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7-Methylguanosine Diphosphate (m^7GDP) Is Not Hydrolyzed but Strongly Bound by Decapping Scavenger (DcpS) Enzymes and Potently Inhibits Their Activity

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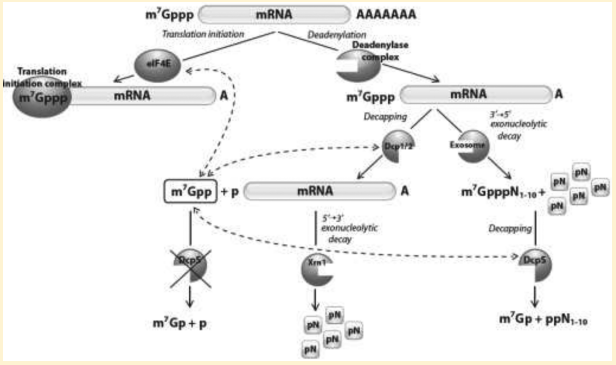
ABSTRACT: Decapping scavenger (DcpS) enzymes catalyze the cleavage of a residual cap structure following 3′ → 5′ mRNA decay. Some previous studies suggested that both m^7GpppG and m^7GDP were substrates for DcpS hydrolysis. Herein, we show that mononucleoside diphosphates, m^7GDP (7-methylguanosine diphosphate) and $m_3^{2,2,7}$GDP (2,2,7-trimethylguanosine diphosphate), resulting from mRNA decapping by the Dcp1/2 complex in the 5′ → 3′ mRNA decay, are not degraded by recombinant DcpS proteins (human, nematode, and yeast). Furthermore, whereas mononucleoside diphosphates (m^7GDP and $m_3^{2,2,7}$GDP) are not hydrolyzed by DcpS, mononucleoside triphosphates (m^7GTP and $m_3^{2,2,7}$GTP) are, demonstrating the importance of a triphosphate chain for DcpS hydrolytic activity. m^7GTP and $m_3^{2,2,7}$GTP are cleaved at a slower rate than their corresponding dinucleotides (m^7GpppG and $m_3^{2,2,7}$GpppG, respectively), indicating an involvement of the second nucleoside for efficient DcpS-mediated digestion. Although DcpS enzymes cannot hydrolyze m^7GDP, they have a high binding affinity for m^7GDP and m^7GDP potently inhibits DcpS hydrolysis of m^7GpppG, suggesting that m^7GDP may function as an efficient DcpS inhibitor. Our data have important implications for the regulatory role of m^7GDP in mRNA metabolic pathways due to its possible interactions with different cap-binding proteins, such as DcpS or eIF4E.



Degradation of mRNA plays a significant role in the post-transcriptional regulation of gene expression.[1] Understanding the control of gene expression requires defining the molecular and cellular basis of mRNA turnover. There are two major pathways that are utilized for degradation of mRNA in eukaryotic cells, both initiated by the removal of the poly(A) tail.[2] In the 5′ → 3′ decay pathway, the cap is hydrolyzed, exposing the mRNA 5′ end to exoribonuclease activity.[3] In the 3′ → 5′ decay pathway, exosome-mediated degradation of mRNA releases a cap containing dinucleotide or a short capped oligonucleotide.[4] Decapping plays an important role in mRNA turnover, and decapping rates can differ significantly for different mRNAs.[5]

Eukaryotic cells contain different classes of enzymes responsible for the cleavage of mono- and trimethylated cap structures. Dcp2 is an RNA decapping enzyme generating m^7GDP from an mRNA chain and enables 5′ → 3′ mRNA decay. In the 3′ → 5′ decay of mRNAs, 5′ capped dinucleotides or short capped oligonucleotides result from the mRNA degradation. The DcpS (decapping scavenger) enzyme acts on them, releasing m^7GMP. DcpS can only hydrolyze the cap on mRNA oligonucleotides up to 10 nucleotides in mammals and 3 nucleotides in nematodes.[6−8] Nematode mRNAs have either monomethylguanosine (MMG) or trimethylguanosine (TMG) caps, and DcpS from *C. elegans* and *A. suum* recognize both caps, whereas human DcpS can degrade only the monomethylated cap structure.[7,8]

A detailed characterization of the DcpS binding affinity and catalytic properties in different eukaryotic organisms, as well as the knowledge of its biological functions in cellular processes, is essential for the design and synthesis of novel cap analogues and improvement of their therapeutic properties. Infections of humans and animals caused by parasitic nematodes are great medical and economic problems (~3 billion people infected),[9] and there is a need for drugs that will specifically block parasite gene expression, without influencing the metabolism of mammalian cells. Nematode DcpS has the unique ability to efficiently hydrolyze the TMG cap whereas the human enzyme cannot.[8]

An important therapeutic aspect of DcpS studies is the regulation of the enzyme activity in human cells in spinal

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muscular atrophy (SMA).[10] Biochemical and structural data indicate that DcpS enzyme could be a molecular target for SMA treatment. It has been demonstrated that the therapeutic effect in this disease might be based on the inhibition of this enzyme by C5-substituted quinazolines, which hold human DcpS in a catalytically inactive conformation.[10]

Several studies have suggested that DcpS in cell extracts (human, yeast, and *Xenopus*) and recombinant human and yeast decapping scavengers were able to efficiently hydrolyze m^7GDP, the product of Dcp2 activity.[11,12] In this context, DcpS proteins were proposed to function not only in the 3′ → 5′ mRNA decay pathway but also to be involved in hydrolyzing m^7GDP generated from cleavage of the capped mRNA in 5′ → 3′ decay pathway. In this scheme, m^7GMP would be the final product of both degradation pathways and a general indicator of eukaryotic mRNA degradation process. However, a detailed analysis of the function of DcpS enzymes in this pathway has not been carefully examined. Moreover, in independent experiments performed by Cohen et al., efficient hydrolysis of m^7GDP to m^7GMP was not observed, using either recombinant human and *C. elegans* nematode DcpS proteins or *A. suum* nematode whole-cell embryo extracts.[8] In *A. suum* extracts, a very small percentage of m^7GDP was converted to m^7GMP, but the activity responsible for this conversion was attributed to nonspecific enzymes, not to DcpS. Liu et al.[6] also reported the hydrolytic stability of m^7GDP in enzymatic assays with the human decapping scavenger. Thus, data regarding the role of DcpS in the hydrolysis of m^7GDP are contradictory. In this paper, we re-examined the hydrolytic activity of four recombinant DcpS enzymes from multiple organisms: human (HsDcpS), *A. suum* nematode (AsDcpS), *C. elegans* nematode (CeDcpS), and *S. cerevisiae* yeast Dcs1 homodimer (ScDcpS), previously evaluated by different research groups.[6,8,11,12] We carried out enzymatic assays using conditions in which an efficient cleavage of m^7GDP was previously reported by van Dijk et al.[11] as well as conditions optimal for HsDcpS binding studies[13] using a highly sensitive high-performance liquid chromatography (HPLC) assay for monitoring the reaction progress and detection of degradation products. We show that recombinant DcpS enzymes have no ability to hydrolyze m^7GDP, regardless of their origin. Furthermore, our DcpS binding and inhibition studies indicate that m^7GDP is strongly bound by all DcpS enzymes and efficiently inhibits their hydrolytic activity toward m^7GpppG. We determined the association constants (K_{AS}) of m^7GDP and $m_3^{2,2,7}$GDP and also the inhibition parameters, the half-maximal inhibitory concentrations (IC_{50}), and the inhibition constants (K_i) of m^7GDP. Overall, the resistance of m^7GDP to DcpS-mediated hydrolysis and its strong binding and inhibitory properties toward decapping scavengers suggest that m^7GDP may play a regulatory role for cap-binding proteins associated with several mRNA pathways. We thus propose an amended model of mRNA degradation.

MATERIALS AND METHODS

Synthesis of Cap Analogues. Cap analogues investigated in this paper (m^7GMP, m^7GDP, m^7GTP, m^7GpppG, m^7Gpppm^{7}G, $m_3^{2,2,7}$GMP, $m_3^{2,2,7}$GDP, $m_3^{2,2,7}$GTP, $m_3^{2,2,7}$GpppG, GpppG) were synthesized according to previously described methods.[14−16] The concentrations of compounds were determined on the basis of their absorption coefficients (ε_{max}) determined in 0.1 M phosphate buffer: ε_{258}(m^7GMP, m^7GDP, m^7GTP, $m_3^{2,2,7}$GMP, $m_3^{2,2,7}$GDP, $m_3^{2,2,7}$GTP) = 11.4 × 10^3 M^{-1} cm^{-1} at pH 6.0, ε_{251}(GpppG) = 25.5 × 10^3 M^{-1} cm^{-1}, ε_{255}(m^7GpppG) = 22.6 × 10^3 M^{-1} cm^{-1}, ε_{259}(m^7Gpppm^{7}G) = 16.0 × 10^3 M^{-1} cm^{-1} at pH 7.0,[17] and ε_{258}($m_3^{2,2,7}$GpppG) = 26.3 × 10^3 M^{-1} cm^{-1} at pH 7.0.[18] Absorption spectra were recorded on Lambda 20 UV/vis spectrophotometer (PerkinElmer, Co., Waltham, MA) at 20 °C.

Protein Expression and Purification. HsDcpS, AsDcpS, CeDcpS, and ScDcpS were expressed in *Escherichia coli* according to the previously described procedures[19,20] and were purified as His-tagged proteins by affinity chromatography using Ni-NTA agarose under native conditions. To obtain homogeneous fractions of enzymes, all recombinant decapping scavengers were further purified by gel filtration at 4 °C through a Pharmacia Superdex-200 gel filtration column (GE Healthcare Bio-Science AB, Uppsala, Sweden), using an AKTA FPLC system (Pharmacia-Biotech, Uppsala, Sweden) and 20 mM Tris HCl buffer containing 50 mM KCl, 0.2 mM EDTA, 20% glycerol (final pH 7.6). The peaks corresponding to the respective decapping scavenger proteins were identified by comparison of their FPLC profiles with reference proteins (BSA, ovalbumin and lysozyme) and stored at −80 °C in 20 mM Tris HCl buffer containing 50 mM KCl, 0.2 mM EDTA, 20% glycerol (final pH 7.6), and 1 mM DTT at ∼50 μM. The concentration of decapping scavenger enzymes was estimated by the Bradford assay[21] and spectrophotometrically from the enzymes' molar absorption coefficient (ε_{280} = 30.4 × 10^3 M^{-1} cm^{-1} for HsDcpS, ε_{280} = 40.3 × 10^3 M^{-1} cm^{-1} for AsDcpS, ε_{280} = 38.9 × 10^3 M^{-1} cm^{-1} for CeDcpS, and ε_{280} = 62.8 × 10^3 M^{-1} cm^{-1} for ScDcpS) calculated from amino acid composition of a monomer using Clustal 2.1 algorithm on the ExPASy Server.

HPLC Analysis of Reaction Progress. The hydrolytic activity of recombinant DcpS was assayed using two experimental conditions: (1) 45 mM Tris HCl containing 9 mM MgAc and 27 mM $(NH_4)_2SO_4$ (final pH 8.0), at 37 °C; (2) 50 mM Tris HCl containing 200 mM KCl, 0.5 mM EDTA, and 1 mM DTT (final pH 7.6), at 20 °C. Initial substrate concentration for DcpS hydrolysis assays was 15 μM. Before each experiment, 1 mL of buffer solution containing the investigated cap analogue was incubated at 20 or 37 °C for 10 min. The hydrolysis process was started by the addition of recombinant DcpS (final concentration either 0.02 or 0.2 μM). At 5, 10, 30, 60 min, and after 24 h of the hydrolysis, 200 μL aliquots of the reaction mixture were withdrawn and the reaction was stopped by heat inactivation of the enzyme (2.5 min at 97 °C). The samples were then subjected to analytical HPLC (Agilent Technologies 1200 Series, Santa Clara, CA) using a reverse-phase Supelcosil LC-18-T column (4.6 mm × 250 mm, 5 μm) with UV/vis and fluorescence detectors. Substrate and products were eluted at room temperature with a linear gradient of methanol from 0% to 25% in aqueous 0.1 M KH_2PO_4 over 15 min at a flow rate of 1.3 mL/min. The fluorescence at 337 nm (excitation at 280 nm) and absorbance at 260 nm were monitored continuously during the analysis. Hydrolysis products were identified by comparison of their retention times with those of the reference standards.

Fluorescence Titration Analysis of Binding Affinity. DcpS-cap binding affinity was determined by monitoring the quenching of intrinsic DcpS Trp fluorescence. The time-synchronized-titration method (TST) was applied for this study.[22] The experiments were performed on LS-50B spectrofluorometer (Perkin-Elmer Co., Waltham, MA) in a quartz cuvette (Hellma, Müllhaim, Germany) with an optical

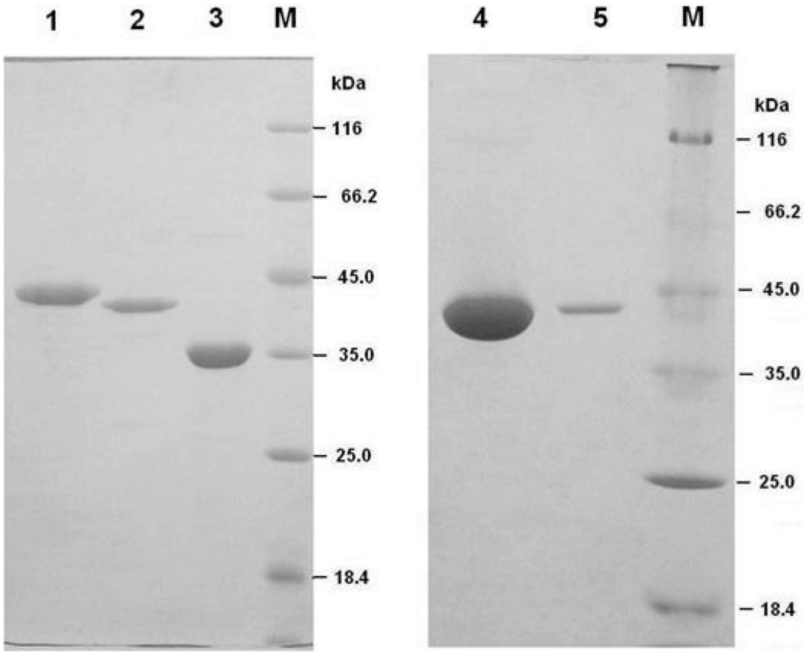

Figure 1. SDS-PAGE of purified recombinant human, nematode, and yeast DcpS proteins. His-tagged decapping scavengers were expressed in *E. coli* and purified using Ni-NTA agarose affinity and gel filtration chromatography. HsDcpS, AsDcpS, and CeDcpS are presented in lanes 1, 2, and 3, respectively. ScDcpS (*S. cerevisiae* Dcs1) is presented in lanes 4 and 5. Protein markers are in lane M. The amount of protein loaded on the gel is 5 μg of HsDcpS, 4 μg of AsDcpS, 7 μg of CeDcpS, and 25 and 2 μg of ScDcpS (lane 4 and 5, respectively). DcpS Coomassie Blue staining of 15% SDS-PAGE gels indicates a high purity of these recombinant proteins.

path length of 4 mm for absorption and 10 mm for emission. All measurements were performed as previously described for HsDcpS: 20 °C, in 50 mM Tris HCl buffer containing 200 mM KCl, 0.5 mM EDTA, and 1 mM DTT (final pH 7.6): experimental condition (2).[13] 1 μL aliquots of cap analogue solutions of increasing concentration (from 5 μM to 2 mM) were added to 1.4 mL of DcpS solution (initial concentration 0.2 μM). The fluorescence intensity was monitored at 340 nm with a 4 nm bandwidth (excitation at 280 nm with a 2.5 nm bandwidth) and corrected for sample dilution and inner filter effects.[23] The equilibrium association constants for single titrations (K_{AS}) were determined by fitting the theoretical dependence of the fluorescence intensity on the total concentration of the cap analogue to the experimental data points using the previously described equation.[22] The final K_{AS} were calculated as weighted averages of 3−5 independent titrations, with the weights taken as the reciprocal standard deviations squared. The numerical least-squares nonlinear regression analysis was performed using ORIGIN 8.0 (Microcal Software Inc.).

Determination of Inhibition Parameters for m^{7}GDP. The inhibitory properties of m^{7}GDP were examined on recombinant DcpS enzymes in decapping assays using m^{7}GpppG as the substrate. Experiments were performed in the binding affinity buffer (50 mM Tris HCl containing 200 mM KCl, 0.5 mM EDTA, and 1 mM DTT, final pH 7.6), at 20 °C: experimental condition (2). The inhibition of the rate of m^{7}GpppG (15 μM) hydrolysis in the presence of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, and 50 μM m^{7}GDP was monitored by HPLC. 1 mL of buffer solution containing the substrate and inhibitor was incubated at 20 °C for 10 min, and the reaction was initiated by the addition of recombinant DcpS. After 10 min, the reaction was stopped by heat inactivation of the enzyme at 97 °C for 2.5 min (200 μL aliquots of the reaction mixture were withdrawn and placed into an Eppendorf tube preincubated at 97 °C). Reaction products were analyzed by HPLC as described above, with a slightly different linear gradient of methanol (from 0% to 15% in aqueous 0.1 M KH_2PO_4 over 15 min). The concentration of DcpS (0.02 μM for CeDcpS, 0.02 μM for AsDcpS, 0.10 μM for ScDcpS, and 0.12 μM for HsDcpS) was optimized to obtain 50% substrate conversion within 10 min of hydrolysis without the inhibitor. IC_{50} values were determined by plotting substrate conversion versus inhibitor concentration (IC_{50} is defined as the concentration of inhibitor causing the decrease of substrate conversion by 50% under fixed enzyme concentration and different substrate concentrations). For calculation of K_i values we used the equation for tight binding competitive inhibitors:[24]

$$IC_{50} = K_i\left(1 + \frac{[S]}{K_m}\right) + \frac{[E]}{2}$$

where K_i is the inhibition constant, [S] is the substrate concentration, K_m is the Michaelis constant, and [E] is the enzyme concentration.

RESULTS

Resistance of m^{7}GDP and m$_3^{2,2,7}$GDP to Hydrolysis Catalyzed by DcpS Enzymes. Naturally occurring mononucleoside diphosphates, m^{7}GDP (all eukaryotes) and m$_3^{2,2,7}$GDP (nematodes), are products of Dcp2 decapping activity on mRNA. Previous reports described conflicting data on the ability of DcpS to convert m^{7}GDP to m^{7}GMP.[6,8,11,12] To re-evaluate whether scavenger decapping enzymes are able to efficiently hydrolyze m^{7}GDP, as well as to examine m$_3^{2,2,7}$GDP susceptibility to DcpS digestion, highly purified human, nematode (*A. suum* and *C. elegans*), and yeast (*S. cerevisiae* Dcs1) recombinant DcpS proteins (Figure 1) were

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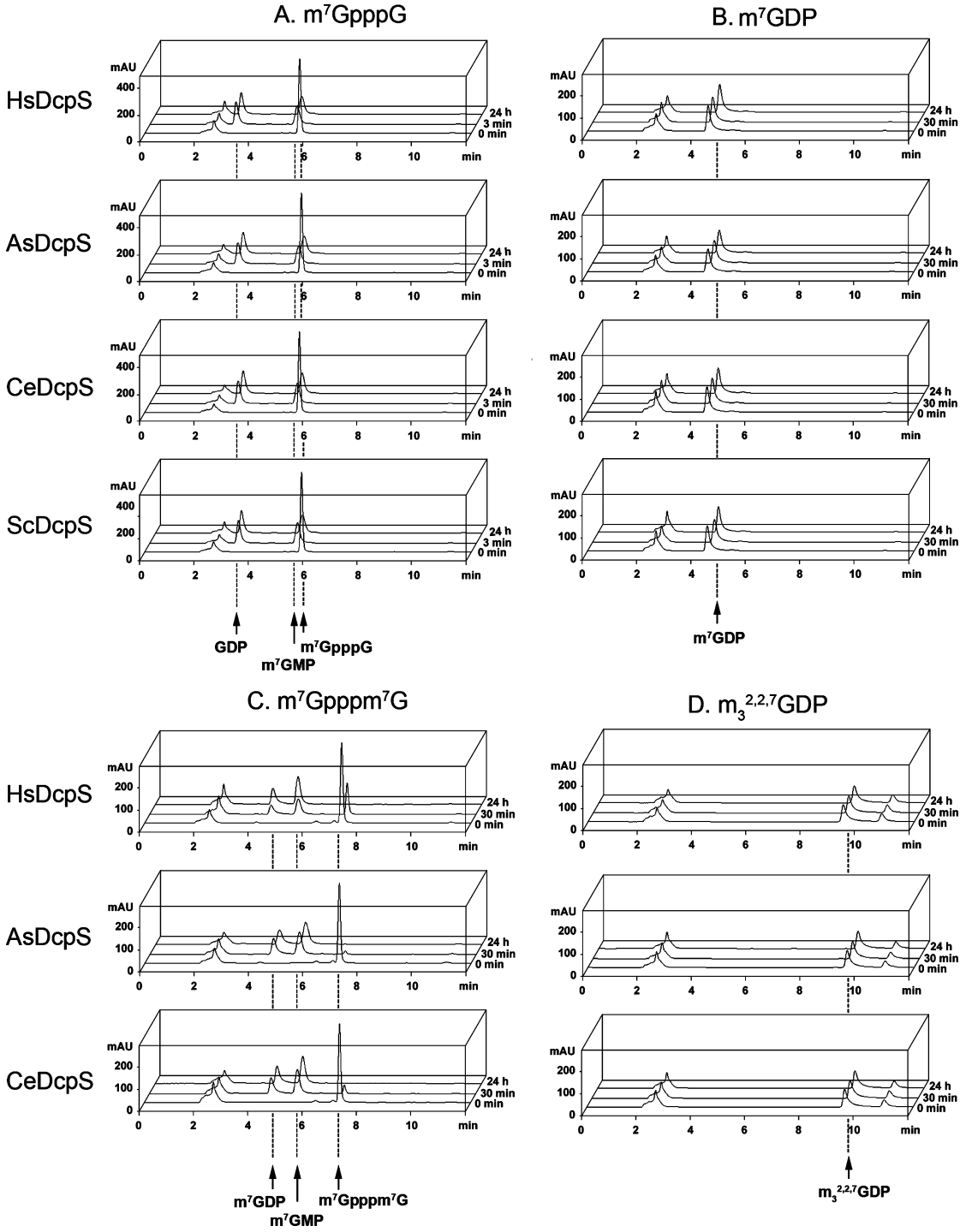

Figure 2. HPLC analysis of hydrolytic susceptibility of m^7GpppG, m^7Gpppm^7G, 7-methylguanosine diphosphate (m^7GDP), and 2,2,7-trimethylguanosine diphosphate ($m_3^{2,2,7}GDP$) to recombinant DcpS. All experiments were performed using condition set (2): 20 °C in 50 mM Tris HCl buffer containing 200 mM KCl, 0.5 mM EDTA, and 1 mM DTT (final pH 7.6) with 0.2 μM DcpS. Initial concentrations of cap analogues were 15 μM. The HPLC peak with a retention time of 2.5 min corresponds to the reaction buffer. (A) DcpS enzymes efficiently hydrolyze m^7GpppG. Complete conversion of the substrate to m^7GMP and GDP products occurs within less than 3 min. (B) and (D) m^7GDP and $m_3^{2,2,7}GDP$, respectively, are not hydrolyzed following 24 h of incubation with DcpS. The HPLC peak with a retention time of 10.8 min in the $m_3^{2,2,7}GDP$ profile corresponds to a substrate contaminant. (C) Hydrolysis of m^7Gpppm^7G is significantly slower than that of m^7GpppG. After 30 min, a small amount of the substrate is still observed in all cases. Chromatographic peaks corresponding to the products, m^7GDP and m^7GMP, retain their intensity after the complete degradation of m^7Gpppm^7G (24 h of investigation), confirming the resistance of m^7GDP to enzymatic cleavage.

examined using two assay conditions: (1) those described by van Dijk et al.[11] for investigation of m^7GDP hydrolysis by DcpS proteins (37 °C, 45 mM Tris HCl containing 9 mM MgAc and 27 mM $(NH_4)_2SO_4$, final pH 8.0) and (2) those proposed by Darzynkiewicz et al.[13] for analysis of human DcpS binding affinity (20 °C, 50 mM Tris HCl containing 200 mM KCl, 0.5 mM EDTA, and 1 mM DTT, final pH 7.6). The first condition was used to replicate van Dijk's experiments, and the second

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one was developed to assay DcpS hydrolytic and binding characterization. In both cases, two concentrations of DcpS were examined, 0.02 and 0.2 μM. The lower concentration was optimal for evaluation of cap analogue degradation during 60 min as described by van Dijk et al.[11] The 10-fold higher enzyme concentration was used to examine the hydrolytic stability of mononucleoside diphosphates toward DcpS. The hydrolytic activity of the decapping scavengers for the m^{7}GpppG cap analogue was also examined.

The rate of hydrolysis of m^{7}GpppG for all the DcpS proteins was very fast at 37 °C under assay condition (1), with the reaction products, m^{7}GMP and GDP, appearing immediately following enzyme addition. In contrast, m^{7}GDP and m$_3^{2,2,7}$GDP were not degraded by the DcpS proteins, as indicated from the HPLC analysis, regardless of the enzyme concentration or incubation time (up to 24 h).

As illustrated in Figure 2, using the alternate assay condition (2), m^{7}GpppG was very efficiently converted to products, within less than 3 min, whereas both mononucleoside diphosphates, m^{7}GDP and m$_3^{2,2,7}$GDP, were resistant to cleavage by all the DcpS enzymes. As shown in Figure 2B,D, the intensity of chromatographic peaks corresponding to the input amounts of m^{7}GDP and m$_3^{2,2,7}$GDP remains unchanged even after long incubation period (24 h).

Taken together, none of the four DcpS enzymes examined, from human, *S. cerevisiae*, *A. suum*, and *C. elegans*, hydrolyzes m^{7}GDP or m$_3^{2,2,7}$GDP, even at high enzyme concentration (0.2 μM) or under different assay conditions. We conclude that the mononucleoside diphosphates, m^{7}GDP and m$_3^{2,2,7}$GDP, are not hydrolyzed by recombinant DcpS.

Stability of m^{7}GDP Confirmed by m^{7}Gpppm^{7}G Incubation with DcpS. To further confirm the stability of m^{7}GDP toward DcpS proteins, we additionally examined the hydrolysis of m^{7}Gpppm^{7}G. As shown in Figure 2C, hydrolysis of m^{7}Gpppm^{7}G by DcpS proteins resulted in the simultaneous appearance of only two reaction products, m^{7}GMP and m^{7}GDP, and the latter was not further converted to any products. The ratio of the chromatographic peak areas corresponding to reaction products (m^{7}GDP:m^{7}GMP) remains constant, even after a long time of incubation (24 h). This is a further demonstration that the mononucleoside diphosphate m^{7}GDP is not hydrolyzed by DcpS to m^{7}GMP.

Activity of Human and Nematode DcpS on m^{7}GpppG, m$_3^{2,2,7}$GpppG, and GpppG. Recombinant DcpS enzyme from *A. suum*, a parasitic nematode of pigs, has not been previously examined. We previously carried out some kinetic analysis of the hydrolysis of m^{7}GpppG and m$_3^{2,2,7}$GpppG by *C. elegans* DcpS.[20] Here, we performed a direct comparison of hydrolysis rates of AsDcpS with human and *C. elegans* DspS enzymes toward the two cap structures, both existing at the 5′ end of nematode mRNAs, as well as toward the unmethylated dinucleotide GpppG. Experiments were performed using assay condition (2), which facilitated simultaneous characterization of DcpS-cap binding affinity and hydrolysis (Figure 3). A low enzyme concentration (0.02 μM) was chosen to be able to analyze reaction progress at several time points with complete degradation of m^{7}GpppG within 60 min (Figure 3).

As presented in Figure 3, m^{7}GpppG hydrolysis occurs very efficiently and at similar rates for all three DcpS proteins, with 95% of the substrate hydrolyzed after 60 min. The comparison of the m$_3^{2,2,7}$GpppG hydrolysis catalyzed by human and nematode decapping scavengers demonstrated that *A. suum* and *C. elegans* DcpS readily hydrolyze a trimethylated

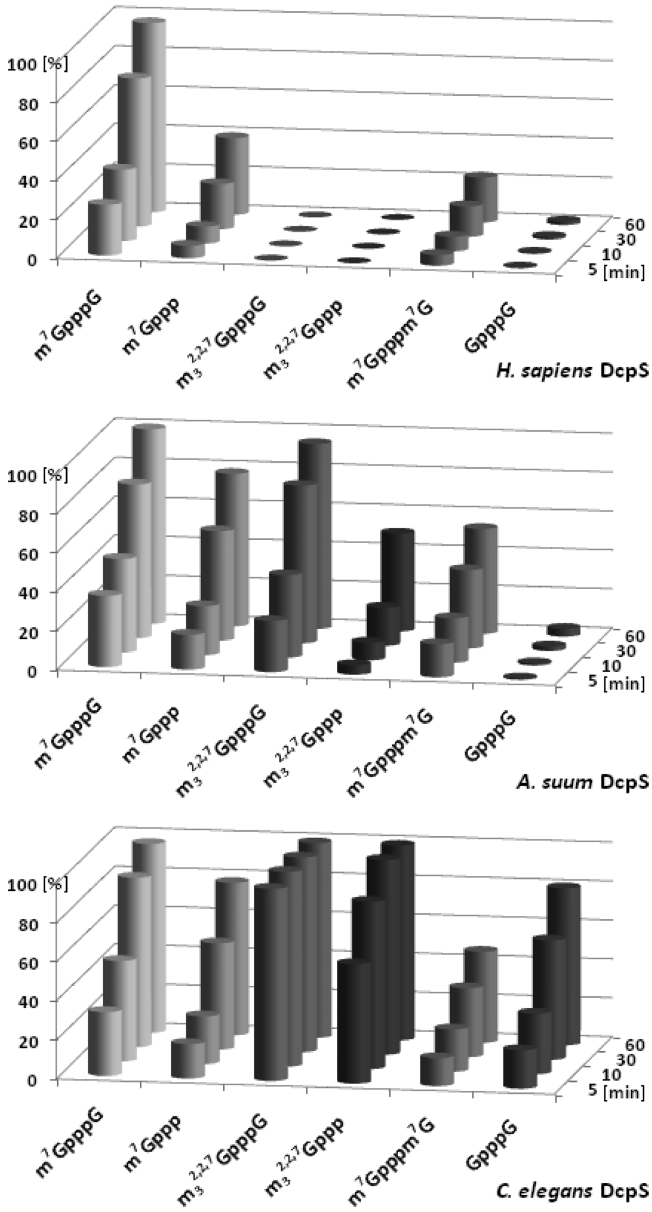

Figure 3. Comparison of the hydrolytic activity of HsDcpS, AsDcpS and CeDcpS. The extent of decapping (x) was determined as the % of hydrolyzed substrate measured by HPLC system. The standard deviation of the mean x value is below 5%. All experiments were performed at 20 °C in 50 mM Tris HCl buffer containing 200 mM KCl, 0.5 mM EDTA, and 1 mM DTT (final pH 7.6) with 0.02 μM DcpS. Initial concentrations of cap analogues were 15 μM.

dinucleotide, but the human enzyme does not (Figure 3). Digestion of m$_3^{2,2,7}$GpppG was not observed after 60 min or 24 h incubation with the human enzyme. The TMG cap is hydrolyzed by *C. elegans* DcpS at a significantly higher rate than that observed for m^{7}GpppG hydrolysis. In contrast, AsDcpS hydrolysis of MMG is slightly greater than that observed for m$_3^{2,2,7}$GpppG. This is the first demonstration of a difference in specificity for a trimethylated cap between nematode DcpS enzymes from different species. The hydrolysis of unmethylated dinucleotide GpppG is much slower as compared to m^{7}GpppG for CeDcpS and does not occur with either AsDcpS or HsDcpS.

Activity of Human and Nematode DcpS on m^{7}GTP and m$_3^{2,2,7}$GTP. Since m^{7}GDP and m$_3^{2,2,7}$GDP were found to

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be resistant to DcpS enzymatic hydrolysis, we next examined DcpS hydrolytic properties toward m^7GTP and $m_3^{2,2,7}GTP$ to gain insight into the contribution of either a second nucleoside or a length of the cap's phosphate chain on the activity of the DcpS enzymes. We found that 7-methylguanosine triphosphates are efficiently hydrolyzed by the human and nematode DcpS proteins, whereas only the nematode decapping scavengers have activity on the $m_3^{2,2,7}GTP$ (Figure 3). The rate of m^7GTP degradation is identical for AsDcpS and CeDcpS, but 2-fold slower for HsDcpS. A significant difference is observed for $m_3^{2,2,7}GTP$ hydrolysis by the two nematode decapping scavengers. $m_3^{2,2,7}GTP$ is a much better substrate for CeDcpS than for AsDcpS, as indicated by a higher hydrolysis rate (Figure 3). This difference is similar to the one observed for $m_3^{2,2,7}GpppG$, which confirms that AsDcpS and CeDcpS differ in their activity for the two additional CH_3 groups at N2 position of 7-methylguanosine.

The rate of mononucleoside triphosphates hydrolysis is slower in comparison with the respective dinucleotides (Figure 3), demonstrating the importance of the second nucleoside on the efficiency of the hydrolysis. We conclude that the second nucleoside is not absolutely required for DcpS activity; however, it enhances DcpS hydrolysis. Furthermore, direct comparison of enzymatic cleavage of mononucleoside di- and triphosphates illustrates the requirement of a triphosphate bridge for efficient hydrolysis by decapping scavengers.

Binding Affinity of Human and Nematode DcpS for Mononucleotide Cap Analogues. Our data demonstrate that m^7GDP and $m_3^{2,2,7}GDP$ are resistant to enzymatic cleavage by DcpS. We next examined whether DcpS proteins were able to bind these nucleotides. To determine the association constants (K_{AS}) for the complexes of DcpS with these mononucleotide cap analogues, we performed fluorescence titration assays (Figure 4). Assay condition (2) was previously determined and optimized to measure cap interaction with human DcpS using quenching of intrinsic Trp fluorescence upon ligand binding.[13]

To further determine the role of the phosphate groups in the interactions with DcpS proteins, we compared the binding affinities of m^7GDP and $m_3^{2,2,7}GDP$ with mononucleoside monophosphates, m^7GMP and $m_3^{2,2,7}GMP$ (Figure 4 and Table 1). We observed that all DcpS proteins have a high binding affinity for m^7GDP, and significant differences were observed between mono- and diphosphate mononucleosides. Human DcpS has a 6-fold higher affinity for m^7GDP (K_{AS} = 114 ± 8 μM^{-1}) compared to m^7GMP (K_{AS} = 19 ± 1 μM^{-1}).[13] An even larger difference between the binding affinities of these two mononucleotides is observed for CeDcpS, m^7GDP (K_{AS} = 97.04 ± 12.52 μM^{-1}) versus m^7GMP (K_{AS} = 8.00 ± 0.65 μM^{-1}). AsDcpS has a higher affinity for m^7GMP (K_{AS} = 26.35 ± 1.84 μM^{-1}) than HsDcpS and CeDcpS; however, it is 1 order of magnitude lower than for m^7GDP (K_{AS} = 185.13 ± 16.48 μM^{-1}). A significantly higher affinity for m^7GDP than for m^7GMP indicates the importance of the second phosphate group for cap recognition by DcpS proteins. The resistance of m^7GDP to enzymatic hydrolysis and its strong binding to human and nematode (*A. suum* and *C. elegans*) decapping scavengers suggested that m^7GDP might be a potential DcpS inhibitor.

Inhibitory Properties of m^7GDP in DcpS-Mediated Hydrolysis of m^7GpppG. To characterize the inhibitory potency of m^7GDP on DcpS proteins hydrolysis of m^7GpppG, IC_{50} values were determined. The results, 3.95 ± 0.23 μM for yeast DcpS, 2.17 ± 0.13 μM for *C. elegans*, 1.44 ± 0.11 μM for *A. suum*, and 4.17 ± 0.50 μM for human enzyme, indicate similar inhibitory properties of m^7GDP toward all the decapping scavengers. To calculate inhibition constants K_i from IC_{50}, we first determined the type of inhibition by comparing DcpS reaction velocities without inhibitor and in the presence of increasing inhibitor concentrations. The observed increasing K_m value without affecting V_{max} indicated m^7GDP is a competitive inhibitor of the decapping scavengers. Inhibition constants were calculated for CeDcpS and ScDcpS using the IC_{50} values determined here and K_m values obtained in the previous studies (1.17 ± 0.14 and 0.14 μM, respectively).[12,20] The inhibition constants (K_i = 0.156 ± 0.020 μM for CeDcpS and 0.036 ± 0.002 μM for ScDcpS) demonstrate that m^7GDP is a strong inhibitor of these enzymes.

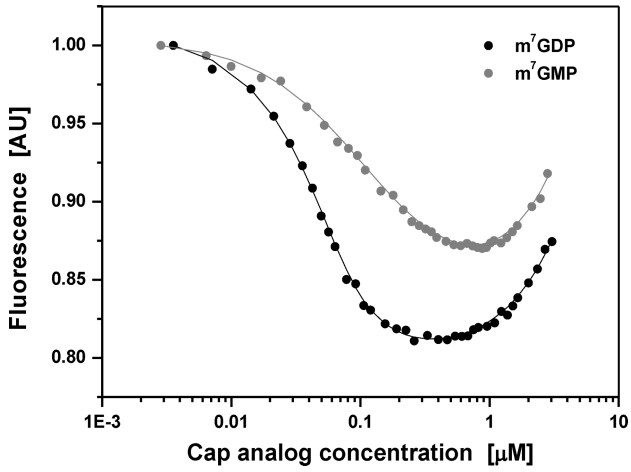

Figure 4. Fluorescence titration curves for binding of mononucleotide cap analogues to *C. elegans* DcpS. Titration experiments were carried out at 20 °C, in 50 mM Tris HCl containing 200 mM KCl, 0.5 mM EDTA, and 1 mM DTT (final pH 7.6). Protein fluorescence was excited at 280 nm and observed at 340 nm. The intensity of fluorescence is represented as relative values (AU: arbitrary units). The observed increasing fluorescence signal at the higher concentration of cap analogues originates from their emission in free forms present in solutions. The shift of the titration curve for m^7GMP toward the higher concentrations, compared to the curve for m^7GDP, indicates weaker binding of m^7GMP by *C. elegans* DcpS.

Table 1. Equilibrium Association Constants (K_{AS}) for the Complexes of Mononucleotide Cap Analogues with the Human and Nematode DcpS, Obtained from Analysis of Steady-State Fluorescence Titration at 20 °C, in 50 mM Tris HCl Buffer Containing 200 mM KCl, 0.5 mM EDTA, and 1 mM DTT (Final pH 7.6)

	K_{AS} [μM^{-1}]		
cap analogue	HsDcpS	AsDcpS	CeDcpS
m^7GMP	19 ± 1	26.35 ± 1.84	8.00 ± 0.65
m^7GDP	114 ± 8	185.13 ± 16.48	97.04 ± 12.52
$m_3^{2,2,7}GMP$	nd[a]	0.28 ± 0.27	2.88 ± 0.67
$m_3^{2,2,7}GDP$	nd	0.53 ± 0.16	1.44 ± 0.26

[a]nd = not determined.

DISCUSSION

Biological Consequences of m^7GDP Resistance toward DcpS Enzymes. DcpS-mediated hydrolysis of m^7GDP to m^7GMP described in previous studies led to the conclusion

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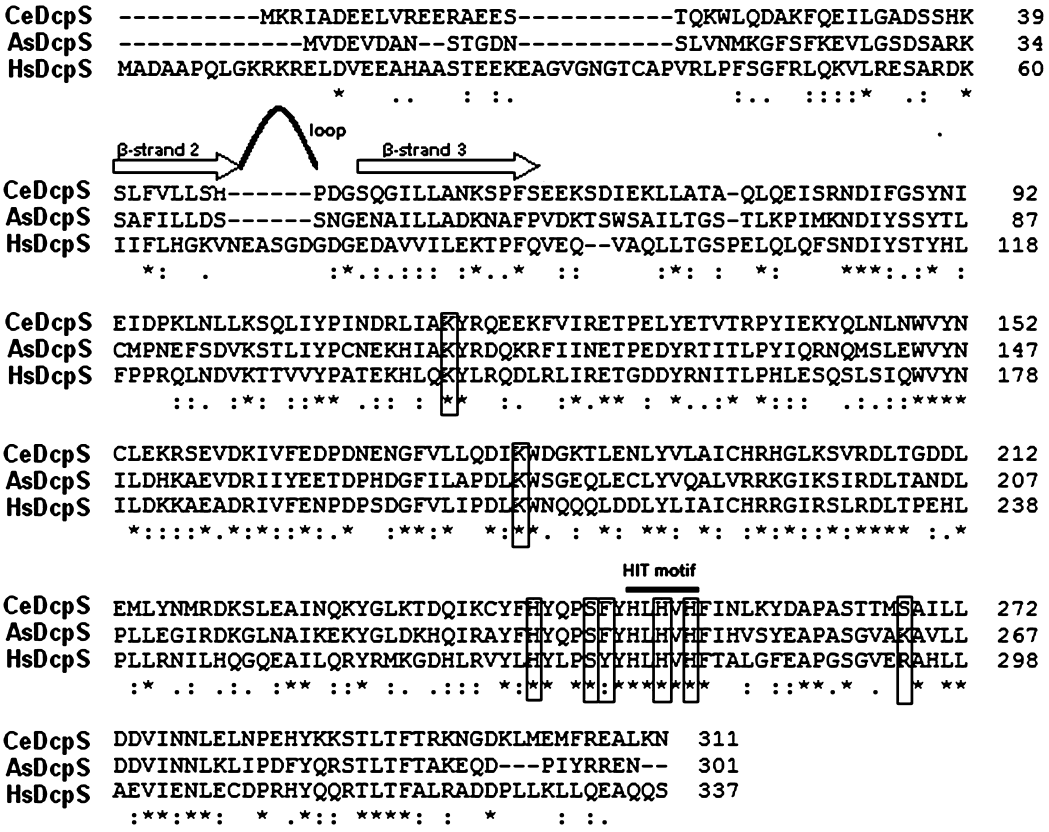

Figure 5. Amino acid alignment of human and nematode *A. suum* and *C. elegans* DcpS obtained using the Clustal 2.0.12 program illustrating conserved residues involved in the cap phosphate recognition and hydrolysis. The amino acids of each decapping scavenger are numbered on the right. Identical residues are indicated by stars below the lines. Conserved substitutions are indicated by (.) and semiconserved by (:). Gaps in the alignment are illustrated as (-). Decapping scavenger enzymes constitute their own branch within the superfamily of pyrophosphatases containing a highly conserved HIT motif (His-φ-His-φ-His-φ, where φ is a hydrophobic residue) utilized to cleave the substrate's phosphate chain. Hydrolysis is performed by a nucleophilic attack on the γ phosphate group and the cleavage of the $\beta-\gamma$ phosphate bond as proposed by Lima et al.[32] The DcpS amino acids involved in a binding of the cap's phosphate chain, according to the resolved structure of HsDcpS,[19] are given in frames. Most of the residues interacting with the triphosphate bridge in the human DcpS−m^{7}GpppG complex are strictly conserved in CeDcpS and AsDcpS. The two nonconserved amino acids are Tyr273 (replaced by Phe in both nematode enzymes) and Arg294 (replaced by Lys in *A. suum* DcpS and Ser in *C. elegans* DcpS). Note that mutation of Tyr273 to Phe in human DcpS results in a protein with 120% of the wild-type enzyme activity,[25] while mutation of Arg294 to Lys increases the activity to 114%,[19] which may explain the higher activity of nematode DcpS enzymes comparing to the human one. Corresponding protein sequence between β-strand 2 and β-strand 3 of HsDcpS[19] (strands are indicated as arrows above the alignment) is shortened by 6 amino acids in *C. elegans* and *A. suum* proteins. In the structure of human DcpS with m^{7}GpppG or m^{7}GpppA, this region forms an extended loop.[19] In the apo-form of human and mouse DcpS proteins, this loop region was not observed in the electron density map and thus was assumed to be disordered.[25]

that scavenger decapping enzymes play roles in both eukaryotic mRNA decay pathways, converting m^{7}GpppN to m^{7}GMP in the 3′ → 5′ pathway and m^{7}GDP to m^{7}GMP in the 5′ → 3′ pathway.[11,12] In contrast, the data from Cohen et al.[8] and Liu et al.[6] indicated that human and nematode DcpS enzymes are not able to hydrolyze m^{7}GDP to m^{7}GMP. Here, we re-examined these contradictory results through a careful analysis of m^{7}GDP hydrolysis using highly purified recombinant decapping scavengers from human, *A. suum*, *C. elegans*, and *S. cerevisiae* and a highly sensitive analytical HPLC assay to monitor reaction progress and hydrolysis products.

All these DcpS enzymes very efficiently hydrolyze their natural substrate, m^{7}GpppG (Figure 3). The m^{7}GDP hydrolysis tests were performed under the same assay conditions as previously chosen by van Dijk et al.[11] and also under conditions adopted for HsDcpS binding studies. Under both conditions, m^{7}GDP and its trimethylated derivative, m$_3^{2,2,7}$GDP, are resistant to DcpS hydrolysis, regardless of DcpS origin, as shown by HPLC analysis. Additional experiments using m^{7}Gpppm^{7}G (that is converted to m^{7}GDP and m^{7}GMP) further support that these scavenger decapping enzymes are not able to hydrolyze m^{7}GDP. On the basis of the analysis of the four DcpS enzymes from different organisms (mammals, nematodes, fungi), we suggest that this is likely a general feature of scavenger decapping enzymes. Therefore, we conclude that DcpS enzymes generate m^{7}GMP only from dinucleotide caps in the 3′ → 5′ decay pathway.

The reason for conflicting results obtained by various research groups with respect to the DcpS ability to hydrolyze m^{7}GDP (Liu et al.[6] and Cohen et al.[8] versus van Dijk et al.[11] and Malys and McCarthy[12]) has not been discussed until now. A definitive explanation for the differences observed is not possible, since some details in the description of experimental procedures are lacking in the previous studies. However, the most likely possible reasons for the differences observed in m^{7}GDP susceptibility to DcpS-mediated degradation include: (1) The concentrations of substrates are not described by van Dijk et al.[11] and Malys and McCarthy;[12] thus, the assays and

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kinetics of the hydrolysis in these cases might overestimate DcpS activity. (2) Our experiments are based on N-terminally His-tagged DcpS proteins compared to C-terminally His-tagged scavengers used by groups that observed the opposite results. Because of the C-terminal localization of the catalytic HIT motif, it is possible that the C-terminally tagged proteins display an altered scavenger decapping activity. (3) The purity of the protein samples from different preparations may not be the same. Gel filtration procedure applied in our studies is an additional control for the homogeneity of DcpS enzymes, not performed in the previously reported procedures.[6,8,11,12] Moreover, we tested in comparative studies four DcpS orthologs (human, *C. elegans*, *A. suum*, and *S. cerevisiae*) under a variety of conditions, including those used by the other groups, clearly observing m^7GDP resistance to DcpS-mediated hydrolysis, regardless of the protein source.

DcpS Enzymes Strongly Bind m^7GDP. Another important finding from the current study is the strong binding of human and nematode DcpS enzymes for m^7GDP. The association constants for the decapping scavengers are 97−185 μM^{-1}. Such high K_{AS} values lead us to conclude that m^7GDP may efficiently bind DcpS proteins and therefore competitively inhibit their hydrolytic activity. A sequence alignment of human and the two nematode DcpS enzymes (Figure 5) and the analysis of the crystallographic structure of HsDcpS−m^7GDP complex[25] suggest that the binding mode of m^7GDP is likely the same, with respect to the interactions of the two phosphate groups with the conserved amino acids of the HIT fold domain.

Remarkably, DcpS binding affinity for 7-methylguanosine diphosphate, m^7GDP, is higher than for its monophosphate counterpart, m^7GMP, as indicated by the association constants (Table 1 and Figure 4). Weaker interactions of m^7GMP with DcpS seem to be very important during the catalytic cycle, triggering the conformational changes of the enzyme that enable product release. In addition, our binding affinity results (Table 1) are in a good agreement with the previously reported differences for inhibition of recombinant *C. elegans* decapping scavenger with m^7GDP and m^7GMP. Cohen et al.[8] noted that competitor concentration for 50% inhibition of CeDcpS hydrolytic activity was about 20-fold higher for m^7GMP (2.5 μM) than for m^7GDP (0.12 μM).

Both trimethylated mononucleotides, $m_3^{2,2,7}$GMP and $m_3^{2,2,7}$GDP, are bound significantly weaker by the nematode decapping scavengers in comparison with their monomethylated counterparts. Binding affinity results (Table 1) are in a good agreement with the previously reported inhibition data for recombinant CeDcpS with mono- and trimethylated cap analogues.[26] K_i values for m^7GDP, m^7GTP, and m^7GpppG were in the range 2.2−3.5 μM, ∼10-fold lower as compared with K_i = 28 μM for $m_3^{2,2,7}$GpppG, indicating a much more efficient inhibition by monomethylated species. Comparison of binding affinity of human and nematode DcpS for monomethylated and trimethylated mononucleotides clearly demonstrates that m^7GDP could be an efficient inhibitor for DcpS.

m^7GDP as a Potent DcpS Inhibitor. To characterize quantitatively the inhibitory potency of m^7GDP, the IC_{50} values were determined. The results obtained for different DcpS enzymes (3.95 ± 0.23 μM for yeast, 2.17 ± 0.13 μM for *C. elegans*, 1.44 ± 0.11 μM for *A. suum*, and 4.17 ± 0.50 μM for human enzyme) indicated that m^7GDP efficiently inhibits the activity of decapping scavengers from different species and acts as a competitive inhibitor. The calculated K_i values (0.156 ± 0.020 for μM CeDcpS and 0.036 ± 0.002 μM for ScDcpS) clearly show the high inhibiting potency of m^7GDP toward decapping scavenger enzymes. Our results are consistent with the previous data indicating the inhibitory properties of m^7GDP and expand these observations to other species in addition to *C. elegans*.[8,26] Furthermore, the inhibition constant for m^7GDP presented by Kwasnicka et al.[26] was determined for *C. elegans* DcpS-mediated hydrolysis of a nonbiological m^7GpppBODIPY cap analogue. Our studies examined the more biologically relevant m^7GpppG substrate.

Comparative Characterization of Hydrolytic Activity of HsDcpS, AsDcpS, and CeDcpS Enzymes. Our experiments on the recombinant *A. suum* DcpS provide an insight into the similarities and differences of the DcpS enzyme from a parasite nematode versus DcpS protein from a potential host organism (HsDcpS) as well as versus DcpS protein from another nematode—*C. elegans* model organism (CeDcpS). Hydrolytic properties of human and nematode (*A. suum* and *C. elegans*) decapping scavengers were compared using non-, mono-, and trimethylated cap analogues, containing two or three phosphate groups (Figure 3). It has been shown previously that human DcpS is not able to hydrolyze TMG caps, in contrast to nematode decapping scavengers.[8] HsDcpS exhibits much higher substrate specificity than nematode decapping scavengers (Figure 3). It hydrolyzes only monomethylated cap analogues containing at least a triphosphate chain, with a preference toward dinucleotide m^7GpppG, which is cleaved with ∼2-fold higher efficiency than m^7GTP. In this study, we present for the first time a significant difference in monomethylated and trimethylated cap analogues specificity between nematode enzymes. *A. suum* DcpS hydrolyzes more efficiently m^7GpppG than $m_3^{2,2,7}$GpppG, in contrast to its *C. elegans* counterpart (Figure 3). These data are supported by activities of the two enzymes on mononucleoside triphosphates, m^7GTP and $m_3^{2,2,7}$GTP (Figure 3).

The previous kinetic studies of human and *C. elegans* DcpS indicated that the rate of the cap hydrolysis catalyzed by DcpS does not depend on the type of the second nucleoside.[20,27] The fact that the rate of m^7GpppG degradation compared to m^7GTP hydrolysis is only 2-fold greater for all the DcpS enzymes studied further confirms that the second nucleoside is not crucial for DcpS activity. The presence of the second nucleoside increases efficiency of the hydrolysis but is not absolutely necessary.

The DcpS enzyme from the parasitic *A. suum* nematode appears much more closely related to human DcpS than the enzyme from *C. elegans*. This is especially illustrated with respect to the substrate specificity for the N7 methyl group of a cap analogue, since GpppG is not a substrate for either HsDcpS or AsDcpS but undergoes some hydrolysis by CeDcpS. Our result is likewise reflected by the higher sequence identity of AsDcpS and HsDcpS (42%) than for CeDcpS comparing to HsDcpS (35%) (Figure 5).

The Role of a Cap's Phosphate Chain Length in the DcpS-Mediated Enzymatic Hydrolysis of Cap Analogues. Our results indicate the importance of the length of the cap's phosphate chain for DcpS binding and hydrolysis. Notably, the DcpS binding pocket does not require a triphosphate chain for cap analogue binding. From the determined K_{AS} constants, we conclude that 7-methylguanosine diphosphate and 7-methylguanosine monophosphate are recognized by different DcpS enzymes. However, the binding affinity is significantly higher for m^7GDP than for m^7GMP. From our studies, it is also clear that although DcpS enzymes

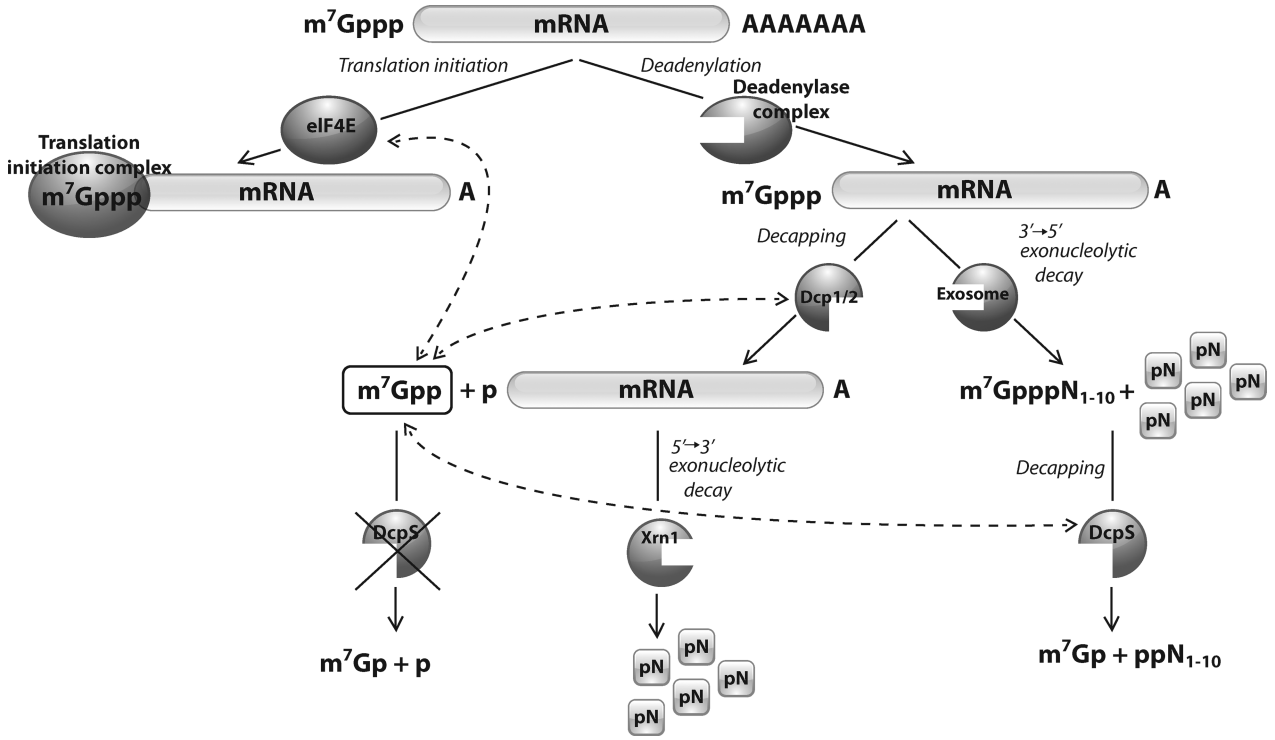

Figure 6. An amended model of mRNA degradation pathways. mRNAs in all eukaryotes possess the m^7GpppN cap at their 5′ ends. In nematodes, a significant portion of the mRNAs possesses a trimethylated cap ($m_3^{2,2,7}$GpppG). mRNA is degraded either at 5′ → 3′ or 3′ → 5′ pathway, both initiated by the deadenylation. The 5′ → 3′ pathway is initiated by Dcp2 pyrophosphatase cleavage of the 5′ mRNA cap. In the 3′ → 5′ pathway, mRNA is progressively cleaved by the exosome, resulting in residual short-capped oligonucleotides that are subsequently degraded by DcpS, producing m^7GMP. In the present study, we show that neither m^7GDP nor $m_3^{2,2,7}$GDP is a substrate for DcpS. However, m^7GDP may interact with cap-binding proteins, as indicated by the dashed lines with arrowheads. The high binding affinity and strong inhibitory properties of m^7GDP toward DcpS make this mononucleoside diphosphate a potential regulator of cap-dependent cellular processes. We suggest that m^7GDP may inhibit DcpS and consequently affect the function of other cap-binding proteins, such as Dcp2[33] or eIF4E,[34] through the increased levels of the free cap species.

have a high affinity for the two phosphate groups of m^7GDP, the diphosphate chain is not sufficient for mononucleotide cap hydrolysis. Consistently, DcpS does not hydrolyze m^7GDP but does degrade the triphosphate m^7GTP. An important role of the triphosphate bridge of the cap for efficient hydrolysis by DcpS is also reflected in the observation that most of the residues interacting with the phosphate groups in human DcpS−m^7GpppG complex (Lys142, Lys207, His268, Ser272, His277, and His279)[19] are strictly conserved in CeDcpS and AsDcpS enzymes (Figure 5), and many of them are crucial for efficient hydrolysis. The two conserved histidine residues of the HIT motif, His277 and His279, are essential for decapping activity. His277 is the nucleophile that attacks the cap's γ phosphate in the catalytic cycle whereas His279 and His268 make direct contacts to the γ phosphate of the substrate. His268Asn mutation and also Lys207Ala substitution (Lys207 is responsible for α phosphate binding) drastically decrease catalytic activity of human decapping scavenger (to 8% and 28%, respectively), whereas both His277Asn and His279Asn substitutions completely inactivate the DcpS enzyme.[19] Moreover, mutation of Tyr273 (interacting with cap's γ-phosphate) suggests that this amino acid also plays an important role in the catalytic cycle. Tyr273 undergoes significant conformational changes upon cap binding, contributing to the formation of the closed conformation, required for the hydrolytic activity.[25] Mutation of Tyr273 to Phe in human DcpS resulted in a protein with 120% of the wild-type enzyme activity, while substitution by Ala reduced the activity to 16%.[25] It was suggested that mutation of Tyr273 to Ala disrupts interactions important for the formation of the closed configuration of the DcpS active site. Mutation of Tyr273 to Phe has little effect on its interactions with residues in the closed state but enhances the interactions in the open conformation facilitating the product release. Interestingly, Tyr 273 is replaced by Phe in both nematode DcpS enzymes (Figure 5). The second nonconserved amino acid involved in binding of a triphosphate chain (β-phosphate) in the human DcpS−m^7GpppG complex, Arg294, is replaced by Lys in *A. suum* and Ser in *C. elegans* DcpS. Remarkably, the Arg294Lys substitution increases human DcpS activity (114%).[19] These few structural differences between nematode and human enzyme's cap triphosphate chain-binding pocket do not significantly change the substrate specificity of nematode decapping scavengers but may explain their higher hydrolytic activity comparing to human DcpS.

Degradation Pathways of mRNA. Efficient conversion of m^7GDP to m^7GMP has been previously demonstrated in yeast, *Xenopus*, and human cell extracts, and decapping scavengers were suggested as the enzymes catalyzing this reaction.[11,28] Furthermore, data have also been described suggesting that recombinant DcpS proteins are also able to hydrolyze m^7GDP.[11,12] These observations led to a model of eukaryotic mRNA degradation in which DcpS acts in both major mRNA decay pathways, generating m^7GMP from m^7GpppN in the 3′ → 5′pathway and from m^7GDP in the 5′ → 3′ pathway.[11]

Our data demonstrate that m^7GDP is resistant to DcpS hydrolysis, regardless of the DcpS origin. Moreover, m^7GDP was found to be an efficient competitive inhibitor of human, nematode, and yeast decapping scavengers. We therefore

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propose an update to the role of DcpS in mRNA degradation pathways. According to our model (Figure 6), m^7GDP, the decapping product of the 5′ → 3′ mRNA decay, is not hydrolyzed by DcpS enzymes. Decapping scavengers produce m^7GMP only from short capped oligonucleotides generated from 3′ → 5′ mRNA decay. As DcpS cannot hydrolyze m^7GDP, there must be another yet to be identified enzymatic activity to protect cells from accumulation of this mononucleotide. The alternate possibility of m^7GDP turnover could potentially be a phosphorylation of m^7GDP to m^7GTP (e.g., by nucleoside diphosphate kinase) and the subsequent DcpS-mediated hydrolysis of m^7GTP to m^7GDP, as it was shown herein.[29,30]

Although DcpS enzymes cannot hydrolyze m^7GDP, they have a high binding affinity for m^7GDP as indicated by the association constants described here (Table 1). The binding affinity of DcpS enzymes from different organisms for m^7GDP is very similar. We have also shown that the m^7GDP inhibitory effect on DcpS depends on the m^7GDP concentration and that m^7GDP is a competitive inhibitor of DcpS at the low micromolar level. The cellular concentration of m^7GDP remains to be determined. To quantify the absolute level of this mononucleotide in a cell, numerous measurements would need to be carried out under a variety of developmental, environmental, and genetic conditions. However, the overall levels of the extractable nucleotides may not provide insight into the local concentrations of the nucleotides in the context of proteins they may interact with. We note that current models of methylated nucleotide inhibition of eIF4E have been widely discussed and presented without discrete data on the nucleotide concentrations.[28,31] Our data suggest the potential importance of m^7GDP as an inhibitor of DcpS, that m^7GDP is not degraded by DcpS, and that this data and its implications alone provide new insight into mRNA turnover.

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Notes

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ABBREVIATIONS

A. suum, *Ascaris suum*; BSA, bovine serum albumin; *C. elegans*, *Caenorhabditis elegans*; DcpS, decapping scavenger; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; IC_{50}, concentration of inhibitor causing the decrease of substrate conversion of 50%; IPTG, isopropyl β-D-1-thiogalactopyranoside; m^7GDP, 7-methylguanosine diphosphate; $m_3^{2,2,7}$GDP, 2,2,7-trimethylguanosine diphosphate; MMG, monomethylguanosine; *S. cerevisiae*, *Saccharomyces cerevisiae*; SMA, spinal muscular atrophy; TMG, trimethylguanosine; TST, time synchronized titration.

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