Metal Determines Efficiency and Substrate Specificity of the Nuclear NUDIX Decapping Proteins X29 and H29K (Nudt16)*

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The Xenopus X29 protein was identified by its high affinity binding to U8 small nucleolar RNA, a small nucleolar RNA required for ribosome biogenesis. X29 and its human homologue H29K (Nudt16) are nuclear nucleoside diphosphatase proteins localized within foci in the nucleolus and nucleoplasm. These proteins can remove m7G and m227G caps from RNAs, rendering them substrates for 5’-3′ exonucleases for degradation in vivo. Here, a more complete characterization of these metal-dependent decapping proteins demonstrates that the metal identity determines both the efficiency of decapping and the RNA substrate specificity. In Mg2+ the proteins hydrolyze the 5’ cap from only one RNA substrate: U8 small nucleolar RNA. However, in the presence of Mn2+ or Co2+ all RNAs are substrates and the decapping efficiency is higher. The x-ray crystal structure of X29 facilitated structure-based mutagenesis. Mutation of single amino acids coordinating metal in the active site yielded mutant proteins confirming essential residues. In vitro assays with purified components are consistent with a lack of protein turnover, apparently due to an inability of the protein to release the decapped RNA, implicating critical in vivo interacting factors. Collectively, these studies indicate that the metal that binds the X29/H29K proteins in vivo may determine whether these decapping proteins function solely as a negative regulator of ribosome biogenesis or can decap a wider variety of nuclear-limited RNAs. With the potential broader RNA substrate specificity, X29/H29K may be the nuclear counterparts of the cytoplasmic decapping machinery, localized in specialized bodies involved in RNA decay.

Although DNA transcription is the first level of regulation of gene expression, more extensive modulation of RNA abundance is post-transcriptional. Various nuclear and cytoplasmic events act together to alter the steady-state balance of cellular RNAs, including RNA capping, RNA splicing/processing, nuclear export, localization within the cytoplasm, translation, and a surveillance mechanism to identify and eliminate aberrant RNAs (1–7).

Two fundamental pathways for RNA turnover are known, with degradation occurring from either the 5′-end or from the 3′-end of the RNA (reviewed in Refs 8–10). Turnover of messages from the 3′-end begins with deadenylation, followed by complete degradation of the RNA by the exosome complex, composed of several exonucleases (11–15). This 3′ to 5′ exonucleolytic activity leaves the 5′-cap structure (m7GpppG), which is degraded via DcpS, a “scavenging” nuclease in the histidine triad (HIT) family of enzymes. DcpS can cleave only free cap structures yielding m7GMP (16–21).

The alternative RNA turnover pathway involves 5′-3′ exonucleases, which degrade the RNA after the 5′-cap has been removed. The “decapping” activity is attributable to a nucleoside diphosphatase hydrolyase acting upon full-length RNA; they release m7GDP and leave a 5′-monophosphate on the 5′-end of the RNA. The decapping NUDIX3 proteins are members of a large evolutionarily conserved family that function as nucleotide diphosphatases acting upon various substrates (X); this motif was first described in MutT (22–24). Two distinct NUDIX proteins capable of cleaving the m7GpppG cap from intact RNA have been described. Dcp2 is cytoplasmic and X29 is a nuclear protein.

The existence of the pathway and mechanism for cytoplasmic RNA turnover was recognized when the catalytic decapping protein, Dcp2, was identified in yeast (19, 25–31). Identification and characterization of the mammalian counterparts of this RNA degradation machinery (15, 32–34) reinforced the importance of this pathway. In vitro, Dcp2 lacks substrate specificity, cleaving all capped RNAs more than 40 nucleotides in length. Cleavage results in accumulation of m7GDP and leaves a 5′-monophosphate on the RNA. In vivo this would be a substrate for 5′-3′ exonucleases (reviewed in Refs. 8 and 29). Decapping is poly(A)-dependent (requires a shortened poly(A) tail) and requires metal (Mg2+ or Mn2+) for hydrolysis. The authentic in vivo substrate, means of substrate recognition, or RNA specificity have not been identified for the cytoplasmic Dcp2 decapping protein, perhaps because there is no RNA substrate specificity.

Subcellular localization of Dcp2 has also provided clues about its role in vivo. Dcp2 is concentrated in cytoplasmic foci, called P-bodies (processing bodies) where it co-localizes with other known or putative RNA decay factors. RNAs destined for turnover are believed to be delivered to these foci for degradation (32, 35, 37–40).

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2 The abbreviations used are: NUDIX, nucleotide diphosphatases acting upon various substrates (X); snoRNA, small nucleolar RNA; DTSSP, 3,3′-dithiodisulfosuccinimidylpropionate; BS3, bis(sulfosuccinimidyl) suberate; EMSA, electrophoretic mobility shift assay; Bis-Tris, 2-(bis(2-hydroxyethyl) amino)-2-(hydroxymethyl)propane-1,3-diol.
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Our recent identification of X29, a nuclear-localized NUDIX protein with decapping activity, suggests that it is the nuclear counterpart to the cytoplasmic decapping enzyme. The Xenopus X29, and its human homologue (H29K), are concentrated within foci in the nucleoplasm and nucleoli (41). The crystal structure of X29 confirmed the presence of a NUDIX fold and demonstrated a homodimeric quaternary structure in this protein (42). It also revealed a requirement for a nucleotide or nucleoside in the recruitment of metals to the active site of X29 and suggested a basis for the observed cooperativity in metal binding to the enzyme.

Here we provide a more complete biochemical characterization of the decapping activity of the Xenopus and the human nuclear decapping proteins. In vitro assay conditions were optimized by altering salts, buffers, and both protein and RNA concentrations to obtain the most efficient decapping activity. Next, the three metals that were found to function as cofactors were titrated, and the enzyme activity and substrate specificity was examined for each metal:protein combination. Although only three metals would support cap hydrolysis by these nuclear NUDIX proteins, each metal had different optimal concentrations resulting in different cleavage efficiency. Most striking and unique are the findings that the metal identity determined both the substrate specificity as well as the efficiency of decapping. In Mg\(^{2+}\) cap hydrolysis is highly specific for U8 snoRNA; in Mn\(^{2+}\) there is broad RNA substrate specificity, and all RNAs are decapped at high efficiency. These data raise the intriguing possibility that the identity of the metal binding the protein in vivo would determine the RNA substrate specificity, and these NUDIX decapping proteins may be the nuclear counterpart of the cytoplasmic decapping machinery involved in RNA decay.

**EXPERIMENTAL PROCEDURES**

**Decapping Reaction**—RNA for the decapping assay was labeled as previously described (41). Unless a specific parameter was being tested (time, temperature, pH, metal, RNA, or protein titrations), the decapping reactions were assembled by combining 0.12 pmol of m\(^7\)GpppG cap-labeled RNA (24 nM) with 45 mM Tris, pH 8.5, and 1 μl of the appropriate metal (5× stock). The reaction volume was added last. Reactions were incubated at 37 °C for 30 min then stopped by addition of EDTA to a final concentration of 50 mM. EDTA, spotted onto a TLC plate, and developed in LiCl/formic acid as described below (41), and then quantitated as above.

**Time Course Experiments**—All time course experiments were performed in a single tube as a scaled-up (typically 3-fold) reaction. The tube was placed at 37 °C at time “0.” At the appropriate time point a 1.8-μl aliquot was removed and transferred to a container containing 0.2 μl of 500 mM EDTA to stop the reaction and stored at 4 °C until all time points were collected. Plates were spotted and developed as usual.

**Experiments involving addition of “more” reagents were performed in a single tube, typically as a 9× (45 μl) reaction. These reactions were initiated by addition of protein in a rate-limiting quantity and incubation at 37 °C (and defined as time 0); aliquots were removed, and the reactions were stopped at the indicated times as described above. After 10 min 30 μl of the reaction was transferred to a fresh tube at 37 °C containing additional protein (from 2- to 10-fold more protein in a volume of <1 μl), and time points were taken as described above. Ten minutes into this “more protein” incubation, 15 μl was transferred to a fresh tube containing additional RNA (from 2- to 10-fold more RNA in a volume of <0.5 μl). All samples were spotted onto a TLC plate, developed, imaged, and quantitated as above.

**Transcription Templates**—Templates for RNA transcription included U8 snoRNA (43), U3 snoRNA (44), U1 small nuclear RNA and U6 small nuclear RNA (45), NO38 mRNA (46), and 5S rRNA (47). Unlabeled, uncapped transcripts were cap-labeled as described (41) and gel purified for use in decapping reactions.

**Inhibition of Decapping by Product**—A standard decapping reaction was set up, except that prior to addition of protein, the putative inhibitor to be assayed was added to the reaction and mixed gently. Final concentrations of 0.1 mM or 2 mM of each putative inhibitor were used. Decapping reactions contained either 200 μM Mn\(^{2+}\), 1 mM Mn\(^{2+}\), or 2 mM Mg\(^{2+}\). Putative inhibitors examined included GMP, GDP, GTP, dGDP, m\(^7\)G, m\(^7\)GDP, and m\(^7\)GTP.

**Generating Mutations in X29 Protein**—The pET19b plasmid encoding X29 cDNA was mutagenized with QuikChange (Stratagene), as per the manufacturer’s recommendations, using the following pairs of deoxyoligonucleotides (provided below in 5’→3’ orientation): R88Ls, CTGGAAGAGGTCTTGAACTGGAGTTGGAAGAGGAACTGGGTCC; R88La, GGACCCAGTTCTCTTTCAACTCCAGTTTGCGACCTTCTTCCAG; R89Qs, CTGGAAGAGGTCTGAAAGGA-
GTGGAAGAGGAACTGGTGCC; E89Q, GGACCAGGTTTCTCTGTCCACGACCTTTCTCCAG; E92Q, GGTCTGAAACGGAGGTGGAAAGGACCTGGGGCTGCTGTCCACGATGAGCAGGAGAAGGGAACCCAGCTGCTGG; E93Q, CCAAGGTGGAGAGCAGGAGCAGGAGAAGGGAACCCAGCTGCTGG. 

Sample was temperature for 30 min. Cross-linkers were quenched with Protein and cross-linker were combined and incubated at room temperature. 

GTCCTGCTCTCGCACGGTGG; E93Q GCAGGACCCAGTTCCTGTTCCAACTCCCGTTTCAGACC; E93Qs, GGTCTGAAACGGAGGTGGAAAGGACCTGGGGCTGCTGTCCACGATGAGCAGGAGAAGGGAACCCAGCTGCTGG. The resulting constructs were verified by sequencing, and BL21 cells were used to overexpress proteins, which were purified via our now-standard methods. 

Optimization of Assay Conditions—Decapping reactions contain “cap labeled” RNA, generated by incubating in vitro transcribed RNA with guanylyltransferase, [α-32P]GTP and S-adenosylmethionine. This results in m7GpppG-RNA with a single radioactive phosphate in the α position (indicated in bold). Ammonium ions, frequently present in decapping assays (26, 28, 32, 49), resulted in decreased decapping efficiency of X29 and the human 29K protein and was omitted from these reactions. Reducing agents had no effect on decapping efficiency. We previously demonstrated that the optimum pH for decapping is near pH 9; all reactions here were performed at pH 8.5 to ensure sufficient buffering capacity of the reagents used. 

EMSA analysis demonstrated that, in the presence of 10-fold molar excess of protein over U8 snoRNA and 250-fold molar excess nonspecific (tRNA) competitor, at least 50% of the input U8 snoRNA was bound by X29 protein. The examination of the ratio of protein to RNA is addressed below. If tRNA was omitted, 100% of the RNA was bound by protein at this molar ratio (data not shown). To assay decapping, substrate (cap-labeled U8 snoRNA, 0.12 pmol) was combined with 1.2 pmol of X29 protein in the presence of metal cation and no tRNA. 

Time course of incubation experiments (below) were performed to ensure the decapping efficiency (defined as the fraction of total RNA substrate converted to product) was examined at a point when the reaction had gone to completion. Thus, for many of these assays, the kinetics were not being measured, rather, the overall efficiency of the reaction was assessed. RNA decapping efficiency was calculated as described under “Experimental Procedures.” 

Reaction Had Gone to Completion When Decapping Efficiency Was Assessed—Time course experiments were performed to determine the required incubation time for some of these experiments. Large, batch reactions containing cap-labeled U8 snoRNA, metal, and X29 protein were assembled. In some cases, lower than optimal amounts of metal were used to slow the reaction, ensure a more accurate determination of efficiency, and determine the minimum amount of metal required for catalysis. At time = 0 an aliquot was removed and EDTA was added. The remainder of the reaction was placed at 37 °C. At the appropriate time, aliquots were removed from the reaction and transferred to a tube containing EDTA to chelate the metal and stop the reaction. When the entire time course was collected, aliquots from each time point were spotted on TLC plates, developed, and quantitated to determine the amount of decapping at each time point. 

U8 snoRNA decapping was metal-dependent and protein concentration-dependent. The product accumulation increased linearly for the first ~10 min of incubation (data not shown, see Fig. 5, A and B). Additional incubation past this time point did not result in significant additional accumulation of m7GDP. Thus, the maximum amount of hydrolysis in the presence of limiting metal was achieved within the first 10 min of incubation. Longer incubations (30 min at 37 °C) were used in some cases to ensure the reaction went to completion. In addition to the longer time point, steady-state analyses to compare the overall efficiency of decapping, kinetic analyses were also per-
formed to address the rate of reaction under conditions where substrate, metal, or enzyme were limiting to more directly address enzyme activity in these reactions (see below).

**Metal Is Required for X29 Catalysis**—The cytoplasmic NUDIX decapping protein, Dcp2, requires metal for hydrolysis of cap from RNA. Mg$^{2+}$ will support cleavage, but Dcp2 has slightly higher efficiency in the presence of Mn$^{2+}$ (15, 26, 33, 34, 49). In contrast, the nuclear NUDIX decapping protein, X29, displayed metal-dependent hydrolysis of both the m$^7$G cap (releasing m$^7$GDP) and the m$^{2,2,7}$G cap (present on mature U8 snoRNPs in vivo) from U8 snoRNA (41). X29 protein can bind U8 snoRNA in the presence of 20 mM EDTA (determined by EMSA) (41), so metal is not required for the RNA binding; however, metal is required for either reorganization of the active site and/or for the chemistry of cap hydrolysis. Titration of metal and assessment of decapping efficiency examined the effect of metal upon catalysis.

To determine which metals are capable of conferring RNA cap-hydrolysis activity on X29 protein under these optimized reaction conditions, a panel of metals was surveyed in the decapping reaction of U8 snoRNA. Results indicated that magnesium, manganese, and cobalt allowed hydrolysis. Nickel, lithium, sodium, and calcium did not confer cleavage activity (data not shown). These ions were of particular interest, because they are present during protein purification in buffers, reagents, or assays, and it was important to determine whether we unintentionally “primed” or loaded the protein with metal by even transiently providing an ion capable of activating the protein. Purified protein incubated with cap-labeled RNA in the absence of added metal was inactive for hydrolysis indicating no metal cofactors co-purified with the protein.

**Mg$^{2+}$ Confers Cap Hydrolysis Activity and Specificity for U8 snoRNA**—The cleavage efficiency and substrate preference of the *Xenopus* and human nuclear decapping proteins were examined in the presence of Mg$^{2+}$. Metal was titrated into otherwise identical reactions, incubated, and spotted on TLC plates, as seen in Fig. 1A. Quantitation to assess the efficiency of decapping demonstrated that the *Xenopus* protein required a minimum of 0.5 mM Mg$^{2+}$ to achieve any detectable decapping of U8 snoRNA. Maximum decapping efficiency averaged 60% and occurred in the presence of 2 mM Mg$^{2+}$. Fig. 1B plots the average decapping efficiency for X29 across the Mg$^{2+}$ concentrations examined. At concentrations over 3 mM Mg$^{2+}$ there is clear and reproducible inhibition of decapping; in 5 mM Mg$^{2+}$ <30% of the input RNA is decapped. At this point it is not known whether this is due to direct inhibition of the protein by Mg$^{2+}$ or is attributable to metal effects on the RNA, which may alter its ability to interact with the protein.

These reactions were performed under these conditions to quantitate and compare relative decapping efficiency. It should be noted that, although there is a 10-fold difference in the molar ratio of protein to RNA, under the conditions correlated with metal identity, the activity of the protein is limiting (see below). The protein was present in 10-fold molar excess (not all of which may be catalytically active) but only about half of the cap-labeled U8 RNA is converted to product in the presence of Mg$^{2+}$. No other cap-labeled RNAs served as substrate (data not shown) demonstrating the *Xenopus* protein is highly specific for decapping U8 snoRNA in the presence of Mg$^{2+}$.

The human H29K protein could cleave U8 snoRNA in the presence of Mg$^{2+}$, but did so at lower efficiency in these protein:RNA ratios (maximum 20% in 2 mM Mg$^{2+}$) (data not shown). These results demonstrate that metal is required for decapping of the U8 snoRNA substrate, but that the ability to cleave is dependent on metal identity.

*FIGURE 1.* Determination of optimal Mg$^{2+}$ concentrations for cap hydrolysis of U8 snoRNA by X29 protein. A, autoradiograph of a TLC showing the titration of increasing concentrations of Mg$^{2+}$ into a decapping reaction containing cap-labeled U8 snoRNA and X29 protein. The migration of known standards is shown on the top. The efficiency of cap hydrolysis was quantitated, and the data from this experiment are shown on the right. The efficiency of cap hydrolysis was quantitated, and the data from this experiment are shown on the right. The arrows indicate the peak of decapping efficiency. B, graph plotting the efficiency of cap hydrolysis (percentage of input RNA that is decapped) in the various concentrations of Mg$^{2+}$, determined in A. The highest level of hydrolysis of U8 snoRNA by X29 protein occurs at 2 mM Mg$^{2+}$; higher concentrations decreased the efficiency of hydrolysis.
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TABLE 1
Decapping efficiency of X29 and H29K proteins

| Metal  | RNA          | X29 (WT) | H29K |
|--------|--------------|----------|------|
| Mg<sup>2+</sup> | U8 | 60% (2 mM) | 20% (2 mM) |
| Mn<sup>2+</sup> | U8 | <2% (2 mM) | 0% (2 mM) |
| Co<sup>2+</sup> | U8 | 95% (0.1 mM) | 80% (0.25 mM) |
|        | U3 | 80% (0.15 mM) | 70% (0.3 mM) |
|        | U3 | 70% (0.15 mM) | 50% (0.3 mM) |

shown, see Table 1). Addition of more protein resulted in more cleavage, but the overall efficiency of U8 snoRNA cap hydrolysis was significantly lower than that for the Xenopus protein. Like X29, in the presence of Mg<sup>2+</sup> the human protein would decap only U8 snoRNA; no other RNAs were substrates (see Table 1).

Thus, Mg<sup>2+</sup> could fulfill the metal requirement for hydrolysis by both the human and Xenopus NUDIX decapping proteins. Cap hydrolysis showed high substrate specificity with only U8 snoRNA recognized as a substrate in 2 mM Mg<sup>2+</sup>. One Metal Increases Cleavage Efficiency and Broadens Substrate Specificity—To examine the effects of metal upon efficiency and specificity of cap hydrolysis, the amount of Mn<sup>2+</sup> or Co<sup>2+</sup> was varied in the reactions. Initial experiments indicated that the decapping proteins displayed relaxed substrate specificity in the presence of these metals.

Variation of Mn<sup>2+</sup> concentration in reactions containing Xenopus X29K protein with U8 snoRNA (Fig. 2A) and other RNA substrates showed that >90% decapping of RNA was achieved in 0.1 mM Mn<sup>2+</sup> at the same RNA:protein ratios used for Mg<sup>2+</sup>. In Mn<sup>2+</sup>, all RNAs were substrates for hydrolysis (Table 1). The human H29K protein demonstrated a significantly higher decapping efficiency in Mn<sup>2+</sup> than in Mg<sup>2+</sup>; up to 80% of the U8 snoRNA was decapped in 0.25 mM Mn<sup>2+</sup> (Fig. 2B). The human protein also displayed broader substrate specificity in Mn<sup>2+</sup>; all RNAs were substrates for hydrolysis (Table 1), including U3 snoRNA (a nucleolar ribonucleoprotein particle), NO38 mRNA, and 5S rRNA (data not shown, summarized in Table 1). The 5S rRNA does not have an m<sup>7</sup>G cap in vivo but is a small structured RNA, demonstrating either a complete lack of substrate discrimination in the presence of Mn<sup>2+</sup> or an increased affinity or recognition of the cap structure (rather than the RNA itself). This scenario may be comparable to the cytoplasmic Dcp2 decapping enzyme where all RNAs are substrates for decapping, including artificial polylinker (12, 34, 49). Although a variety of RNA substrates were examined and found to be decapped by H29K and X29 proteins in Mn<sup>2+</sup>, only U3 snoRNA is completely characterized here with metal titrations to determine the optimal decapping efficiency by both proteins in the presence of Mn<sup>2+</sup> (Table 1).

Both the Xenopus and human proteins decapped U8 snoRNA in the presence of Co<sup>2+</sup>. However, slightly higher concentrations of Co<sup>2+</sup> (relative to Mn<sup>2+</sup>) were required for which slightly lower overall decapping efficiencies were observed (Fig. 2C and Table 1). In the standard decapping reaction with X29, 0.15 mM Co<sup>2+</sup> yields >80% hydrolysis of cap from the U8 snoRNA present in the reaction; decapping efficiency was monitored by quantifying the m<sup>7</sup>GDP accumulated. Addition of 0.3 mM Co<sup>2+</sup> to a reaction containing the human H29K protein results in hydrolysis of 70% of the U8 snoRNA in the reaction and 50% decapping of U3 snoRNA. Although the cap hydrolysis supported by cobalt is not as efficient as Mn<sup>2+</sup>, the overall decapping efficiency is higher than that seen in Mg<sup>2+</sup>.

In the presence of Co<sup>2+</sup> and Mn<sup>2+</sup>, both the Xenopus and human proteins demonstrate broad RNA substrate specificity and decap a wide range of RNAs. Mn<sup>2+</sup> supports that most efficient, decapping a higher percentage of the RNA present with the least amount of metal, followed closely by Co<sup>2+</sup> with Mg<sup>2+</sup> demonstrating the lowest efficiency of hydrolysis. Table 1 summarizes the averages of data collected for two RNA substrates and the three metals for both the human and Xenopus proteins. These results show that the metal not only affected the efficiency of hydrolysis but also altered the substrate specificity.

X29 Mutants Identify Amino Acids Involved in Cooperative Binding of Metal—The x-ray crystal structure of the X29 protein was obtained with no bound metal or nucleotide or nucleoside. Subsequently, complexes with nucleotide of nucleoside or cap analogue together with metal bound in the active site were determined (42). These data identified the amino acids that directly or indirectly coordinate metal ions and are therefore inferred to be essential for catalysis. The crystal structure of X29 indicated that the carboxylates of several glutamic acid residues within the active site (Glu-89 and Glu-93) and also outside (Glu-150) the NUDIX motif form cross bridges to the Mn<sup>2+</sup> ions, which are also coordinated to the nucleotide of nucleoside or cap analogue (42). Fig. 3A shows the primary sequence of X29 and Fig. 3B displays the structural arrangement of these amino acids within the active site, determined with metal and cap analogue soaked into the crystal (42). Although Glu-150 is neither proximal in the linear sequence nor part of the NUDIX motif; it appears to be pointed away from the active site in the apoprotein, is repositioned in the cap-and-metal bound structure, and is pointing into the active site. It was hypothesized from the crystal structure that Glu-150 is the first ligand that binds metal and that reorientation of its side chain is the switch that confers cooperativity on X29. It is not unusual to have general base catalysis by a glutamate outside the NUDIX motif (24) and Glu-150 may play this role in the X29-catalyzed reaction.

Site-directed mutagenesis was performed to alter single glutamic acid residues. Constructs encoding X29 mutant proteins denoted E89Q, E92Q, E93Q, and E150Q, as well as a double mutant E92Q,E93Q were generated. Sequencing the full-length construct after mutagenesis verified the targeted residues were the only changes induced. Mutant proteins were overexpressed in bacteria and purified as per the now-standard three-column protocol used to purify protein for crystallography (50); this ensured the proteins were >98% pure as validated by SDS-PAGE (data not shown). Fig. 3C shows an EMMSA demonstrating that all but one protein were competent in forming a U8 RNA-protein complex that co-migrated with the WT X29:U8 RNA complex. This indicated that the altered proteins folded
FIGURE 2. Determination of optimal metal concentrations for cap hydrolysis of U8 snoRNA by H29K and X29 proteins. A, autoradiograph of a TLC showing the titration of increasing concentrations of Mn$^{2+}$ into a decapping reaction containing cap labeled U8 snoRNA and X29 protein. The migration of known standards is shown on the right. B, graph plotting the efficiency of cap hydrolysis in the various concentrations of Mn$^{2+}$ for X29 protein and the H29K protein, as indicated. The efficiency of cleavage increases to 100% hydrolysis at $\sim$0.1 mM Mn$^{2+}$ for the Xenopus protein. Up to 0.3 mM Mn$^{2+}$ is required for efficient cap hydrolysis by the human protein. C, graph plotting the efficiency of cap hydrolysis in the various concentrations of Co$^{2+}$, for X29 protein and the H29K protein, as indicated. X29 protein reaches a maximum efficiency of cleavage at $\sim$0.15 mM Co$^{2+}$. The H29K protein requires higher levels of metal, with maximum hydrolysis efficiency occurring at 0.3 mM Co$^{2+}$. 
correctly and bound U8 snoRNA with high affinity. The E150Q mutant protein generated a complex that migrated slightly slower than the wild-type X29-U8 complex (Fig. 3C, lanes 8 and 9). One interpretation is that the glutamine at position 150 has an affinity for metal that is so much lower than that of glutamic acid that metal capture does not occur at the cap binding site. Because the metal plays a dominant role in binding the cap structure, its absence may leave the cap free and unconstrained, altering mobility of the complex. Other interactions between U8 snoRNA and the protein appear to be sufficient to stabilize the complex; U8 snoRNA lacking cap generates an EMSA gel shift. This further strengthens the hypothesis that U8 binding to X29 occurs through multiple recognition sites, and the cap binding is coordinated by amino acids through the metals.

The decapping efficiency of each of the mutants was quantitated in the presence of various concentrations of metal. The E89Q and E150Q mutants were inactive under all conditions assayed (summarized in Table 2). Mutant proteins E93Q and the E92Q,E93Q double mutation displayed <5% decapping activity in Mn<sup>2+</sup> under the optimized decapping conditions. A 10-fold increase in the amount of metal present in the reaction (to 1 mM Mn<sup>2+</sup>) only marginally increased the efficiency of cap hydrolysis; the decapping efficiency increased 2-fold but was still <15% of input RNA (see Table 2).

The E92Q mutant displayed weak decapping activity under the standard decapping conditions in both Mg<sup>2+</sup> and Mn<sup>2+</sup>, but a significant increase in hydrolysis was seen in the presence of higher concentrations of metal. In the crystal structure of the complex containing X29 plus cap, Glu-92 is coordinated to the Mn<sup>2+</sup> through a water molecule (42), although it is not certain whether this is the water facilitating hydrolysis. Under the standard assay conditions with 1.2 pmol of wild-type or E92Q mutant, higher concentrations of Mn<sup>2+</sup> increased the efficiency of hydrolysis by the E92Q protein in a metal concentration-dependent manner (Fig. 3D and Table 2). A change in the affinity of the E92Q mutant for Mn<sup>2+</sup> (relative to wild type) is consistent with charge transfer from Glu-92 to the water, which is greatly diminished by substitution of glutamine at this position. Wild-type X29 binds Mn<sup>2+</sup> cooperatively (42) with a Hill coefficient of 3, consistent with the crystal structure and a catalytic requirement for all three Mn<sup>2+</sup> ions. These data may reflect a loss of cooperativity or a shift of the Mn<sup>2+</sup> binding curve to the right for the E92Q mutant. Up to 75% of input RNA was cleaved in 1 mM Mn<sup>2+</sup> for the E92Q.

![FIGURE 3. Examination of proteins with mutations in the metal binding site of X29 protein. A, amino acid sequence of X29 protein showing the NUDIX sequence (underlined), and the amino acids at the positions that were changed to generate R88L, E89Q, E92Q, E93Q, E150Q, and the double mutant E92Q,E93Q. B, crystal structure showing the amino acids (in green, identity as labeled) that appear to coordinate binding of the metal (Mn<sup>2+</sup>, purple spheres) that was soaked into the crystal along with the cap analogue (in blue). C, mutant proteins indicated in A were overexpressed in bacteria and purified to near homogeneity. The proteins were each combined with internally labeled (uncapped) U8 snoRNA in the standard gel shift buffer, incubated, and then loaded onto a non-denaturing gel for analysis of complex formation via electrophoretic mobility shift assay. This autoradiograph shows free RNA and the RNA-protein complex, migrating as indicated. All proteins are capable of generating a shifted RNA-protein complex, indicating all are correctly folded and can bind the RNA with high affinity, similar to the wild-type protein. D, plot of the decapping efficiency of the wild-type X29 protein and two of the mutants, as a function of increasing concentration of Mn<sup>2+</sup>. These were the only two mutants that showed significant levels of hydrolysis activity. Increased hydrolysis activity was seen in the presence of higher metal concentrations.](https://www.jbc.org/article/S0021-9525(07)00480-6/abstract)
mutant, whereas very little hydrolysis was seen in the presence of 300 μM Co⁴⁺ and only 25% in 1 mM Co⁴⁺ (Table 2). Controls demonstrated that accumulation of m⁷GDP was protein-dependent.

These data are consistent with the inferences from the crystal structures that identified Glu-89, -92, -93, and -150 as inner or outer sphere metal ligands and thereby implicated them in catalysis. Variation in the efficiency and substrate specificity of decapping among the metals may reflect differences in coordination chemistry or in their ability to activate water to provide the specific base for the decapping reaction. In this complex reaction, it may be difficult to distinguish effects of metal identity on the catalytic reaction from influences on substrate RNA conformation. The broader substrate range and higher activity in the presence of different metals stresses the importance of identifying the metal that is bound to the nuclear decapping protein in vivo.

Table 2 shows the decapping efficiency of mutant X29 proteins. Cap-labeled U8 snoRNA was incubated with wild-type X29 protein or the mutant protein indicated, in the presence of various amounts of those metals indicated. The decapping efficiency for each combination under these conditions was determined by TLC and quantitated. Values represent the average of a minimum of at least three replicates using at least two independent preparations of RNA.

| Metal | WT X29 | E89Q | E92Q | E93Q | E92Q,E93Q | E150Q |
|-------|--------|------|------|------|------------|-------|
| Mg²⁺  | 60     | 0    | 10   | <1   | ND         | <1    |
| Mn²⁺  | 90     | <2   | 35   | 5    | 8          | <2    |
| Co²⁺  | 85     | ND   | 75   | 10   | ND         | <2    |

* ND, not determined.

### FIGURE 4. Titration of protein to address protein turnover in a decapping reaction.

Decapping reactions containing cap-labeled U8 snoRNA and metal (the identity and amount as indicated) were incubated with various amounts of the human or Xenopus 29K protein for 30 min. Reactions were spotted in TLC plates that were developed, exposed to a Fuji phosphor plate, and quantitated. Percent decapping is plotted as a function of protein concentration in the presence of the metal indicated.

60% of the RNA and 0.6 pmol will cleave 30% of the input RNA, addition of more protein under these conditions does not increase the decapping of input RNA even after these prolonged incubations.

There are several alternative, not mutually exclusive interpretations of these data. 1) The active fraction of protein is undergoing only a single round of decapping under the conditions used here; decapped U8 RNA may not be released after the reaction or the protein may not “reset” to be cleavage-competent. 2) There may be multiple rounds of enzyme turnover in Mn²⁺ but only a single round of hydrolysis in Mg²⁺. 3) The protein binds all the RNA present, but only a subset of the RNA-protein complexes are cleavage-competent, perhaps due to metal-induced (and metal-specific) structural changes in the protein or the RNA (or both) that enhance cap cleavage. The protein was crystallized as a dimer, but it is not yet known whether the monomer or the dimer is the catalytically active form of the protein. 4) Accumulation of product (m⁷GDP) may inhibit the cleavage reaction, perhaps in a metal-specific manner.

### Titration of Protein in Decapping Reaction Demonstrates Protein Is Limiting—To determine the rate of decapping under different protein concentrations, a time course was performed where parallel decapping reactions differed only by the amount of protein present. Protein was varied from 120 ng (2.4 pmol) down to 0.01 ng (0.2 fmol) per standard (5 μl) reaction containing 0.12 pmol of RNA. Aliquots removed at the appropriate points generated a time course of decapping for each protein concentration in either Mg²⁺ (Fig. 5A) or Mn²⁺ (Fig. 5B). TLC analysis and quantitation demonstrated that less protein results...
in less m^7GDP being released. For Mg\(^{2+}\), addition of more protein still did not result in 100% decapping of the RNA substrate.

Independent of metal identity, limiting amounts of protein resulted in lower rates of decapping. In all cases, the maximal rate of decapping occurred within the first 5–7 min of the reaction. After this time, the rate slowed but additional release of cap was detected through the 30-min incubation. Above a certain ratio of protein:RNA (>5-fold excess of protein), the initial
The rate of the reaction did not increase over the first 7 min, although there were slight differences in overall decapping efficiency at 30 min. In Mg\(^{2+}\), the maximal rate of decapping yielded 3 fmol of product per minute, whereas in Mn\(^{2+}\) it was calculated to be 5.2 fmol of product per minute given the amount of RNA substrate (0.12 pmol) in the reaction. These values were determined using the rate of product accumulation within the first 7 min of the reaction, because after that time the rate slows.

Although the end-point efficiency of the reaction appeared to be dependent upon the amount of protein present, the rate of the reaction did not vary significantly (as long as the protein was present in a 5- to 10-molar fold excess over RNA) (Fig. 5, A and B). Increasing the amount of substrate present in the reaction (by >10-fold) had an inhibitory effect upon the efficiency of decapping unless more protein was added (data not shown). Titration of RNA and protein levels present indicated that the absolute ratio of RNA:protein needed to be maintained (at a 10:1 excess of protein over RNA) for optimal decapping. This may be related to the presence of a lower affinity, nonspecific RNA binding previously described for this protein (48) resulting in non-productive aggregation.

Thus, while the numbers indicated the protein was present in molar excess, the decapping activity was limiting in the reaction. We do not know the precise activity of the protein (percentage of active molecules in the preparation) nor the stoichiometry of the protein-RNA interaction. However, it is possible the protein multimerizes and is capable of only one round of cleavage in vitro.

**X29 Protein Decaps as a "Single Turnover Reaction" in Vitro—** To examine whether the decapping protein was able to turn over in the reaction, a single, large scale reaction was assembled containing U8 snoRNA, X29 protein, and 150 mM Mn\(^{2+}\). Decapping was assayed over a time course, under conditions of limiting protein, as determined above. In the first stage or the reaction, the amount of protein present was that previously determined to result in decapping of a maximum of 30% of the RNA in the reaction (see Fig. 5B). After 10 min of incubation, an aliquot of the "first stage" reaction was removed and transferred to a tube containing more protein, comprising the "second stage." The additional protein was added in a small volume so as to not significantly alter concentrations of other components. Decapping of RNA was assessed in this more protein stage over a time course. Ten minutes into this more protein reaction, an aliquot was removed and transferred to a fresh tube containing additional RNA to generate the third stage. Again this was performed with minimal volume increase and aliquots taken for the time course. All samples were spotted on a TLC plate. After developing the TLC plate, the percentage of total RNA decapped was quantitated and plotted as a function of time (Fig. 5C).

The first stage of the reaction has limiting protein; decapping plateaus at 25% within 10 min and no additional decapping is evident, despite the remaining capped RNA present in the reaction. This RNA is not defective since addition of more protein (in the second stage of the reaction) does result in additional decapping and ~85% of the RNA is decapped. When more RNA is added (in the third stage), there is no additional decapping detected, although the protein is present in molar excess. If the protein was capable of turning over there should have been efficient decapping in the first and the third stages of the reaction. Similar data were obtained for the mammalian protein (data not shown) demonstrating that this inability of the protein to turnover is a characteristic of this protein in the three-component system conditions assayed in vitro under the conditions used here.

**Uncapped RNA Can Compete for U8 snoRNA Decapping by X29—** If the inability of the X29 protein to turn over was due to an inability to release the U8 snoRNA “product” of decapping then uncapped U8 snoRNA would be predicted to effectively compete for, and inhibit decapping of cap-labeled U8 snoRNA. To examine this, uncapped U8 snoRNA or U3 snoRNA was added to standard decapping reactions containing cap-labeled U8 or U3 snoRNA, and the effects upon decapping were monitored. Protein was always added last, prior to incubation for 15 min at 37 °C. The data, summarized in Table 3, demonstrate that uncapped U8 snoRNA effectively competed for the decapping of cap-labeled U8 snoRNA in the presence of Mg\(^{2+}\) or Mn\(^{2+}\). Uncapped U3 snoRNA is less effective but does compete with U8 for decapping by X29. Likewise, uncapped U8 snoRNA is very effective at competing decapping of U3 snoRNA and is more efficient than the ability of U3 to compete with itself. Collectively, these results support the earlier conclusions that there is a higher relative affinity for U8 than U3 snoRNA and that uncapped RNAs can bind X29 and inhibit the decapping of cap-labeled substrate (41).

Collectively, these experiments demonstrate that the nuclear decapping protein is active but does not turn over under the conditions used in this artificial, purified, three-component in vitro system (containing RNA, protein, and metal). There are at least two possible reasons for this. 1) There is product inhibition, either by the m\(^7\)GDP that is generated in the reaction or from the decapped RNA. The m\(^7\)GDP does migrate away from the decapping protein.

**Table 3**

| Cap-labeled RNA | Competitor | Molar excess | Decapped % |
|----------------|------------|--------------|------------|
| U8 (in Mg\(^{2+}\)) | U8         | None         | 58         |
|                 | 2×         | 67           |
|                 | 25×        | 35           |
| U3             | None       | 71           |
|                 | 2×         | 65           |
|                 | 25×        | 60           |
| U8 (in Mn\(^{2+}\)) | U8         | None         | 89         |
|                 | 2×         | 87           |
|                 | 25×        | 47           |
| U3             | None       | 89           |
|                 | 2×         | 87           |
|                 | 25×        | 80           |
| U3 (in Mn\(^{2+}\)) | U8         | None         | 79         |
|                 | 2×         | 55           |
|                 | 25×        | 6            |
| U3             | None       | 75           |
|                 | 2×         | 63           |
|                 | 25×        | 36           |
the origin in the TLC system arguing it is weakly bound. However, uncapped RNA does prevent X29-mediated decapping of cap-labeled substrate indicating the decapped RNA does bind tightly and has the potential to be a competitor in the decapping reaction. 2) There may be a higher multimerization of the protein in vitro affecting the stoichiometry of the complex and preventing product disassociation. In vivo, the association of other interacting proteins or components of a putative nuclear decapping complex (that would parallel the one characterized in the cytoplasm) may alleviate this situation. An examination of these points and the biological relevance of the in vitro data are discussed below.

**Does Accumulation of Product Inhibit the Decapping Reaction?**—Although these decapping enzymes are the catalytic component involved in removing m’GDP from capped RNAs, titration of protein and RNA indicated an absence of enzyme turnover (Fig. 5C). To address whether accumulation of released m’GDP product has the ability to inhibit decapping, reactions were performed in the presence of a variety of nucleotide of nucleosides and metal combinations. Decapping reactions contained buffer, 0.12 pmol of U8 snoRNA, metal (0.2 mM or 1 mM Mn\(^{2+}\) or 2 mM Mg\(^{2+}\)), and the nucleotide of nucleosides that were examined as putative inhibitors, added to a final concentration of 100 μM or 2 mM. X29 protein (2.5 pmol) was added last; the reaction incubated at 37 °C for 30 min and spotted on a TLC plate. Autoradiography of the developed TLC plate (Fig. 6) demonstrated that addition of GMP (data not shown) or m’G, even at the highest (2 mM) concentration, did not affect decapping. Normal decapping was seen with lower levels (0.1 mM) of m’GDP and m’GTP, but higher concentrations (2 mM) showed an effect on decapping (Fig. 6). In contrast, even the lowest levels of GDP, GTP, and dGDP completely inhibited decapping. The metal identity did not affect the results. Pyrophosphate did inhibit the decapping reaction (data not shown), quite likely through the formation of insoluble metal pyrophosphates that remove essential metal from the reaction mixture. Taken together, these data indicate that, although product inhibition by released m’GDP may occur, the amount of m’GDP released from RNA by X29 in these decapping reactions is well below the amount found to inhibit X29 decapping activity in the assay (Fig. 6). Protein is in excess, and EMSA indicated that, under identical conditions, all the U8 RNA present in these reactions is bound by protein (data not shown). The variable efficiency of decapping may reflect differences in the efficiency of RNA release or in the chemistry of the reaction, both of which may be affected by the metal present.

**Do the Nuclear Decapping Proteins Form Higher Order Multimers?**—The X29 crystal structure clearly demonstrated formation of X29 homodimers (42). The interface has a contact area of ~2500 Å\(^2\), mediated by multiple hydrogen bonds between the β-sheets that constitute this surface. The extensive, non-hydrophobic nature of the interaction, the apparent contribution of each subunit to cap recognition, and the implications for RNA binding increase the possibility that X29 acts as a dimer (42). Although the x-ray crystal structure shows homodimer formation, the crystallography conditions were neither physiological nor typical of those used in these biochemical analyses. Biochemical data were sought to address the oligomeric state of the protein under the buffer conditions and significantly lower protein concentrations used in the in vitro binding and decapping assays.

Four different types of analyses have been performed, and all demonstrate dimer formation, if not higher order multimerization of the X29 protein in solution. The protein crystallized as a homodimer (42). We previously described the results from a dynamic light scattering analysis of the protein used in the crystallographic analysis (50). These data were also consistent with formation of X29 homodimers in solution.

Gel-filtration analysis was performed with bacterial overexpressed, purified X29 protein as well as protein purified directly from *Xenopus* ovary tissue. Using an S300 column on a SMART system, and calibrating with standard gel filtration molecular mass markers, the elution profile of the purified X29 proteins was nearly identical to that of the 60-kDa bovine serum albumin standard (data not shown). There was a significantly much smaller (~10% of the total protein) protein peak eluting at ~30 kDa in the protein samples. Western blot analysis of all fractions identified the proteins that eluted at both 30 kDa and that at 60 kDa were X29 migrating on PAGE gels as a 30-kDa band. These data indicate the X29 protein forms a stable homodimer in solution but is monomeric in reduced denaturing conditions.

Protein cross-linking was a fourth method employed to address whether the X29 protein can form oligomeric complexes at lower protein concentrations. The nuclear decapping
proteins were incubated in the presence of chemical cross-linkers. DTSSP, a cross-linker that is reversible by a reducing environment, or BS³, a non-reversible cross-linking agent, was used. No nucleic acids or metals were added to these reactions. Both cross-linking reagents mediate cross-links between primary amines (primarily lysine) and have eight carbon spacer arms (~12 Å).

X29 or H29K proteins were incubated in the presence of either cross-linking agent for the previously determined optimal time period. After incubation, the reaction was terminated with Tris, pH 8, and aliquots of the sample were added to PAGE loading dyes that either contained or were lacking reducing agents. Proteins were resolved by SDS-PAGE, and the gels were stained with Coomassie Blue to directly visualize the migration of the proteins in the gel.

![Figure 7. Protein chemical cross-linkers demonstrate the 29K proteins form dimers in solution. Xenopus (lanes 1–6) or human (lanes 8 and 9) proteins were incubated in the presence of BS³, DTSSP, or untreated, as indicated. After the incubation half of the reaction received SDS sample buffer with no reducing agents, and the other half received SDS sample buffer plus dithiothreitol prior to heating and loading the gel. The gel was stained with Coomassie Blue to directly visualize the migration of the proteins in the gel.]

Collectively, these protein-protein cross-linking data demonstrate that both the human and the Xenopus proteins can form homodimers under our standard in vitro (decapping and EMSA) conditions. This dimerization is not a unique property of the Xenopus protein, nor is it induced by crystallization conditions. With the protein forming a dimer under our “standard” assay conditions, there is a strong likelihood that the dimer (rather than the monomer) binds the RNA for cap hydrolysis.

**DISCUSSION**

X29, the Xenopus NUDIX protein demonstrated to be a nuclear decapping protein, and H29K (AKA Nudt16), its human homologue, remove 5' cap structures from RNA in vitro (41). Here we better characterized the metal-dependent catalytic activity of the human and Xenopus wild-type proteins and mutant Xenopus protein site mutants in the active site. The decapping efficiency and substrate specificity are determined by the identity of the metal, which is required for cleavage. These data confirm the identities of functional residues in the mechanism that were inferred from the crystal structure of X29 and its complexes and demonstrate that, under the conditions used here, the protein undergoes a single round of catalysis. The protein appears to be unable to release the RNA and thus fails to turn over in the reaction. While providing a better understanding of how these proteins function, these in vitro data raise still more intriguing questions about the substrate specificity and identity of the metal cofactor in vivo and how these together determine the biological role of the nuclear decapping proteins.

*Metal, Required For Catalysis, Determines RNA Substrate Specificity—When Mg²⁺ was added to X29 protein present in a 10-fold molar excess over RNA substrate, the protein could decap a maximum of 60% of input RNA resulting in the release of m⁷GDP; only U8 snoRNA was a substrate for decapping. The human protein homologue demonstrated the same limited substrate specificity in Mg²⁺ but decapped a significantly smaller fraction of the input U8 snoRNA. In contrast, in the presence of Mn²⁺ or Co²⁺, both proteins demonstrated high activity (100% of the input RNA was decapped under identical RNA:protein ratios) and broader substrate specificity. Although it is not unusual for Mn²⁺ or Co²⁺ to alter the activity level of a NUDIX enzyme (23, 24, 34, 49, 51, 52), alteration of the specificity of cleavage by the protein is not typical. Furthermore, the increased level of hydrolysis seen in the presence of Co²⁺ at efficiency nearly equal to that of Mn²⁺ had not been reported. The only other NUDIX protein reported to use Co²⁺ for hydrolysis had significantly lower activity, compared with the Mg²⁺-containing form (51).

Mutagenesis experiments tested the effects of changing amino acids that were predicted by the x-ray crystal structure to be involved in coordinating metal and/or functioning in the cleavage reaction. The glutamic acids at positions 89, 92, and 93 point into the active site in the apo-enzyme while the side chain of Glu-150 does not (42). In the presence of Mn²⁺ and nucleotide of nucleoside the side chain of Glu-150 rotates into the...
active site and coordinates two metals in the cap-and-metal bound form (42). It was proposed that binding of the first Mn\(^{2+}\) to the side-chain carboxylate of Glu-150 induces the side-chain rotation that brings it into position for simultaneous coordination to a second Mn\(^{2+}\), through cross coordination with Glu-89, which in turn coordinates to a third metal. This inferred scenario was proposed as the basis for the observed cooperation of Mn\(^{2+}\) binding to X29 (42).

Changing the active site glutamic acid residues that are inner sphere ligands of the Mn\(^{2+}\) to glutamine eliminated most or nearly all the decapping activity in those mutants (E89Q, E93Q, and E150Q), but the proteins did fold correctly because a specific EMSA complex could be formed with all mutant proteins. Because Glu-150 was predicted to coordinate the first metal binding (42) it is not surprising that altering this residue completely eliminated decapping activity. At this point we do not fully understand the altered mobility by EMSA when E150Q binds U8 RNA. As noted above, a plausible explanation for this is that the 5’ terminal cap is bound to X29 primarily through coordination to Mn\(^{2+}\) in the active site. Failure to fix metal at this site would likely allow the 5’-end of the RNA to remain unbound, which could affect the mobility via EMSA.

Although the E92Q mutant retained some hydrolysis activity, it lost the ability to cooperatively bind metal. Addition of higher concentrations of metal to the reaction resulted in significantly increased decapping activity (~70% of input RNA) seen at 7-fold the Mn\(^{2+}\) concentration required by the wild-type protein (Figs. 2 and 3 and data not shown). This supports a role for the charge transfer via the inner sphere water ligand that mediates the interaction of the side-chain carboxylate of Glu-92 with a Mn\(^{2+}\). Although the uncharged glutamine amide group can orient this water by hydrogen bonding, the higher Mn\(^{2+}\) concentration required to achieve maximum decapping efficiency with loss of cooperativity in Mn\(^{2+}\) binding argue for a charge transfer interaction of Glu-92 with Mn\(^{2+}\) through the bound water in the wild-type enzyme. Mutagenesis of structurally equivalent conserved amino acids in other NUDIX proteins have variable effects upon activity (26, 33, 49, 53–56). These reports did not note changes in the activity and specificity of corresponding mutants in response to metal concentration.

Although Mn\(^{2+}\) is known to increase the activity of several of the NUDIX protein reactions, including Dcp2 (34, 51, 57), we know of no case where Mn\(^{2+}\) and Co\(^{2+}\) broaden the substrate specificity of an enzyme. The molecular basis for how each metal differentially affects the activity and substrate specificity of hydrolysis by the nuclear decapping protein is unknown, but additional biophysical studies will be undertaken to directly examine this.

**Multimerization: Function and Modulation of Activity?**—The ability to dimerize is characteristic of many, but not all NUDIX proteins. While the stoichiometry of the cytoplasmic NUDIX decapping protein, Dcp2, is not known, it does assemble in vivo as a heteromultimer, in direct contact with Dcp1 protein in yeast or with Dcp1 mediated by Headless in humans (3, 37, 58). There are several pieces of data that support the nuclear decapping protein also forms multimers. The light scattering data or overexpressed protein (42), gel filtration data of both overexpressed protein and protein isolated from *Xenopus* ovary (data not shown), and the x-ray crystal data (42) all indicate/are consistent with homodimer formation of X29. The cross-linking data here demonstrated that the X29 protein was very efficient at forming dimers in vitro, under conditions identical to those used in decapping assays. The human protein was less efficient at dimer formation but at least 50% of the protein could be cross-linked as a dimer. It may be noteworthy that the fraction of protein that can form a dimer parallels the decapping efficiency in Mn\(^{2+}\).

**What Metal Mediates Cleavage in Vivo and Why Would It Matter?**—These biochemical studies were performed to better understand decapping on a molecular level and to gain information about how these nuclear decapping proteins may function in vivo. The in vitro data demonstrate that the identity of the bound metal affects the specificity of the substrate permitted to participate in the reaction, and it seems likely the same will be true in vivo. At 10-fold lower concentrations than Mg\(^{2+}\), both Mn\(^{2+}\) and Co\(^{2+}\) broaden the substrate specificity and increase the protein’s catalytic activity at least 2-fold. Although it is true that intracellular levels of Mn\(^{2+}\) and Co\(^{2+}\) are quite low, the apparent cooperative binding of Mn\(^{2+}\) and a high local concentration of X29 protein in subnuclear foci may generate microenvironments where a sufficiently high concentration of Mn\(^{2+}\) (or Co\(^{2+}\)) would be available to mediate efficient catalysis (59, 60), but this remains to be demonstrated.

The potential broader substrate specificity of this nuclear decapping protein would be of interest because it could have an expanded role in vivo, affecting ribosomal subunit maturation (if it turns over U3 snoRNA, required for small ribosomal subunit maturation) and U8 snoRNA. The nucleoplasmic foci could be novel sites of nuclear (pre-)mRNA surveillance where the decapping machinery would eliminate messages not fit for export to the cytoplasm or destroy m\(^{2+}\)G-capped small nuclear RNAs that are not correctly assembled and need to be turned over. Thus the nuclear X29-containing foci may be the nuclear counterpart of the cytoplasmic P-bodies, involved in degradation of cytoplasmic mRNAs (3, 36, 38–40).

Although these nuclear decapping proteins do not appear to turn over in vitro, it is not clear this is a unique situation as comparable kinetic studies have not been performed on the cytoplasmic decapping protein. For X29 and its homologues, it is quite likely that in vivo there are other, as yet unidentified binding partners that may have various roles, including, but not limited to, regulating activity by altering RNA access or facilitating protein turnover after catalysis, possibly by enhancing RNA release after cleavage. Restricting these decapping proteins to foci in the nucleus would be one effective means of regulating their activity, but additional RNA-binding proteins that either regulate access or deliver RNAs to the sites of decay are alternative scenarios. Regardless, the potential broad substrate specificity of these nuclear decapping proteins opens the potential for this evolutionarily conserved nuclear decapping protein to regulate gene expression on a fundamental level, before mRNAs leave the nucleus.

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