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The African swine fever virus g5R protein possesses mRNA decapping activity

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ABSTRACT

The African Swine Fever Virus (ASFV) encodes a single Nudix enzyme in its genome, termed the g5R protein (g5Rp). Nudix phosphohydrolases cleave a variety of substrates, such as nucleotides and diphosphoinositol polyphosphates. Previously, ASFV g5Rp was shown to hydrolyze diphosphoinositol polyphosphates and GTP, but was unable to cleave methylated mRNA cap analogues. In vaccinia virus (VACV), a distant relative of ASFV, the D9 and D10 Nudix enzymes were shown to cleave the mRNA cap, but only when the cap was attached to an RNA body. Here, we show that recombinant ASFV g5Rp hydrolyzes the mRNA cap when tethered to an RNA moiety, liberating m^7GDP as a product. Mutations in the Nudix motif abolished mRNA decapping activity, confirming that g5Rp was responsible for cap cleavage. The decapping activity of g5Rp was potently inhibited by excess uncapped RNA but not by methylated cap analogues, suggesting that substrate recognition occurs by RNA binding.

Introduction

The Nudix hydrolase motif is a signature sequence characteristic of a diverse group of phosphohydrolases found in viruses, prokaryotes, and eukaryotes (reviewed in McLennan, 2006). Nudix enzymes cleave a broad group of substrates that are generally comprised of a *nu*cleoside *di*phosphate linked to another moiety, *X* (Koonin, 1993; Bessman et al., 1996). Interestingly, the Nudix enzymes found in viruses are restricted almost exclusively to the five viral families that belong to the monophyletic lineage of large nucleocytoplasmic DNA viruses, comprised of poxviruses, asfarviruses, iridoviruses, phycodnaviruses and mimiviruses, suggesting possible overlapping functions for these proteins (Iyer et al., 2001; Iyer et al., 2006).

The D9 and D10 proteins of vaccinia virus (VACV), the prototypic poxvirus, are Nudix hydrolases that share 25% sequence identity to each other (Shors et al., 1999). D9 and D10 are expressed at different times during virus infection; D9 is expressed early whereas D10 is expressed during the late phase of viral infection (Lee-Chen and Niles, 1988; Parrish and Moss, 2006). Previous genetic studies demonstrated that over-expression of the D9R (VACV-WR_114) or the D10R (VACV-WR_115) gene resulted in enhanced turnover of mRNA molecules containing a 5′ m^7GpppNm cap, a stabilizing component of both VACV and cellular transcripts (Shors et al., 1999). Moreover, deletion of the D10R gene from the VACV genome resulted in the persistence of cellular and viral transcripts and a delay in the shutoff of host protein synthesis (Parrish and Moss, 2006). These two genetic observations led to the hypothesis that D9 and D10 cleave the mRNA cap, thereby accelerating viral and cellular mRNA turnover and promoting the sequential cascade of viral gene expression and the shutoff of host protein synthesis. In support of this hypothesis, Dcp2, a Nudix enzyme conserved from yeasts to mammals, has been shown to be an mRNA decapping enzyme (Wang et al., 2002; van Dijk et al., 2002; Steiger et al., 2003; Cohen et al., 2005; Xu et al., 2006).

More recent biochemical studies confirmed that both VACV D9 and D10 contain intrinsic mRNA decapping activity, releasing m^7GDP as a reaction product (Parrish et al., 2007; Parrish and Moss, 2007). Similar to eukaryotic Dcp2, D9 and D10 were unable to efficiently cleave a free methylated cap analogue (m^7GpppNm); robust decapping activity was only observed when the methylated cap structure was tethered to an RNA moiety (Wang et al., 2002; van Dijk et al., 2002; Piccirillo et al., 2003; Steiger et al., 2003; Cohen et al., 2005; Parrish et al., 2007; Parrish and Moss, 2007). In accord with this observation, uncapped RNA inhibited D9 and D10 decapping activity, suggesting RNA binding is required for these proteins to locate and cleave the cap structure (Parrish et al., 2007; Parrish and Moss, 2007). In addition, free methylated cap derivatives inhibited cap cleavage by D9 and D10, indicating that these proteins may also interact with the cap structure during substrate recognition (Parrish et al., 2007; Parrish and Moss, 2007).

African Swine Fever Virus (ASFV), the lone representative of the *Asfarviridae* virus family, encodes a single Nudix enzyme in its genome, denoted as the g5R protein (g5Rp) (NCBI ID: NP_042795) (Cartwright et al., 2002). Intriguingly, ASFV g5Rp shares greater sequence similarity to the *Schizosaccharomyces pombe* Dcp2 mRNA decapping enzyme than either VACV D9 or D10 (McLennan, 2007). Previous biochemical studies demonstrated that g5Rp hydrolyzes a

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broad range of substrates, most efficiently cleaving diphosphoinositol polyphosphates but also hydrolyzing nucleotide substrates such as GTP (Cartwright et al., 2002). Despite its broad substrate range, g5Rp was unable to efficiently cleave free methylated cap analogues, which led to the conclusion that g5Rp was not an mRNA decapping enzyme (Cartwright et al., 2002). However, in light of the recent observations that cap attachment to an mRNA body is required for Nudix-mediated mRNA decapping, the role of g5Rp in this process needs to be reevaluated (Wang et al., 2002; van Dijk et al., 2002; Piccirillo et al., 2003; Steiger et al., 2003; Cohen et al., 2005; Parrish et al., 2007; Parrish and Moss, 2007).

To examine if ASFV g5Rp possesses mRNA decapping activity, a g5Rp fusion protein was expressed in bacteria and subsequently purified by affinity chromatography. In contrast with the limited activity of g5Rp on free methylated cap analogues, g5Rp was able to robustly cleave a cap structure attached to an mRNA moiety in a manner dependent on the Nudix motif, releasing m^7GDP as a product. g5Rp-decapping activity was inhibited by uncapped RNA but not methylated cap analogue derivatives, suggesting that g5Rp recognizes the RNA moiety to find target substrates.

Results

Recombinant ASFV g5Rp decaps mRNA

Although it was previously shown that ASFV g5Rp cannot cleave a free methylated cap structure, recent studies demonstrated that mRNA decapping mediated by Nudix enzymes is dependent on the methylated cap structure being tethered to an mRNA body (Wang et al., 2002; van Dijk et al., 2002; Piccirillo et al., 2003; Steiger et al., 2003; Cohen et al., 2005; Parrish et al., 2007; Parrish and Moss, 2007). To determine if the ASFV g5Rp can cleave a cap structure on an intact mRNA, a maltose binding protein (MBP)–g5R fusion protein containing a C-terminal His_{10} tag (MBP-g5R-HIS) was expressed in *Escherichia coli* and purified by affinity chromatography through amylose and nickel-nitrilotriacetic acid columns. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) analysis of the purified protein fractions revealed a single ~75-kDa band corresponding to the predicted mass of MBP-g5R-HIS (Fig. 1A).

Next, the recombinant g5Rp was incubated with a 309-nt ^{32}P-cap-labeled RNA substrate and the products of the reaction were resolved by polyethyleneimine (PEI)-cellulose thin layer chromatography (TLC) and detected by autoradiography. Unlabeled nucleotide standards were visualized by UV shadowing. In the absence of recombinant g5Rp, the ^{32}P-cap-labeled RNA substrate remained at the origin of the plate (Fig. 1B). However, inclusion of recombinant g5Rp in the decapping reaction resulted in the release of a product that co-migrated with an unlabeled m^7GDP standard (Fig. 1B). To confirm the identity of the released m^7GDP product, a portion of the decapping reaction was incubated with nucleoside diphosphate kinase (NDPK), an enzyme that specifically adds a phosphate group to nucleoside diphosphate substrates, thereby producing nucleoside triphosphate products. Following treatment with NDPK and resolution by PEI-cellulose TLC, the m^7GDP product shifted to co-migrate with the unlabeled m^7GTP standard, verifying that the product originally released by g5Rp was m^7GDP (Fig. 1B). The amount of product liberated by g5Rp cap cleavage increased with increasing enzyme concentration and incubation time (Figs. 2A and B, respectively).

The Nudix motif of ASFV g5Rp is required for mRNA decapping

The Nudix hydrolase motif consists of the highly conserved amino acid sequence GX_5EX_5[UA]$XREX_2EEXGU$ where U represents an aliphatic, hydrophobic residue and X represents any amino acid

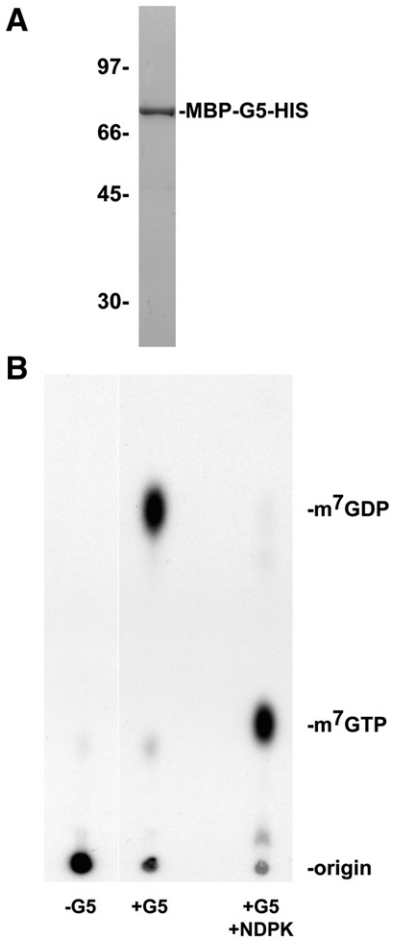

Fig. 1. Recombinant ASFV g5Rp catalyzes RNA cap cleavage. (A) ASFV g5Rp was expressed as an MBP-g5R fusion protein appended with a C-terminal His_{10} tag in *Escherichia coli* and then purified over amylose and nickel-nitrilotriacetic acid columns. Purified recombinant g5Rp was resolved by SDS/PAGE and detected by staining with Coomassie blue. Protein mass standards (in kDa) are labeled on the left whereas the ~75-kDa recombinant MBP-g5R-HIS band is indicated on the right. (B) 75 ng of recombinant ASFV g5Rp was incubated with 0.02 pmol of ^{32}P-cap-labeled actin RNA in decapping buffer for 30 min at 37 °C. A portion of this reaction was treated with 2 U of nucleoside diphosphate kinase (NDPK) and 1 mM ATP for an additional 30 min at 37 °C. NDPK adds a phosphate group exclusively to nucleoside diphosphates, resulting in the production of nucleoside triphosphates. Reaction products were resolved by PEI-cellulose TLC and detected by autoradiography. Unlabeled nucleotide standards were visualized by UV shadowing and are designated on the right.

(Koonin, 1993; Bessman et al., 1996). For several Nudix hydrolases, the glutamic acid residues in the EX_2EE sequence have been shown to be essential for catalytic activity, coordinating divalent cation binding and nucleophilic attack of the phosphate bond (reviewed in Mildvan et al., 2005). To demonstrate that recombinant g5Rp was solely responsible for cap cleavage in a manner dependent on the Nudix motif, mutations were introduced in the critical EX_2EE residues of this sequence. Specifically, one g5R mutant protein was synthesized in which the glutamic acid at residue 147 was converted into a glutamine (E147Q). A second g5R mutant protein was created in which the two glutamic acid residues at positions 150 and 151 were changed to glutamine residues (E150Q/E151Q). These two mutant proteins were expressed and purified concomitantly with wild-type recombinant g5Rp and resolved by SDS/PAGE (Fig. 3A). As expected, incubation of the ^{32}P-cap-labeled RNA substrate with wild-type g5Rp resulted in cap cleavage, as observed by m^7GDP release (Fig. 3B). When equivalent amounts of the two mutant versions of the g5Rp were