate], at a total initial MOI of 0.1 PFU/cell. Passages were performed in the absence of ribavirin (empty symbols) or in the presence of 5 mM ribavirin (filled symbols). The ratio of RNA of the two competing viruses was determined using discriminatory RT-PCR amplifications, and plotted against passage number. The experimental points were adjusted to a straight line whose equation is given in each panel. The relative fitness value (f) in the absence or presence of ribavirin is indicated in a white and black box, respectively. Statistical significances are computed through the *t*-test, to evaluate if the slope of the ratio of RNA of the two competing viruses versus the passage number (4 points) is different from zero; significances are represented as (* $p < 0.05$; ** $p < 0.01$). Procedures are further detailed in Section 4.

was extinguished at passage 7 in the presence of ribavirin [no infectivity or virus-specific RT-PCR amplified material were detected or rescued after three blind virus passages(Agudo et al., 2010)]. In contrast, FMDV 2C(I248T) passaged in the presence of ribavirin was not extinguished in the course of at least 10 passages since the supernatants from passages 9 and 10 regained the capacity to produce infectious progeny after one passage in the absence of ribavirin. Also, the specific infectivity of the FMDV 2C (I248T) populations passaged in the absence or presence of ribavirin remained constant, while FMDV Wt displayed a decrease in specific infectivity in the presence of 5 mM ribavirin (Fig. 3) which is a feature of lethal mutagenesis (Agudo et al., 2010; Baranovich et al., 2013; Crotty et al., 2001; González-López et al., 2004; Grande-Pérez et al., 2005; Ortega-Prieto et al., 2013; Pariente et al., 2003, 2001; Perales et al., 2009). Thus, substitution I248T in 2C was sufficient to impede or delay the extinction of FMDV by high ribavirin concentrations.

2.3. *Effect of 2C substitution I248T on the mutant spectrum composition of FMDV passaged in the presence of ribavirin*

The effect of 2C substitution I248T on mutant spectrum complexity was examined by comparing FMDV Wt and FMDV 2C (I248T) populations passaged in the absence or presence of ribavirin. The results (Table 1) show a 2–7-fold increase of mutation frequency in the viruses passaged in the presence of ribavirin, in agreement with previous studies with FMDV (Agudo et al., 2010; Airaksinen et al., 2003; Perales et al., 2011). Remarkably, the presence of 2C substitution I248T, either alone or together with the triple replacement SSI in 3D prevented the ribavirin-mediated increase in G→A and C→U transitions (Transition bias in Table 1). The increase was 22-fold for the wild type virus ($P < 0.001$; χ^2 test) and 0.9-fold for FMDV-2C(I248T) (Table 1). Therefore substitution I248T in 2C can modulate nucleotide incorporation and contribute to virus survival under enhanced mutagenesis without the need of amino acid substitutions in the viral polymerase.

2.4. *Effect of substitution I248T on the ATPase activity of 2C*

It is intriguing that the same amino acid substitution in 2C facilitated FMDV survival under disparate conditions: presence of guanidinium hydrochloride (Pariente et al., 2003), adaptation to alternative animal host species (Núñez et al., 2001; Sanz-Ramos et al., 2008), and enhanced mutagenesis as described in the present report. Obviously, amino acid substitution I248T is not phenotypically neutral, and it was important to identify some functional difference between the wild type and mutant 2C. A well characterized biochemical activity of FMDV 2C is its ATPase activity (Sweeney et al., 2010). To test if substitution I248T affected the ATPase activity of FMVD 2C, the wild type and substituted 2C [termed 2C Wt and 2C(I248T), respectively] were expressed in *E. coli*, purified, and kinetic parameters for ATP hydrolysis measured in the absence and presence of RTP. In the absence of RTP, the presence of substitution I248T resulted in a 13-fold decrease in the rate of ATP hydrolysis, but a 5-fold increase in the affinity to ATP (relative to 2C Wt) (Table 2). RTP produced a competitive inhibition of the ATPase activity of 2C Wt, but not of 2C(I248T); the $K_{i,\ app}$ for 2C Wt is 273 μM, of the same order of magnitude than the $K_{m,\ app}$ for ATP (112 μM) (Fig. 4; Table 2). GuH inhibits the ATPase activity of poliovirus (PV) 2C in vitro(Pfister and Wimmer, 1999a). The ATPase activity of FMDV 2C Wt, but not that of 2C (I248T) was inhibited by GuH (Fig. S1), as expected from the previously documented selection of GuH-resistant FMDV mutants harboring substitution I248T in 2C (Pariente et al., 2003). The results indicate at the biochemical level a functional alteration of 2C, and suggest that I248T may confer a selective advantage by attenuating the inhibitory activity of RTP on 2C (see also Section 3).

We conclude that FMDV can acquire a polymerase-independent level of resistance to ribavirin through a single amino acid replacement in non-structural protein 2C. Resistance is based on modulation of nucleotide incorporation to prevent the excess of G→A and C→U transitions associated with ribavirin mutagenesis. The results have uncovered a new, polymerase-independent mechanism of FMDV escape to lethal mutagenesis (Pogolotti and Santi, 1982).

3. Discussion

Resistance to the mutagenic activity of ribavirin has been mainly associated with the RdRp of the target viruses (Agudo et al., 2010; Arnold et al., 2005; Pfeiffer and Kirkegaard, 2003, 2005a, b; Sierra et al., 2007; Vignuzzi et al., 2006; Young et al., 2003). This is expected because this nucleoside analogue is intracellularly converted into

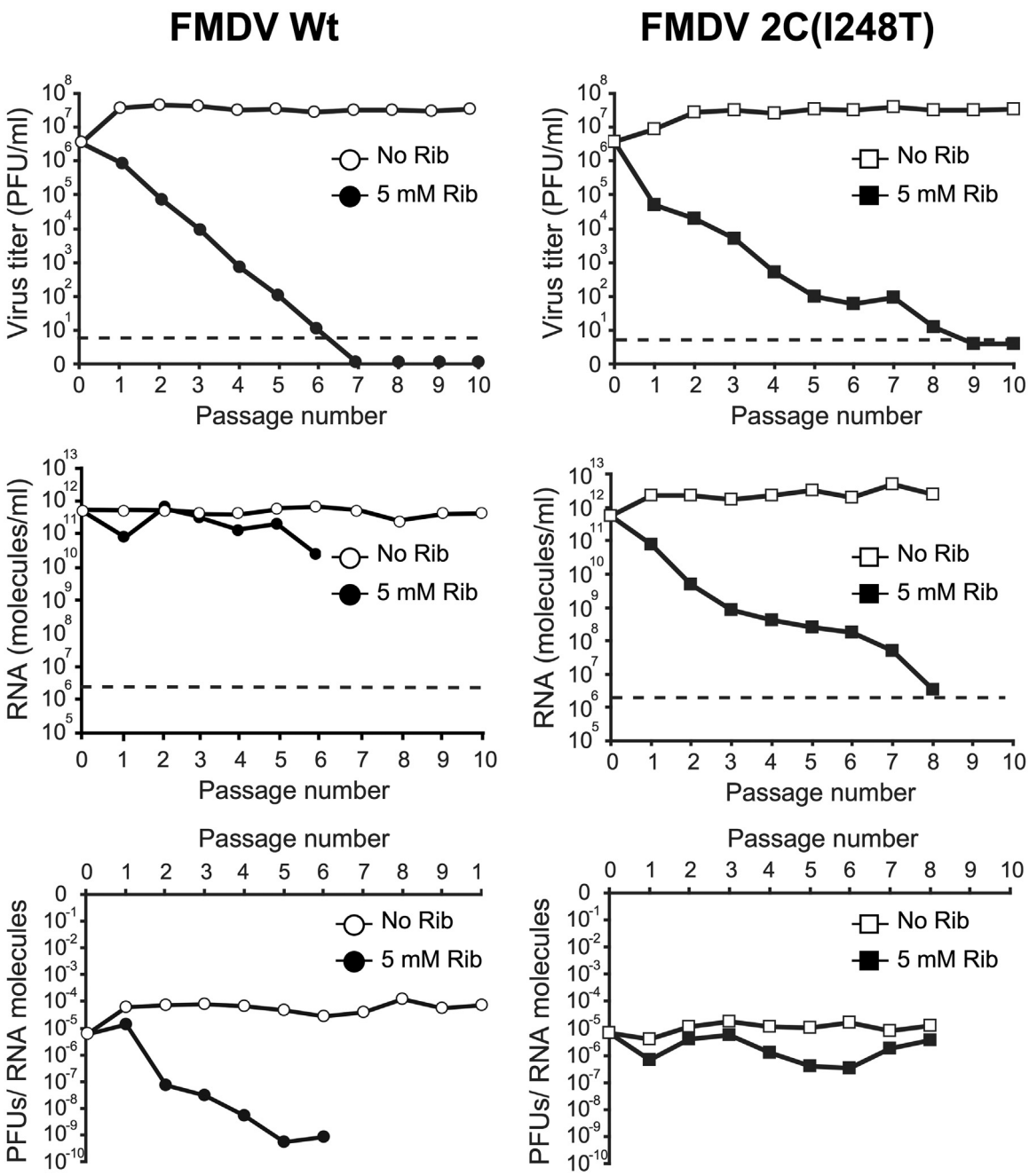

Fig. 3. Response of FMDV Wt and FMDV 2C(I248T) to ribavirin. BHK-21 cells (2×10^6) were infected with either FMDV Wt (left panels) or FMDV 2C(I248T) (right panels) at a MOI of 0.4 PFU/cell. In successive passages, the same number of cells was infected with the supernatant from the previous passage in the absence or presence of ribavirin (5 mM), as indicated in each panel. Virus titer, RNA molecules in the cell culture supernatant, and the specific infectivities are shown in the three panels from top to bottom; discontinuous lines indicate the limits of detection. RNA molecules and specific infectivities at passages 7–10 of FMDV Wt in the presence of ribavirin could not be determined because the RNA was not amplified by RT-PCR. For the FMDV 2C(I248T) the specific infectivity at passages 9 and 10 in the presence of ribavirin could not be calculated due to undetectable RNA viral titer. Procedures are further detailed in Section 4.

RTP, which is the active mutagenic substrate for some RdRPs (Agudo et al., 2010; Beaucourt and Vignuzzi, 2014; Crotty et al., 2001; Crotty et al., 2000; Day et al., 2005; Freistadt et al., 2004; Lanford et al., 2001; Maag et al., 2001; Sierra et al., 2007). Three molecular mechanisms of ribavirin resistance associated with amino acid substitutions in the polymerase have been described in picornaviruses: (i) a general increase of polymerase fidelity that limits the incorporation of RTP into viral RNA (Arnold et al., 2005; Pfeiffer and Kirkegaard, 2005a; Vignuzzi et al., 2006); (ii) a specific decrease of RTP incorporation without significant change of the general copying fidelity of the enzyme(Arias et al., 2008; Sierra et al., 2007); and (iii) modulation of relative nucleotide incorporation to avoid the excess of G→A and C→U transitions evoked by ribavirin (Agudo et al., 2010).

When FMDV 3D(SSI) was challenged with the need to further replicate in the presence of 5 mM ribavirin, no additional substitutions in 3D were selected, but a new phenotypic transition involving substitution I248T in 2C came into play (Fig. 1). The fact that the three substitutions P44S, P169S and M296I, when present in the same polymerase molecule inflicted a fitness cost upon the virus in the absence of ribavirin suggests that 3D may have limitations to accept additional substitutions to respond to ribavirin and to maintain RNA synthesis activity, and consequently, viral fitness. Limitations of viral proteins to accept amino acid substitutions and to remain functional have been suggested by theoretical

Table 1
Mutant spectrum composition of FMDV populations passaged in the absence or presence of ribavirin.

FMDV[a]	Rib (5 mM)	Mutation frequency[b]	Number of nucleotides (clones)	Mutation types[c]									Transition bias[d]
				Transitions				Transversions					
				G→A	A→G	C→U	U→C	C→A	G→U	U→A	C→G	Total	
Wt	–	6×10^{-4}	29,810 (22)	2	7	1	8	0	0	0	0	18	0.18
Wt	+	13×10^{-4}	32,520 (24)	20	2	16	6	0	0	0	0	44	3.95
2C(I248T)	–	6×10^{-4}	28,455 (21)	4	3	2	3	1	1	1	1	16	0.88
2C(I248T)	+	22×10^{-4}	29,810 (22)	10	11	21	24	0	0	0	0	66	0.79
TSSI	–	2×10^{-4}	35,230 (26)	0	2	1	5	0	0	0	0	8	0.09
TSSI	+	13×10^{-4}	29,810 (22)	8	11	9	9	1	1	0	0	39	0.70

[a] Each virus was subjected to 4 serial passages in BHK-21 cells, except FMDV Wt, that was passaged 5 times in the absence of Rib. Values for FMDV Wt were previously reported (Agudo et al., 2010), and are included here for completeness and comparative purposes.
[b] Mutation frequencies are minimum values (repeated mutations counted only once), and are based on the sequencing of a total of 28,000 to 36,000 nucleotides per population, comprising genomic residues 6610–7964 (3D-coding region).
[c] The types of mutations not included were not found in the mutant spectra analyzed.
[d] Trasition bias is defined here as the ratio between the frequency of transitions G to A plus C to U, relative to the frequency of transitions A to G plus U to C: $\left[\left(\frac{G\rightarrow A}{G}\right)+\left(\frac{C\rightarrow U}{C}\right)\Big/\left(\frac{A\rightarrow G}{A}\right)+\left(\frac{U\rightarrow C}{U}\right)\right]$.

Table 2
Kinetic parameters K_m and V_{max} for the ATPase activity of 2C Wt and 2C(I248T) in the absence or in the presence of RTP.[a]

RTP [μM]	2C Wt			2C (I248T)		
	$K_{m,\ app}$ (μM)	V_{max} (s^{-1})	V_{max}/K_m (μM^{-1} x s^{-1})	$K_{m,\ app}$ (μM)	V_{max} (s^{-1})	V_{max}/K_m ($\mu M^{-1} \times s^{-1}$)
0	112 ± 17	0.0020 ± 0.0001	1.8×10^{-5}	19 ± 3	0.00015 ± 0.00001	0.8×10^{-5}
250	237 ± 30	0.0020 ± 0.0001	0.8×10^{-5}	16 ± 4	0.00013 ± 0.00001	0.8×10^{-5}
500	317 ± 66	0.0020 ± 0.0002	0.6×10^{-5}	15 ± 4	0.00013 ± 0.00001	0.8×10^{-5}

[a] The procedures used are those described in Section 4. Optimal pH, temperature and ionic conditions for the assay were established in experiments described in Section 4. The K_m and V_{max} values of the ATPase assay are those calculated from the plots represented in Fig. 4 (A and B).

studies and by the observed high proportion of lethal or highly deleterious mutations in viral enzymes (Parera et al., 2007, 2009; Sanjuan et al., 2004; Wilke et al., 2005). There are several reasons why the triple SSI substitution was preferentially selected over the 2C I248T. One is that in our previous study (Agudo et al., 2010) the initial selection of ribavirin-resistant mutants was carried out using a high fitness FMDV biological clone termed MARLS while the present work was initiated with our standard molecular clone in which the three polymerase substitutions were introduced by site-directed mutagenesis. Also, the triple polymerase mutant might confer a greater fitness advantage than the 2C mutation alone in the presence of 5 mM ribavirin (Agudo et al., 2010).

Proteins other than the viral polymerase can also affect the template copying fidelity of RNA viruses. Coronavirus nsp10 is involved in replication fidelity, probably via regulation of nsp14-ExoN activity (Smith et al., 2013, 2015). The alphavirus helicase/protease nsP2 affects polymerase fidelity, perhaps as a response to depletion of intracellular nucleotides that may occur under mutagenic conditions (Stapleford et al., 2015). The present study with FMDV shows that amino acid substitution I248T in non-structural protein 2C can modulate mutation types so as to counteract the mutational tendencies of ribavirin and mediate FMDV survival.

Picornavirus non-structural protein 2C belongs to the superfamily 3 (SF3) helicases. 2C and 2BCE participate in formation of membrane structures in the replication complex (RC) (Cho et al., 1994), and they are probably involved also in RNA synthesis (Banerjee et al., 1997; Jurgens and Flanegan, 2003; Lyons et al., 2001), capsid assembly and uncoating. 2C is also the target of several inhibitors (DePalma and Neyts, 2010). However, the pivotal role of 2C in the picornaviruses life cycle remains elusive [2C functions reviewed in Gorbalenya and Lauber (2010), Kirkegaard and Semler (2010), Palmenberg et al. (2010), and Rozovics and Semler (2010)].

Both PV and FMDV 2C are active as oligomers and are endowed with ATPase and RNA-binding activities (Adams et al., 2009; Mirzayan and Wimmer, 1994; Rodriguez and Carrasco, 1995; Sweeney et al., 2010). 2C has several subdomains (Teterina et al., 2006) and substitution I248T is located 6 residues upstream of the beginning of a Cys-rich motif (Pfister et al., 2000) (although in a sequence alignment this domain in FMDV 2C includes only one Cys residue), and far from the membrane-binding, divalent ion-binding NTPase and RNA-binding domains (Pfister and Wimmer, 1999b; Sweeney et al., 2010; Teterina et al., 1997b, 2006). The hypothesis that I248T was selected to counteract the mutagenic effect of ribavirin is supported by three findings: (i) FMDV harboring substitution I248T does not display a loss of specific infectivity upon treatment with ribavirin (Fig. 3). (ii) There is an increase in fitness value when FMDV 2C(I248T) is competed with FMDV Wt in the presence of ribavirin. (iii) Although ribavirin confers a 1.7-fold higher mutation frequency to the mutant virus than to the Wt, the proportion of G→A and C→U transitions remains unaltered in FMDV 2C (I248T) progeny. We cannot exclude that selection of I248T in 2C might have been favored also by some effect of high ribavirin concentration on a cellular function required for virus progeny production.

The effect of substitution I248T selected in response to ribavirin has revealed a new function for 2C in FMDV: an effect on the selectivity of nucleotide incorporation. Under high ribavirin concentration, I248T in 2C might have been selected because this substitution renders the ATPase activity of 2C more resistant to the inhibition by RTP. However, it is not clear if maintenance ATP hydrolysis relates to modulation of transition types. Infection of BHK-21 cells with FMDV results in a two-fold decrease of the intracellular levels of GTP and ATP but not of UTP and CTP (Airaksinen et al., 2003). Since the ratio of nucleoside-triphosphates to nucleoside-diphosphates that reaches locally the replication complex is unknown (Koonin and Agol, 1984), it is not

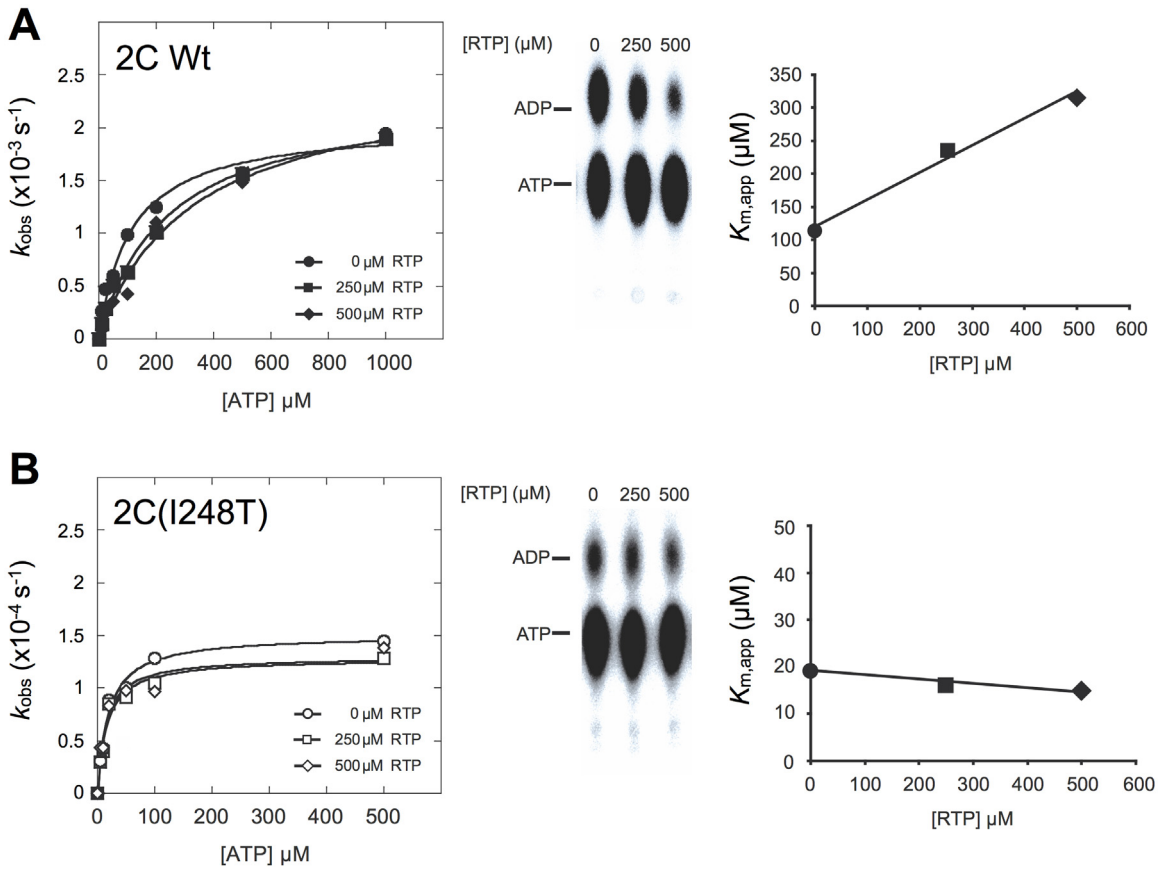

Fig. 4. Inhibition of ATPase activity of 2C by ribavirin-triphosphate. (A) Production of ADP from ATP catalyzed by 2C Wt in the absence or presence of ribavirin-5′-triphosphate (RTP). The middle panel depicts a representative thin layer chromatogram used to measure ADP and ATP concentrations. The right panel represents the $K_{m,\ app}$ values obtained by analysis of the nonlinear graph represented on the left as a function of RTP concentration. The slope of this line is $K_m/K_{i,\ app}$, consequently, the inhibitory constant $K_{i,\ app}$ = 273 μM. (B) Same as (A) using 2C(I248T). No inhibition by RTP is observed. The kinetic constants for the ATPase activity deducted from analysis of these plots are detailed in Table 2. Similar results were obtained with independent preparations of 2C Wt or 2C(I248T). 2C. Reaction conditions, analytical procedures and kinetic parameters are described in Section 4.

possible to relate ATP hydrolysis with viral fitness in the presence of RTP. 2C might influence nucleotide incorporation by mechanisms not directly related to ATP hydrolysis. Assuming that FMDV replication resembles that of PV, both 2C and 3D may be found in the replication complex, along with other non-structural viral proteins and host proteins (Gladue et al., 2012; Takegami et al., 1983; Teterina et al., 2011; Yin et al., 2007). In particular, documented direct interactions 2C-3AB and 3D-3AB may permit an effect of substitutions in 2C on 3D conformation. Relatively modest conformation changes at the N-terminal region of FMDV 3D are sufficient to modify nucleotide incorporation and resistance to nucleotide analogues (Agudo et al., 2010; Ferrer-Orta et al., 2007, 2015; Ferrer-Orta et al., 2010). It may be that I248T in 2C is selected under different environmental circumstances with and without a mutagenic milieu because it produces several compatible phenotypic changes. In the present study, the conjoined effects might be continuing replication, ribavirin resistance and an adequate pattern of nucleotide recognition. The notion of joker mutation may have as its basis a very restricted set of mutations (and their corresponding amino acid substitutions) that happen to influence multiple traits in a positive manner.

In conclusion, we have documented a new role of FMDV protein 2C consisting in its capacity to modify the relative incorporation of nucleotide substrates, dependent on a single amino acid substitution. Thus, the results have unveiled a new mechanism of escape to lethal mutagenesis and may help to elucidate the biological role of protein 2C.

4. Materials and methods

4.1. Cells, viruses, and infections

The origin of BHK-21 cells, procedures for cell growth and for infection with FMDV in the presence or absence of ribavirin (Sigma), have been previously described (Agudo et al., 2010; Perales et al., 2009; Sierra et al., 2007; Sobrino et al., 1983). FMDV C-S8c1 is a plaque-purified derivative of natural isolate C1 Santa-Pau Spain 70 (Sobrino et al., 1983), a representative of European serotype C FMDV. Ribavirin exerted a cytostatic effect on BHK-21 cells as measured by cell viability using trypan blue staining. The maximum reduction of cell viability in the presence of ribavirin was around 40% in the presence of 5 mM ribavirin at 48 h post-treatment, in agreement with our previous results(Perales et al., 2009).

4.2. Extraction of RNA, cDNA synthesis, PCR amplification, molecular cloning and nucleotide sequencing

RNA was extracted from the culture supernatants of infected cells as previously described(Arias et al., 2005; Sierra et al., 2007). Reverse transcription (RT) was carried out using AMV reverse transcriptase (Promega), and PCR amplification was performed using EHF DNA polymerase (Roche) as specified by the manufacturer. RT-PCR amplification intended for the cloning of individual cDNA molecules was carried out using *Pfu ultra* DNA polymerase (Stratagene), using primers and procedures that have

been previously described (Airaksinen et al., 2003; Sierra et al., 2007). To avoid repeated sequencing of the same template molecules that may produce a bias in mutation frequency values, RT-PCR amplifications were carried out with dilutions 1:10, 1:100 and 1:1000 with each template preparation. Only when at least the template diluted 1:100 produced a visible DNA amplification band, was molecular cloned performed using DNA amplified from undiluted template sample. Nucleotide sequencing was carried out as previously described (Sierra et al., 2007).

4.3. Quantification of viral RNA

Extracellular FMDV RNA was quantified by real-time RT-PCR (RT-qPCR) amplification using the Light Cycler instrument (Roche) and the RNA Master SYBR green I kit (Roche) as previously described (Sierra et al., 2007). To quantify intracellular FMDV RNA at early time post-infection, 7×10^5 BHK-21 cells were infected as described above with the same amount of PFUs for all viruses (MOI = 1 PFU/cell). Cell culture supernatants were withdrawn immediately after the 1 h adsorption period and at 2 h post-adsorption; the corresponding cell monolayers were washed with isotonic phosphate buffer (pH 6.0) to inactivate remaining virions, and harvested with 400 μL of Trizol (Sigma). RNA extraction and RT-qPCR were carried out as described above.

4.4. Preparation of FMDV C-S8c1 with substitutions in 2C and 3D

Plasmid pMT28 encodes an infectious transcript of FMDV C-S8c1(García-Arriaza et al., 2004). Plasmid pMT28-3D(SSI) is an infectious clone expressing 3D with substitution P44S, P169S and M296I in the context of the C-S8c1 (Agudo et al., 2010). The preparation of plasmid pMT28-2C(I248T) has been previously described (Sanz-Ramos et al., 2008). To construct pMT28-TSSI, an infectious clone expressing FMDV with amino acid substitutions I248T in 2C, and P44S, P169S and M296I in 3D, in the context of the C-S8c1 genome, plasmid pMT28-3D(SSI) was digested with *Rsr*II (position 5839) and *Nde*I (the *Nde* I site was engineered at the 3′ side of the viral poly(A)) at position 8140 (García-Arriaza et al., 2004). The DNA was purified and ligated to pMT28-2C(I248T), previously linearized with the same enzymes. Restriction enzymes were from New England Biolabs. Ligation, transformation of *Escherichia coli* DH5α, colony screening, nucleotide sequencing, preparation of infectious RNA transcripts, and RNA transfection using lipofectin were carried out as previously described (Arias et al., 2005; Sierra et al., 2007). Virus rescued from transfection of pMT28, pMT2-3D(SSI) and pMT28-2C(I248T) was termed FMDV Wt, 3D(SSI) and 2C(I248T), respectively.

4.5. Quasispecies analysis

To determine the complexity of mutant spectra, FMDV RNA was extracted from infected cell culture supernatants as described above, and subjected to RT-PCR using primers PolC-*Kpn*I (GTTGG**T**AC**C**CACTCTGCTGGAGGC; sense, 5′ position 6502) and Pol1-*Xba*I (AAT**CTA**GATGTTTGGGGGATTATGCG; antisense, 5′ position 8060). The underlined residues indicate the sequences recognized by restriction enzymes *Kpn*I and *Xba*I, respectively, and bold-face letters indicate modifications introduced in the genomic sequence. cDNA was digested with *Kpn*I and *Xba*I enzymes, and ligated to plasmid pGEM-3Z Vector (Promega), previously digested with de same enzymes. Transformation, colony screening and nucleotide sequencing were carried out as described above. The region sequenced spanned residues 6508–8036 and includes the entire 3D-coding region (residues 6610–8020). The mutant spectrum complexity was determined by quantifying the mutation frequency, expressed as the number of different mutations divided by the total number of nucleotides sequenced.

4.6. Fitness assays

Relative fitness was measured by growth-competition experiments in the presence or absence of ribavirin, as previously described(Agudo et al., 2010; Arias et al., 2004; Sierra et al., 2007). The proportion of the two competing genomes at different passages was determined by RT-qPCR, employing primers specifically designed to discriminate the two RNAs in the competition. Specifically, the discriminatory forward primers were I248(-1) (ACAACAAATTGGACATCA**T**C) for pMT28 Wt and pMT28-3D(SSI), and T248(-1) (ACAACAAATTGGACATCA**C**C) for pMT28-2C(I248T) and pMT28-TSSI (the 5′ nucleotide is at position 5069 for both primers; the bold-face residue indicates the position that is specifically discriminated). The reverse primer was 2CD4 (ACACAGATTTTTGGGAAGGT; 5′ position 5326) in both cases. The hybridization temperature was 65 °C for primer I248(-1) and 63 °C for primer T248(-1).

4.7. Molecular cloning of FMDV 2C and 2C(I248T)

The genomic region encoding 2C of FMDV in plasmid pMT28 was amplified by PCR with *Pfu* ultra DNA polymerase (Stratagene) using primers 2C*Nde*I5′, with an introduced *Nde*I restriction site (AGAGCAGAGAAACATATGAAAGCACGTGACATCAACGACATC; sense 5′ position 4330; bold-face letters indicate modifications introduced in the genomic sequence and underlined symbols indicate the nucleotide sequence recognized by *Nde*I), and 2C*Eco*RI3′, containing two stop codons upstream an *Eco*RI restriction sequence (CACAGATTTTTGGGA**GAATTC***CTATCA*TTGCTTAAAAATTGGGTGGCTTGACAC; antisense, 5′ position 5325; underlined residues indicate the nucleotide sequence recognized by *Eco*RI; residues in italic indicate the two stop codons). The PCR product was digested with *Nde*I and *Eco*RI, and ligated to pET-28a digested with the same enzymes. The *Nde*I restriction site introduced during PCR, changes the first amino acid of 2C from Leu to Met. To recover the initial sequence, plasmid DNA was subjected to a site-directed mutagenic PCR with oligonucleotides containing the corresponding mutated nucleotides, using the QuikChange site-directed mutagenesis kit (Stratagene). The plasmid obtained, termed pET28-2C, encodes the 2C protein of FMDV C-S8c1 preceded by an amino acid tract which sequence is GSSHHHHHHSSGLVPRGSH.

To construct pET28-2C(I248T) (a plasmid encoding 2C of C-S8c1 with the amino acid replacement I248T), plasmid pET28-2C was subjected to site-directed mutagenesis with the oligonucleotides mutCTu (AAATTGGACATCA**C**CAAAGCACTTG; sense, 5′ position 5074) and mutCTd (GGGTGTCTTCAAGTGCTTTG**G**TGATGTCC; antisense, 5′ position 5107) using the QuikChange site-directed mutagenesis kit (bold-face letters indicate modifications in the genomic sequence to express 2C with I248T).

4.8. Expression and purification of protein 2C

Kanamycin-resistant *E. coli* colonies harboring plasmid pET28-2C were grown in LB medium until an optical density of 0.6 at 600 nm was reached. Then, isopropyl 1-thio-β-D-galactopyranoside (IPTG) (Fermentas) was added to a final concentration of 0.5 mM and the culture grown for 2.5 additional hours. After induction with IPTG, the synthesis of a 35–49-kDa was observed, concordant with the expected size of FMDV 2C (38 kDa). Cells were pelleted by centrifugation at 4000 g for 15 min and stored at −20 °C. Purification of 2C was carried out as previously described (Banerjee et al., 1997) with minor modifications. Briefly, *E.coli* cells were suspended in buffer A [20 mM Tris HCl, pH 8.0, 100 mM NaCl, 2 mM imidazol and 0.1% Triton X100 (Sigma)] and incubated with

gentle stirring during 15 min at 4 °C in the presence of 0.1 mg/mL of lysozyme (Sigma) and 1 mM phenylmethylsulfonyl fluoride (Boehringer). The cells were then sonicated for 5 cycles of 30 s alternating with 5 cycles of 30 s, on ice. After centrifugation of cellular debris at 10,000 rpm for 15 min at 4 °C, the 2C protein remained in the insoluble fraction. Proteins were solubilized by adding buffer A containing 6 M urea, followed by centrifugation at 4 °C. The supernatant (containing solubilized 2C) was loaded onto a column with Ni^{2+}-charged resin (Invitrogen) equilibrated with buffer A containing 6 M urea, and allowed to flow under gravity. The column was successively washed with buffer A containing 6 M urea and 10 mM imidazole, and then with buffer A containing 6 M urea, 50 mM imidazole and 10% glycerol. Finally, the His-tagged 2C was eluted with buffer A containing 6 M urea, 500 mM imidazole and 10% glycerol. Fractions of 2 mL were collected and purity was tested by SDS-PAGE (12%) and Coomassie Blue staining. Fractions containing His-2C protein were pooled and dialyzed three successive times (3 h at 4 °C each) against buffer B (50 mM Tris HCl, pH 7.4, 100 mM KCl, 1 mM DTT, 1 mM EDTA and 20% glycerol) containing 4 M, 2 M and 0 M urea, respectively. Samples were stored in aliquots at −70 °C for further use. The enzymes were > 95% pure, as judged by SDS-PAGE and Coomassie blue staining, and reacted positively with monoclonal antibody 1c8 which is specific for FMDV 2C (kindly provided by Dr. Emiliana Brocchi).

4.9. 2C ATPase activity assays

To measure the ATPase activity of 2C, the reaction mixtures contained 50 mM MOPS (pH 7.5), 5 mM DTT, 10 mM NaCl, 2 mM $MgCl_2$ and 100 μM [α-^{32}P]ATP (0.01 mCi/mL; Amersham). The reaction was started by addition of 2 C diluted in buffer B (3 μM His-2C, final concentration) and was carried out at 37 °C. At different times, the reaction was quenched by EDTA (83 mM final concentration); then 2 μL were applied on a polyethyleneimine cellulose-coated TLC plastic sheet (Merck). The chromatogram was developed in 0.75 M NaH_2PO_4 and dried at 65 °C. Radioactivity corresponding to ATP and ADP, was visualized and quantitated with a Phosphorimager (BAS-1500; Fuji). The migration of [α-^{32}P]ADP in the chromatograms was determined by digestion of [α-^{32}P]ATP with alkaline phosphatase. To determine the kinetic constants K_m and V_{max} of ATPase activity of 2C, the corresponding activities were measured with increasing concentrations (0.1–1000 μM) of [α-^{32}P]ATP. Data were fit by nonlinear regression using the program KaleidaGraph (Synergy Software). ATPase activity in the presence of RTP (Moraveck), was measured as described above, but including different concentrations of substrate and RTP in the reaction mixtures, as indicated for each experiment in the Results section.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2016.04.023.

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