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Novel interactions at the essential N-terminus of poly(A) polymerase that could regulate poly(A) addition in *Saccharomyces cerevisiae*

Chukwudi Ezeokonkwo, Mohamed A. Ghazy [1], Alexander Zhelkovsky [2], Pei-Chun Yeh, Claire Moore *

Tufts School of Medicine and the Sackler Graduate School of Biomedical Sciences, 136 Harrison Avenue, Boston, MA 02111, USA

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ABSTRACT

Addition of poly(A) to the 3′ ends of cleaved pre-mRNA is essential for mRNA maturation and is catalyzed by Pap1 in yeast. We have previously shown that a non-viable Pap1 mutant lacking the first 18 amino acids is fully active for polyadenylation of oligoA, but defective for pre-mRNA polyadenylation, suggesting that interactions at the N-terminus are important for enzyme function in the processing complex. We have now identified proteins that interact specifically with this region. Cft1 and Pta1 are subunits of the cleavage/polyadenylation factor, in which Pap1 resides, and Nab6 and Sub1 are nucleic-acid binding proteins with known links to 3′ end processing. Our results suggest a novel mechanism for controlling Pap1 activity, and possible models invoking these newly-discovered interactions are discussed.

Structured summary of protein interactions:
PAP1 binds to **Fip1** by anti bait coimmunoprecipitation (View interaction)
PAP1 binds to **Fip1** by pull down (View interaction)
PAP1 physically interacts with **PTA1** by two hybrid (View interaction)
PAP1 binds to **Sub1** by pull down (View interaction)
PAP1 physically interacts with **Fip1** by two hybrid (View Interaction: 1, 2)
PAP1 binds to **Nab6** by pull down (View interaction)
Nab6 physically interacts with **PAP1** by two hybrid (View interaction)
Cft1 binds to **PAP1** by pull down (View interaction)
PTA1 binds to **PAP1** by pull down (View interaction)

1. Introduction

Polyadenylation of eukaryotic mRNA involves cleavage of precursor and addition of adenosines onto the new 3′ end, and is catalyzed by a conserved complex of over 20 proteins [1]. Acquisition of the poly(A) tail promotes release from the site of transcription and efficient mRNA export, and the length affects turnover rate and translation [2,3].

The poly(A) polymerase (PAP) that adds the tail is tightly controlled during a cycle of 3′ end processing so that initiation of poly(A) synthesis occurs promptly after cleavage yet terminates once the tail is sufficiently long. Because of this central role, PAPs have become targets for regulatory proteins and post-translational modifications such as acetylation, phosphorylation and sumoylation [1,4] that potentially alter the network of interactions made by PAP within the processing complex. Mammalian PAP interacts with the CPSF-160 and hFip1 subunits, which direct the enzyme to the correct RNA substrates by interacting with signal sequences that define the poly(A) site [5,6]. Several proteins that are not part of the basic processing complex, such U1A, U1-70K and hnRNP H, can regulate polyadenylation by direct interaction with PAP [7–9].

Less is known about contacts made with yeast Pap1. Fip1 acts as a flexible tether to incorporate the enzyme into the cleavage/polyadenylation factor (CPF) [10–12] through a Fip1 binding site in the

Abbreviations: CF I, cleavage factor I; CPF, cleavage/polyadenylation factor; CPSF, cleavage/polyadenylation specificity factor; DTT, dithiothreitol; GAD, gal activation domain; GBD, gal binding domain; GST, glutathione S-transferase; PAP, poly(A) polymerase; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate

* Corresponding author. Address: Dept. of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA. Fax: +1 617 636 6935.
E-mail address: Claire.moore@tufts.edu (C. Moore).
[1] Present address: Ain Shams University, Cairo, Egypt.
[2] Present address: New England Biolabs, Ipswich, MA, USA.

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Pap1 C-terminal domain [13]. Deletion of the first 18 amino acids of Pap1 is lethal in yeast and causes defective polyadenylation of pre-mRNA when assayed using purified factors [14]. Surprisingly, this mutation had no effect on the catalytic activity of the enzyme when it is separated from CPF [11,14]. The truncated Pap1 was also normal for interaction with Fip1. The same region of vertebrate enzymes has 56% similarity to this yeast sequence, but has not been correlated with a particular function. These findings suggest that contacts between the N-terminus of Pap1 and unknown subunits of the mRNA 3′ end processing complex are critical for its essential function in mRNA maturation. In this study, we report the identification of proteins that interact with this part of Pap1 and possibly modulate its activity.

2. Materials and methods

2.1. Yeast strains

The yeast strains used in this study were W303 (*MATa* leu2-3, 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15) and PJ69-4A (*MAT* a leu2-3, 112 ura3-52 trp1-901 his3-200 gal4Δgal80Δ GAL-ADE2 lys2::GAL1-HIS3 met2::GAL7-LacZ). *KANMX* or *HIS3* replacements of the *SUB1* and *NAB6* genes were made in W303.

2.2. Two-hybrid analysis

Pap1-GBD and ΔN-Pap1-GBD [15] and gal4 activation domain (GAD)-Pta1 constructs [16] were described previously. Two-hybrid analysis was performed by transforming plasmid pairs into the PJ69-4A strain [15,16]. Transformants were selected on medium lacking leucine and tryptophan to ensure that both the GAD and GBD plasmids were present. Protein–protein interactions were scored by the ability of cells to grow in the absence of histidine.

2.3. Peptide columns

A Pap1 peptide, MSSQKVFGITGPVSTVGA, or an Npl3 peptide, RGGYDSPRGGY, was coupled to column matrix (Pierce UltraLink EDC/DADPA Immobilization Kit), incubated for 2 h at 25 °C with nuclear extract prepared as described [17] except using IPP-150 buffer (10 mM Tris, pH 7.9, 150 mM NaCl, 1 mM MgOAc, 2 mM $CaCl_2$, 0.1% NP-40, 1 mM DTT and the protease inhibitors PMSF, leupeptin, antipain and pepstatin-A), washed using 12 column volumes of IPP-150, and eluted using 100 mM glycine, pH 3. Eluted proteins were analyzed by mass spectroscopy through the Tufts Core Facility.

2.4. In vitro protein–protein interaction assays

For protein expression in *Escherichia coli* Rosetta (DE3), the Pap1-GST, ΔN-Pap1-GST, and Nab6-V5-His_6 plasmids were made by modifying pJPAP1 [14]. Other expression plasmids were described previously: Pap1-His_6 and ΔN-Pap1-His_6 [14], Fip1(1-206) [11], Sub1-V5-His_6 [18], Pta1 and Pta1 truncations [16], and Cft1-GST [19]. Expression and purification was performed as described [14]. GST pull-downs followed a modification of a previous protocol [16], using 50 μl glutathione-Sepharose beads in 200 μl of IP-150 for 2 h at 4 °C with gentle shaking, followed by four washes with IP-150, and elution with IP-150 containing 50 mM glutathione at 4 °C for 1 h with gentle shaking. Protein–protein interactions were analyzed by resolving proteins on an SDS–10% polyacrylamide gel, followed by Western blotting with the following antibodies: monoclonal Pap1 antibody at 1:100 dilution, V5 antibody (Invitrogen) at 1:5000 dilution, and polyclonal Fip1 antibody at 1:7500 dilution.

2.5. In vitro 3′ end processing

Yeast extract was prepared and used for processing assays as described [11,16], except that the final dialysis was twice against 2 l of buffer D for 2 h, and then overnight.

3. Results

3.1. The Pap1 N-terminus interacts with the first 300 amino acids of Pta1 and with Cft1

Pta1 has been shown to interact with in vitro translated Pap1 [16]. We used two-hybrid analysis to confirm this interaction in vivo and identify interaction domains by pairing full-length and truncations of Pta1 (Fig. 1A) with full-length Pap1 or Pap1 lacking the N-terminus (ΔN-Pap1). The Pta1 constructs have been previously shown to express protein and interact with other proteins [16]. Consistent with the known C-terminal Fip1 binding site, both Pap1 and ΔN-Pap1 interact with Fip1, but no interaction was seen between Pap1 and full-length Pta1 (Fig. 1B). Analysis using Pta1 truncations revealed an interaction between Pta1 (Δ300–785) and full-length Pap1 that was lost with ΔN-Pap1 or when another 25 amino acids of Pta1 were removed (Δ275–785). The lack of interaction between full-length Pta1 and Pap1 may be due to steric constraints that prevent a two-hybrid signal when both proteins are part of CPF.

We also assessed physical contact using recombinant GST-Pta1 and His_6-tagged Pap1 or ΔN-Pap1. Pap1 interacted with full-length Pta1 and the 1–300 amino acid fragment of Pta1, but not with the 1–275 fragment of Pta1 (Fig. 2A). ΔN-Pap1 did not interact with any forms of Pta1, but bound to Fip1 (Fig. 2B), indicating that unfolding of the protein does not explain a lack of interaction. These findings extend the two-hybrid results by showing a direct interaction between Pap1 and Pta1 mediated by amino acids 1–300 of Pta1 and the N-terminus of Pap1.

To study the function of the Pta1/Pap1 interaction in 3′ end processing, the 1–300 Pta1 fragment was added to a poly(A) addition assay using yeast extract and radiolabeled *GAL7* RNA substrate ending at the poly(A) site. Extract supplemented with the Pta1 fragment showed reduced accumulation of polyadenylated product compared to extract receiving an equal amount of GST (Fig. 2C), suggesting a role for this Pta1 domain in regulating Pap1 activity.

To ask if the interaction of CPSF160, the mammalian Cft1 homologue, with poly(A) polymerase [6] is conserved in yeast, pull-downs were performed using GST-Cft1 and His_6-tagged Pap1 or ΔN-Pap1. Cft1 bound full-length Pap1, but not Pap1 lacking the N-terminus (Fig. 3). Thus, like its mammalian homologue, Cft1 interacts with Pap1, but in yeast, this interaction is localized to the Pap1 N-terminus.

3.2. Sub1 and Nab6 interact with the Pap1 N-terminus

To find other proteins that interact with the Pap1 N-terminus, a column coupled to a peptide comprising the first 18 amino acids of Pap1 was used to pull down proteins from yeast nuclear extract. Sub1, a protein implicated in both transcription and 3′ end processing [20], and references therein), was one of the proteins identified from mass spectroscopy analysis of bound proteins. Sub1 was not found on a control column using a peptide from a different yeast protein, Npl3. Pull-downs showed that Sub1 interacts directly with