Deadenylation is prerequisite for P-body formation and mRNA decay in mammalian cells

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Deadenylation is the major step triggering mammalian mRNA decay. One consequence of deadenylation is the formation of nontranslatable messenger RNA (mRNA) protein complexes (messenger ribonucleoproteins [mRNPs]). Nontranslatable mRNPs may accumulate in P-bodies, which contain factors involved in translation repression, decapping, and 5’-to-3’ degradation. We demonstrate that deadenylation is required for mammalian P-body formation and mRNA decay. We identify Pan2, Pan3, and Caf1 deadenylases as new P-body components and show that Pan3 helps recruit Pan2, Ccr4, and Caf1 to P-bodies. Pan3 knockdown causes a reduction of P-bodies and has differential effects on mRNA decay. Knocking down Caf1 or overexpressing a Caf1 catalytically inactive mutant impairs deadenylation and mRNA decay. P-bodies are not detected when deadenylation is blocked and are restored when the blockage is released. When deadenylation is impaired, P-body formation is not restorable, even when mRNAs exit the translating pool. These results support a dynamic interplay among deadenylation, mRNP remodeling, and P-body formation in selective decay of mammalian mRNA.

Introduction

Regulation of mRNA turnover plays an essential role in modulating gene expression (Meyer et al., 2004; Parker and Song, 2004; Garneau et al., 2007). For all major paths of mRNA decay yet recognized in mammalian cells, including mRNA decay directed by AU-rich elements (AREs) in the 3’ untranslated region (Chen and Shyu, 1995), decay mediated by destabilizing elements in protein-coding regions (Grosset et al., 2000; Chang et al., 2004), nonsense-mediated decay (NMD; Chen and Shyu, 2003), decay directed by microRNAs (miRNAs; Wu et al., 2006), and decay of stable mRNAs such as β-globin mRNA (Yamashita et al., 2005), the first major step is deadenylation.

Mammalian deadenylation is mediated by the concerted action of two different poly(A) nuclease complexes (Yamashita et al., 2005). Poly(A) tails are first shortened to ~110 nt by Pan3 in association with Pan3. In the second phase of deadenylation, a complex composed of Ccr4 and Caf1 catalyze further shortening of the poly(A) tail to oligo(A). Decapping by the Dcp1–Dcp2 complex (Lykke-Andersen, 2002; van Dijk et al., 2002; Wang et al., 2002; Piccirillo et al., 2003) may occur during and/or after the second phase of deadenylation (Yamashita et al., 2005). Although Pan3 and Caf1 associate with Pan2 and Ccr4 poly(A) nucleases, respectively (Brown et al., 1996; Albert et al., 2000; Tucker et al., 2001; Temme et al., 2004; Uchida et al., 2004), their in vivo roles in mammalian mRNA turnover remain unclear. In yeast, Pan3 does not exhibit poly(A) nuclease activity but its association with Pan2 is required for proper function of Pan2 (Brown et al., 1996; Mangus et al., 2004). In vitro experiments using recombinant human Pan2 and Pan3 proteins (Uchida et al., 2004) suggest that Pan3 plays a role in enhancing the poly(A) nuclease activity of Pan2 in mammalian cells. However, ectopic overexpression of Pan2 alone in mouse NIH3T3 cells results in highly rapid and processive deadenylation of an otherwise stable reporter mRNA or a premature translation-termination codon (PTC)–containing mRNA (Yamashita et al., 2005), indicating that Pan3 is not required for the nuclease activity of Pan2 for mammalian mRNA turnover. Instead, Pan3 may modulate the activity of Pan2 poly(A) nuclease or link deadenylation to subsequent decay of the mRNA body. Unlike Pan3, Caf1 exhibits poly(A) nuclease activity (Daugeron et al., 2001; Dupressoir et al., 2001; Temme et al., 2004; Bianchin et al., 2005; Molin and Puisieux, 2005). However, studies in yeast show that Caf1 poly(A) nuclease activity per se is not required for general deadenylation in vivo, although the presence of Caf1 is necessary for proper deadenylation by
Are these factors necessary for P-body formation, and are their functions in deadenylation crucial for P-body formation? Is deadenylation a prerequisite for P-body formation? In this paper, we address these questions by examining the subcellular localization and functions of poly(A) nucleases and their cofactors in relation to the formation of P-bodies and mRNA turnover in mammalian cells.

**Results**

Pan2 and Pan3 are components of P-bodies

We first determined whether Pan2 and Pan3, the factors involved in initiating mammalian mRNA deadenylation (Uchida et al., 2004; Yamashita et al., 2005), are found in P-bodies of mouse NIH3T3 fibroblasts (the cell line used for monitoring mRNA decay kinetics by the well-established transcriptional pulsing system; Xu et al., 1998; Chen et al., 2007). Dcp1a, a well-characterized component of P-bodies which is necessary for mRNA decapping in eukaryotes (Beelman et al., 1996; Lykke-Andersen, 2002; Cougot et al., 2004; Kedersha et al., 2005), was used as a marker to visualize P-bodies by immunofluorescence microscopy. Both endogenous Dcp1a and ectopically expressed Dcp1a fused with green fluorescent protein (GFP-Dcp1a) distributed in a focal pattern that disappeared upon translation blockage by cycloheximide in NIH3T3 cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200801196/DC1), as expected for P-bodies (Andre et al., 2005; Brengues et al., 2005; Ferraiuolo et al., 2005). The results show that cytoplasmic foci detected by an anti-Pan2 antibody colocalize with P-bodies either marked by GFP-Dcp1a (Fig. 1 A) or detected by an anti-Dcp1a antibody (Fig. 1 B, top). Upon cycloheximide treatment, neither P-bodies nor Pan2 foci were detected (Fig. 1 B, bottom). These results indicate that Pan2 is a component of P-bodies.

We further substantiated this finding by showing that Pan2 foci colocalize with P-bodies in COS7 cells, using both Dcp1a and Xrn1 as markers to visualize P-bodies (Fig. 1 C, top and middle). Moreover, the endogenous Pan3 also distributes in cytoplasmic foci that colocalize with the Pan2 foci in COS7 cells (Fig. 1 C, bottom). Because of lack of an anti-Pan3 antibody suitable for immunofluorescence microscopy of mouse NIH3T3 cells, DNA coding for HA-Pan3 (HA-tagged Pan3) was delivered into NIH3T3 cells via transient transfection. Our data show that HA-Pan3 also distributes in a focal pattern colocalized with P-bodies (Fig. 1 D). It is worth noting that the number of P-bodies increased appreciably when HA-Pan3 was overexpressed (Fig. 1 D), suggesting a role of Pan3 in initiating or enhancing the formation of P-bodies. The HA-Pan3 foci are not stress granules, as they can be detected regardless of whether or not the cells were treated with arsenite (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200801196/DC1), a cellular stress inducer (Kedersha et al., 2002). Stress granules were only found in the arsenite-treated cells, as visualized by a stress granule marker G3BP1 (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200801196/DC1), a cellular stress inducer (Kedersha et al., 2002). Stress granules were only found in the arsenite-treated cells, as visualized by a stress granule marker G3BP1 (Fig. S2, bottom), and not in cells without arsenite treatment (Fig. S2, top). Importantly, when stress granules were induced, they did not colocalize with the HA-Pan3 foci (Fig. S2, bottom, right). Collectively, our results identify both Pan2 and Pan3 as being two new components of P-bodies in mammalian cells.
Pan3 helps Pan2, Ccr4, and Caf1 localize to P-bodies

Although Pan2 and Pan3 can form a complex in the cytoplasm, the finding that HA-Pan3 can be detected in P-bodies when it is overexpressed alone (Fig. 1 D) suggests that Pan3 can associate with P-bodies without a coordinated expression of Pan2. In contrast, ectopically expressed Pan2 did not distribute in a focal pattern when expressed alone (Fig. 2 A, top and middle) but did colocalize with P-bodies when coexpressed with HA-Pan3 (Fig. 2 A, bottom). In contrast, ectopically expressed myc-PABP, a non–P-body component (Kedersha et al., 2005) which has the ability to bind Pan3 (Brown et al., 1996; Mangus et al., 2004; Uchida et al., 2004), displayed a uniform distribution in the cytoplasm without forming foci even when coexpressed with HA-Pan3 (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200801196/DC1). These results further corroborate our conclusion that HA-Pan3 foci correspond to P-bodies and indicate that Pan3 helps Pan2 localize to P-bodies.

As Ccr4 has been observed to colocalize with P-bodies in mammalian cells (Cougot et al., 2004; Andrei et al., 2005), we then tested whether Caf1 can also be found in mammalian P-bodies by examining subcellular localization of endogenous Caf1 in NIH3T3 cells (Fig. 2 B). Immunofluorescence microscopic results show a strong staining in both nuclei and cytoplasm of cells (with two to six foci colocalized with P-bodies marked by GW182 in each cell) when the anti-Caf1 antibody was used (Fig. 2 B, top), whereas only a weak and nonspecific background staining was detected when preimmune serum was used (Fig. 2 B, bottom). Thus, our data indicate that only a small portion of Caf1 colocalizes with P-bodies, which is similar to the staining patterns previously observed for Ccr4 (Cougot et al., 2004; Andrei et al., 2005). Although GFP-Ccr4 was observed to colocalize with P-bodies in HEK293 cells (Cougot et al., 2004), ectopically expressed Ccr4 and Caf1 did not form foci that colocalized with P-bodies in NIH3T3 cells, regardless of whether they were overexpressed separately (Fig. 2, C [top and middle] and D [top, left]) or together (Fig. 2 C, bottom). Because Pan3 is able to enhance localization of Pan2 to P-bodies (Fig. 2 A), we then tested whether Pan3 also helps Ccr4 and Caf1 localize to P-bodies. After coexpressing HA-Pan3 with GFP-Ccr4 (Fig. 2 D, top, right) or Caf1-V5 (Fig. 2 D, bottom), we observed that both proteins distributed in a focal pattern colocalized with the P-bodies. These experiments show that Pan3 also enhances localization of Ccr4 and Caf1 to P-bodies.

Pan3 knockdown affects P-body formation and has differential effects on mRNA decay

The findings in the previous sections (Figs. 1 D and 2) suggest that Pan3 plays a critical role in mammalian P-body formation. We knocked down Pan3 in NIH3T3 cells using siRNAs and examined the effects on P-body formation (Fig. 3 A). Immunofluorescence microscopy revealed a reduction of P-bodies in cells treated with Pan3-specific siRNAs but not in cells treated with control siRNAs (Fig. 3 A, top and middle). Western blotting analysis indicated that an ~80% knockdown efficiency was achieved (Fig. 3 A, bottom). These experiments demonstrate that Pan3 not only is an integral component of P-bodies but also is important for the formation of P-bodies.

We next examined the effect of Pan3 knockdown on mRNA deadenylation and decay in NIH3T3 cells. Decay of a nonsense-containing mRNA (BBB+PTC), a c-fos ARE-containing mRNA (BBB+ARE), and the stable β-globin (BBB) message, representing three distinct pathways, was tested. The transcripts were expressed under the control of an inducible Tet-off promoter.
P-bodies (Fig. 3A) and has differential effects on mRNA decay (Fig. 3B) suggest that different roles of Pan3 and P-bodies in different mRNA decay pathways exist in mammalian cells (see Discussion).

Pan2–Pan3 and Ccr4–Caf1 complexes can interact with each other in vivo

The observations that Pan3 helps recruit Pan2, Ccr4, and Caf1 to P-bodies (Fig. 2) suggest that the Pan2–Pan3 and Ccr4–Caf1 complexes can communicate with each other in vivo. To test this hypothesis, we performed a series of coimmunoprecipitation/Western blotting experiments using RNase A–treated lysates prepared from cells overexpressing the components of these complexes (Fig. 4). When HA–Pan3 was expressed alone, endogenous Pan2 and PABP were both coprecipitated along with P-bodies (Fig. 3A) and has differential effects on mRNA decay (Fig. 3B) suggest that different roles of Pan3 and P-bodies in different mRNA decay pathways exist in mammalian cells (see Discussion).
The notion that the Pan2–Pan3 and Ccr4–Caf1 complexes can associate with each other was further confirmed by the results showing that Pan3 is a genuine PABP binding protein (Siddiqui et al., 2007) and that Pan2 and Pan3 can form complexes in vivo (Mangus et al., 2004). In contrast, endogenous Caf1 was not found in the HA-Pan3 precipitate (Fig. 4A), indicating a lack of direct interaction between Pan3 and Caf1 or an interaction too weak to be detected. However, when HA-Pan2 was ectopically expressed alone (Fig. 4B), endogenous Caf1, but not PABP, was coprecipitated with HA-Pan2, indicating a specific interaction between Pan2 and Caf1, either directly or indirectly. Therefore, Pan2–Pan3 and Ccr4–Caf1 complexes may communicate with each other in vivo via the interaction between Pan2 and Caf1.
Caf1 overexpression led to extremely rapid deadenyl-
ating of BBB+PTC, BBB+ARE, and BBB mRNAs in NIH3T3 cells.

To better address the role of Caf1 in mammalian mRNA turnover, we took the approach of inhibiting endogenous Caf1 activity by ectopically expressing a catalytically inactive mutant of Caf1 and monitoring the effects on the decay of BBB+PTC, BBB+ARE, and BBB mRNAs in NIH3T3 cells (Fig. 6, A and B [bottom, left] and C [middle, middle]). We changed a critical aspartate residue, which is necessary for metal binding and Caf1 nuclease activity (Viswanathan et al., 2004), to alanine by site-directed mutagenesis to create the mutant Caf1. Northern blotting results show that the Caf1 mutant exhibits a profound dominant-negative effect, blocking deadenylation of all the reporter mRNAs tested and leading to drastic stabilization of these transcripts. It is worth noting that the broad bands normally detected for the internal control α-globin–GAPDH hybrid message constitutively transcribed from the SV40 early promoter also became tight and migrated much more slowly than the poly(A)⁺ band. These observations indicate that overexpression of mutant Caf1 has a general and very profound dominant-negative effect on deadenylation in mammalian cells.

As Caf1 and Ccr4 work as a complex (Tucker et al., 2002; Temme et al., 2004; Behm-Ansmant et al., 2006) and ectopically overexpressing both Ccr4 and Caf1 had an additive effect on accelerating poly(A) shortening (Fig. 6 C, bottom, left), we then tested whether coexpressing wild-type Ccr4 can release the deadenylation blockage caused by the Caf1 mutant. The results (Fig. 6 C, bottom, middle) show that the dominant-negative effect exerted by the Caf1 mutant was greatly diminished when wild-type Ccr4 was coexpressed. The deadenylation kinetics of BBB mRNA were now similar to those observed in cells coexpressing wild-type Caf1 and Ccr4 (Fig. 6 C, bottom, left). Thus, the poly(A)ₗ shortenings activity of Caf1 can be complemented by that of Ccr4 for deadenylation of the mRNA substrate. Previously, we had shown that overexpressing a dominant-negative catalytically inactive Ccr4 mutant slowed the deadenylation of BBB mRNA (Fig. 6 C, middle, left; Yamashita et al., 2005). We now tested whether the poly(A)ₗ nuclear activity of Caf1 can also complement that of Ccr4 by coexpressing wild-type Caf1 and the Ccr4 mutant. The results show that the deadenylation kinetics of BBB mRNA in the presence of both wild-type Caf1 and Ccr4 mutant (Fig. 6 C, bottom, right) are similar to those observed in cells coexpressing wild-type Ccr4 and Caf1 mutant (Fig. 6 C, bottom, middle) or coexpressing wild-type Ccr4 and Caf1 (Fig. 6 C, bottom, left). Collectively, we conclude that the poly(A)ₗ nuclear activities of Ccr4 and Caf1 in the Ccr4–Caf1 complex have complementary roles in mammalian deadenylation.

Caf1 is required for deadenylation and can accelerate poly(A) shortening in mouse NIH3T3 cells

To address the role of Caf1 in mammalian mRNA turnover, we first determined whether mRNA deadenylation and decay requires Caf1. We knocked down endogenous Caf1 in NIH3T3 cells by siRNA (Fig. 5) and examined the effects on the deadenylation and decay kinetics of BBB+PTC, BBB+ARE, and BBB mRNAs. As shown in Fig. 5, Caf1 knockdown significantly impairs the deadenylation and decay of all the mRNAs tested, demonstrating that Caf1 plays a key role in mRNA deadenylation and decay in mouse NIH3T3 cells.

As Caf1 was shown to exhibit poly(A) nucleus activity in vitro (Viswanathan et al., 2004; Bianchin et al., 2005), we next tested whether Caf1 can accelerate deadenylation in vivo by examining the effects of overexpressing Caf1 on deadenylation of BBB+PTC, BBB+ARE, and BBB mRNAs in NIH3T3 cells (Fig. 6). Caf1 overexpression led to extremely rapid deadenylation of all the reporter mRNAs tested. Poly(A) shortening was detected as early as the 0-h time point (Fig. 6, A–C, top, right). These results demonstrate that Caf1 exhibits a robust poly(A) shortening activity that enhances the deadenylation of mRNAs in the cytoplasm of mammalian cells. Interestingly, ectopically overexpressing both Ccr4 and Caf1 had an additive effect on accelerating poly(A) shortening (Fig. 6 C, bottom, left), indicating that coordinated expression of both proteins further promotes the deadenylation function of Ccr4–Caf1 poly(A) nuclease complex in NIH3T3 cells.

The poly(A)ₗ nuclease activities of Caf1 and Ccr4 have complementary roles in mammalian deadenylation

As Caf1 and Ccr4 work as a complex (Tucker et al., 2002; Temme et al., 2004; Behm-Ansmant et al., 2006) and ectopically overexpressing both Ccr4 and Caf1 had an additive effect on accelerating poly(A) shortening (Fig. 6 C, bottom, left), we then tested whether coexpressing wild-type Ccr4 can release the deadenylation blockage caused by the Caf1 mutant. The results (Fig. 6 C, bottom, middle) show that the dominant-negative effect exerted by the Caf1 mutant was greatly diminished when wild-type Ccr4 was coexpressed. These results demonstrate that Caf1 exhibits a robust poly(A) shortening activity that enhances the deadenylation of mRNAs in the cytoplasm of mammalian cells. Interestingly, ectopically overexpressing both Ccr4 and Caf1 had an additive effect on accelerating poly(A) shortening (Fig. 6 C, bottom, left), indicating that coordinated expression of both proteins further promotes the deadenylation function of Ccr4–Caf1 poly(A) nuclease complex in NIH3T3 cells.
P-body formation requires active deadenylation

Our findings that expression of the dominant-negative HA-Caf1 mutant completely blocked deadenylation (Fig. 6 C, middle, middle) and coexpression of wild-type HA-Ccr4 released the deadenylation blockage (Fig. 6 C, bottom, middle) provided an approach to study how alteration of deadenylation may impact P-body formation without knocking down any deadenylase that may be physically required for the structural integrity of P-bodies. We performed immunofluorescence microscopy to test for the presence of P-bodies in cells overexpressing wild-type Caf1, mutant Caf1, or coexpressing the Caf1 mutant with wild-type or mutant Ccr4 (Fig. 7). It should be noted that without a coordinated expression of Pan3, ectopically expressed Ccr4 and Caf1 proteins did not form foci that colocalized with P-bodies (Fig. 2 C and Fig. 7, green staining). To test the presence of P-bodies, endogenous Pan2 and Dcp1a were used as markers to visualize P-bodies. The results show that P-bodies could hardly be detected in NIH3T3 cells overexpressing the Caf1 mutant (Fig. 7 A, right, dashed lines), whereas overexpressing wild-type Caf1 did not have any obvious effect (Fig. 7 A, left). Similar results were also observed in COS7 cells (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200801196/DC1). Remarkably, P-bodies were restored when wild-type Ccr4 was coexpressed with the Caf1 mutant (Fig. 7 B, left), a situation in which the negative effect of the Caf1 mutant on deadenylation was mitigated (Fig. 6 C, bottom, middle). Conversely, P bodies remained undetectable when cells were cotransfected with both mutant Ccr4 and mutant Caf1 (Fig. 7 B, right, dashed lines). Collectively, these results demonstrate that P-body formation is dependent on active deadenylation in mammalian cells. This conclusion was further substantiated by the observation that P-bodies were also undetectable when Caf1 was knocked down (Fig. 8 A, bottom, left), a condition that also severely impaired deadenylation (Fig. 5).

Several previous studies have shown that both the number and size of P-bodies are increased by treating eukaryotic cells with the translation inhibitor puromycin, which increases the amount of nontranslatable mRNPs (Cougot et al., 2004; Maroney et al., 2006; Eulalio et al., 2007a; Yang and Bloch, 2007). To further substantiate the finding that P-body formation is dependent on active deadenylation, we tested whether P-body formation can be induced by puromycin treatment of the cells in which deadenylation has already been impaired. As shown in Fig. 8, puromycin treatment increases the number and size of P-bodies when deadenylation is not blocked, namely in cells transfected with nonspecific siRNA (Fig. 8 A, top, right), overexpressing wild-type Caf1 (Fig. 8 B, top, right, dashed line), or nontransfected (Fig. 8 B, top and bottom, right, area surrounding the enclosed region). Strikingly, when deadenylation was blocked by knocking down Caf1 (Fig. 8 A, bottom) or by overexpressing the Caf1 dominant-negative mutant (Fig. 8 B, bottom, dashed line), puromycin treatment failed to induce P-body formation. These results demonstrate that P-bodies cannot form as long as deadenylation is impaired, even when mRNPs exit the translating pool.
Moreover, knocking down Dcp2 does not have any appreciable stabilizing effect on a PTC-containing mRNA (Yamashita et al., 2005). In the present study, we further show that NMD, ARE-mediated mRNA decay, and the default decay pathway for a stable message are all impaired when deadenylation is inhibited either by knocking down Caf1 (Fig. 5) or by overexpressing a Caf1 dominant-negative mutant (Fig. 6). Thus, deadenylation is a necessary initial step for all major paths of mRNA decay yet recognized in mammalian cells, including NMD.

Deadenylation is a prerequisite and precursor for P-body formation

Several lines of evidence from this study demonstrate that deadenylation is a prerequisite and precursor for P-body formation in mammalian cells. First, impairment of deadenylation by knockdown of Caf1 (Fig. 5) leads to loss of P-bodies (Fig. 8 A).

Discussion

Deadenylation is a necessary initial step in all major pathways of mammalian mRNA decay

Deadenylation is known as the first major step triggering general mRNA decay in mammalian cells (Meyer et al., 2004; Parker and Song, 2004). The importance of deadenylation for initiating NMD in mammalian cells is a recent insight (Chen and Shyu, 2003; Yamashita et al., 2005) because previous studies in yeast supported a decapping-dependent NMD pathway (Muhlrad and Parker, 1994). Several findings demonstrate that mammalian NMD occurs in the cytoplasm and is triggered by deadenylation but not by decapping. A PTC at codon 39 of β-globin (BBB+PTC) mRNA induces accelerated deadenylation and subsequent rapid decay of the RNA body. Blocking translation initiation stabilizes the transcript, confirming involvement of the NMD pathway in the cytoplasm (Chen and Shyu, 2003). Moreover, knocking down Dcp2 does not have any appreciable stabilizing effect on a PTC-containing mRNA (Yamashita et al., 2005). In the present study, we further show that NMD, ARE-mediated mRNA decay, and the default decay pathway for a stable message are all impaired when deadenylation is inhibited either by knocking down Caf1 (Fig. 5) or by overexpressing a Caf1 dominant-negative mutant (Fig. 6). Thus, deadenylation is a necessary initial step for all major paths of mRNA decay yet recognized in mammalian cells, including NMD.

Deadenylation is a prerequisite and precursor for P-body formation

Several lines of evidence from this study demonstrate that deadenylation is a prerequisite and precursor for P-body formation in mammalian cells. First, impairment of deadenylation by knockdown of Caf1 (Fig. 5) leads to loss of P-bodies (Fig. 8 A).
In contrast, Pan3 knockdown results in P-body reduction (Fig. 3 A) but has little or no impairing effect on deadenylation (Fig. 3 B). These results show that blocking deadenylation impairs P-body formation but that the converse is not true, thereby establishing the cause–effect relationship between deadenylation blockage and P-body loss. Second, the inhibitory effects of a dominant-negative mutant of Caf1 on deadenylation and P-body formation (Figs. 6, 7, and S4) demonstrate that P-body loss caused by knocking down Caf1 mutant impaired both the first and the second phases of deadenylation in a concerted manner (Yamashita et al., 2005). Importantly, coexpression of wild-type top or mutant Caf1 followed by puromycin treatment for 1 h. Immunofluorescence staining was performed as described in Materials and methods. Endogenous Dcp1a was used as a marker to visualize P-bodies. Bars, 15 μm.

Deadenylation may induce mRNP remodeling required for P-body formation
Several observations in this study suggest that an mRNP containing a poly(A)-shortened mRNA undergoes remodeling before it appears in P-bodies. First, Pan2–Pan3 and Ccr4–Caf1 complexes can interact with each other in vivo (Fig. 4), suggesting that they form a super complex to direct the two consecutive phases of deadenylation in a concerted manner (Yamashita et al., 2005) and to elaborate subsequent mRNP remodeling. This notion is further supported by our observation that overexpression of the Caf1 mutant impaired both the first and the second phases of deadenylation (Fig. 6). It appears that the two phases of deadenylation, although they take place sequentially, are functionally linked (Yamashita et al., 2005). Second, key components of both poly(A) nuclease complexes are all found in P-bodies (Figs. 1 and 2), suggesting a direct role for them in P-body formation. Third, although PABP can enhance Pan2 nuclease activity (Boeck et al., 1996; Mangus et al., 2004; Uchida et al., 2004) and can interact with Pan3 (Fig. 4), PABP does not colocalize with P-bodies (Fig. S3; Kedersha et al., 2005), suggesting that the first phase of deadenylation, as well as that of mRNP remodeling involving dissociation of PABPs, occurs before appearance of an mRNP in P-bodies.

Because PABPs are not found in P-bodies and one major change in mRNPs after deadenylation is the loss of PABPs, one important implication of our observations is that PABPs play an inhibitory role and prevent mRNPs from joining existing P-bodies or nucleating P-body formation in mammalian cells. It is plausible that the 3′ poly(A) tail of an mRNA must first be shortened, not only to permit dissociation of PABPs or factors that are important for efficient translation of the mRNP but also to allow joining of translation repressors or other P-body components to the mRNP.

P-bodies play differential roles in mRNA turnover and are not required for all mRNA decay pathways in mammalian cells
A central issue concerns whether all mRNA decay requires P-bodies. Our results (Fig. 3) show that knocking down Pan3, a manipulation that has little effect on deadenylation but significantly reduces P-bodies, slows decay of BBB+PTC mRNA but not BBB+ARE or BBB mRNA, supporting a differential involvement of P-bodies in mammalian mRNA decay. These results are consistent with observations that several NMD-required factors can accumulate in P-bodies in yeast and D. melanogaster (Barbee et al., 2006; Sheth and Parker, 2006; Eulalio et al., 2007a) and that knocking down GW182 effectively abolishes P-bodies but has little effect on ARE-mediated decay in some human cells (Stoecklin et al., 2006). Thus, even though decay of ARE-containing mRNAs could involve or occur in P-bodies (Ferraiuolo et al., 2005; Franks and Lykke-Andersen, 2007), ARE-mediated decay does not always require P-bodies.

Our finding that reduction of P-bodies by Pan3 knockdown enhances the decay of BBB and BBB+ARE mRNAs (Fig. 3) suggests a possible role for P-bodies in preventing mRNAs from degradation. In this case, P-bodies provide a storage site for mRNAs before they are degraded or reenter translatable pool (for reviews see Eulalio et al., 2007b; Kedersha and Anderson, 2007; Parker and Sheth, 2007). On the other hand, for aberrant mRNAs, such as PTC-containing transcripts, sequestration in P-bodies may provide a rapid means to prevent accidental translation before degradation. Collectively, our results indicate that P-bodies play differential roles in mammalian mRNA turnover and are not required for all mRNA decay pathways in mammalian cells.

Pan3 and Caf1 have distinct roles in mammalian mRNA turnover
Mammalian deadenylation is mediated by a concerted action of Pan2–Pan3 and Ccr4–Caf1 poly(A) nuclease complexes.

Figure 8. Puromycin treatment does not induce P-body formation when deadenylation is impaired. (A) Puromycin treatment enhances P-body formation (top, right) in NIH3T3 cells but does not induce P-body formation when Caf1 is knocked down (bottom, right). The cells transfected with either the control nonspecific siRNA (top) or Caf1 siRNA (bottom) were treated with (right) or without (left) puromycin for 1 h. Endogenous GW182 was used as a marker to visualize P-bodies. (B) Puromycin treatment does not induce P-body formation in cells overexpressing the mutant Caf1 (bottom, right, dashed line). NIH3T3 cells were transfected with plasmids encoding either wild-type (top) or mutant (bottom) Caf1 followed by puromycin treatment for 1 h. Immunofluorescence staining was performed as described in Materials and methods. Endogenous Dcp1a was used as a marker to visualize P-bodies. Bars, 15 μm.
mutant promotes the formation of functional deadenylase complexes because the deadenylase activity of the wild-type protein can complement its partner in the same complex. In contrast, HA-Ccr4 mutant and HA-Caf1 mutant cannot form a functional deadenylase complex with each other, as both mutants are catalytically inactive. It is worth noting that although Caf1 knockdown severely impairs mRNA deadenylation and decay (Fig. 5), Ccr4 knockdown only modestly affects the second phase of deadenylation without significantly impairing overall mRNA decay (Yamashita et al., 2005). Therefore, the role of Caf1 in mammalian turnover is distinct from that of Ccr4 and is more significant than previously recognized.

A model linking deadenylation, P-bodies, and mRNA decay

Based on previous and the current findings, we envisage the following scenario for deadenylation, P-body formation, and differential mRNA decay in mammalian cells (Fig. 9). Pan2–Pan3 and Ccr4–Caf1 complexes first form a super complex on mRNAs in the cytoplasm. The 3' poly(A) tail–PABP complex then stimulates poly(A) shortening by the Pan2–Pan3 in the super complex but inhibits the activity of Ccr4–Caf1 (Tucker et al., 2002), allowing the first phase of deadenylation to proceed. mRNP remodeling occurs during or after the first phase of deadenylation. Remodeling might involve dissociation of PABPs and some translation initiation factors and association of translation repressors (Coller and Parker, 2005; for reviews see Eulalio et al., 2007b; Parker and Sheth, 2007) to promote the transition of mRNPs to a nontranslatable state. A remodeled mRNP may associate with existing P-bodies or nucleate formation of new P-bodies, in which the second phase of deadenylation by Ccr4–Caf1 and/or decapping would proceed. For some mRNPs, such as a PTC-mRNP, this process may take place with the help of Pan3. Sequestration within P-bodies at this point may provide a
quick means of keeping aberrant mRNPs, such as PTC-mRNPs, from being translated before their poly(A) tail or cap is removed and the RNA body degraded. Alternatively, a remodeled mRNP may undergo the second phase of deadenylation outside of P-bodies, which could induce another mRNP remodeling that determines whether the oligo(A)-mRNP would be degraded inside or outside of P-bodies.

The observation that four component proteins of the two major poly(A) nucleic complexes can be found in mammalian P-bodies links all major mRNA decay factors except the 3′ exosome complex to P-bodies. The present results argue for a biological role for P-bodies in coupling deadenylation and mRNP remodeling to translation repression in an effective manner. One important conclusion from our findings in this study is that although P-bodies may be assembled via different mechanisms, deadenylation is always a necessary step that enables mRNPs to enter existing P-bodies or to initiate the formation of P-bodies. It will be interesting to see how knockdown of Pan3 or Caf1 or overexpression of the Caf1 dominant-negative mutant might impact different posttranscriptional mechanisms regulating mammalian mRNA expression, such as those mediated by miRNAs, whose functions were found to be linked to P-bodies (Jackson and Standart, 2007; Nilsen, 2007; Pillai et al., 2007; for reviews see Eulalio et al., 2007b; Parker and Sheth, 2007).

Materials and methods

Plasmids
To construct a plasmid encoding HA-tagged Pan3 or Caf1, a 2.24-kb Pan3 cDNA amplified by RT-PCR from pSR-HA-Caf1 as the template. To construct pcDNA6-Acfl-V5, a plasmid encoding catalytic inactive Caf1 mutant [D40A] was created using the QuikChange site-directed mutagenesis kit (Stratagene) with pSR-HA-Caf1 as the template. To construct pcDNA6-Pan2-V5, a 3.6-kb Pan2 cDNA was amplified from IMAGE clone clone 3357890 and inserted between the AflII and XbaI sites of pcDNA6/V5-HisA (Invitrogen). To generate pcDNA6-Caf1-V5, pcDNA6-Pan2-V5, and pcDNA6-Pan3-V5, the coding region of PABP was amplified by PCR from mouse NIH3T3 or monkey COS7 cells, the corresponding polyclonal antibodies were generated in rabbits immunized with gene-specific peptides using the custom antibody service from Bethyl Laboratories, Inc. Affinity-purified anti-peptide antibodies were used at the following dilutions: rabbit anti-Pan3 peptide antibody at 1:2,000; rabbit anti-Caf1 peptide at 1:1,000; or rabbit anti-Pan2 peptide at 1:2,000. Rabbit anti-PABP antibody (gift from R. Lloyd, Baylor College of Medicine, Houston, TX) was used at a 1:4,000 dilution. HRP-conjugated donkey anti-rabbit IgG antibodies (1:4,000; GE Healthcare) or goat anti–mouse (1:10,000; Bio-Rad Laboratories) were used as secondary antibodies for detection with a chemiluminescence reagent (peroxide/luminol enhancer; Thermo Fisher Scientific).

For coimmunoprecipitation, COS7 cells expressing HA-Pan2, Ccr4-V5, HA-Pan3, or V5-Pan3 were lysed at 48 h after transfection by incubation for 10 min at 4°C in 600 μl of lysis buffer (20 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl, 1 mM MgCl2, 0.5% NP-40, and 1 mM NaF supplemented with a protease inhibitor cocktail [Roche]). 50 μl of cell lysate was preserved as input and the remainder was incubated with a monoclonal anti–V5-agarose conjugate (Sigma-Aldrich) or a rat monoclonal anti-HA Affinity Matrix (Roche) in the presence of 0.1 μg/ml RNase A. Immunoprecipitations were performed at 4°C for 4 h. The beads were washed five times with the lysis buffer. Coimmunoprecipitated proteins were detected by Western blotting using the indicated antibodies. The protein samples were separated by 7–10% SDS-PAGE and analyzed by Western blot analysis as described in the previous paragraph.

Western blot analysis and immunoprecipitation
Cytoplasmic and nuclear lysates were prepared as described previously (Peng et al., 1998). Total cell lysates (5–40 μg) were resolved on a 7 or 10% polyacrylamide gel and analyzed using an ECL Western blotting kit (GE Healthcare). The PVDF blots were blocked with specific antibodies as indicated in each figure and detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Membranes were incubated with one of the primary antibodies at the indicated dilutions: HRP-conjugated monoclonal Anti-V5 antibody at 1:5,000 (Invitrogen); HRP-conjugated monoclonal anti-HA antibody at 1:1,000 (Roche); mouse anti-HA monoclonal antibody at 1:4,000 (Roche); mouse anti–ubiquitin monoclonal antibody at 1:10,000 (Sigma-Aldrich); rabbit anti-Dcp1a serum at 1:4,000 (Bethyl Laboratories, Inc. or gift from S. Ohno); rabbit anti-Rck/p54 serum at 1:1,000 (Bethyl Laboratories, Inc.); mouse monoclonal antibody against GAPDH at 1:10,000 (Research Diagnostics, Inc.); and mouse antibody against lamin A/C at 1:1,000 (Santa Cruz Biotechnology, Inc.). To detect endogenous Caf1, Pan2, and Pan3 in mouse NIH3T3 or monkey COS7 cells, the corresponding polyclonal antibodies were generated in rabbits immunized with gene-specific peptides using the custom antibody service from Bethyl Laboratories, Inc. Affinity-purified anti-peptide antibodies were used at the following dilutions: rabbit anti-Pan3 peptide antibody at 1:2,000; rabbit anti-Caf1 peptide at 1:1,000; or rabbit anti-Pan2 peptide at 1:2,000. Rabbit anti-PABP antibody (gift from R. Lloyd, Baylor College of Medicine, Houston, TX) was used at a 1:4,000 dilution. HRP-conjugated donkey anti-rabbit IgG antibodies (1:4,000; GE Healthcare) or goat anti–mouse (1:10,000; Bio-Rad Laboratories) were used as secondary antibodies for detection with a chemiluminescence reagent (peroxide/luminol enhancer; Thermo Fisher Scientific).

Immunofluorescence microscopy
NIH3T3 cells were seeded in 6-well plates at a density of 0.4 x 10⁶ cells per well, 24 h before transfection using Lipofectamine 2000 (Invitrogen). At 22–26 h after transfection, cells were reseeded to slide chambers (BD Biosciences) and incubated overnight. For cycloheximide, puromycin, or arsenite treatment, cells were incubated in media containing either 10 μg/ml cycloheximide, 10 μg/ml puromycin, or 100 μM arsenite
of rabbit antibody was incubated for 5 min at RT with 5 μl Zenon rabbit camera (Coolsnap HQ; Roper Scientific). Stacks of 20 images were processed without DAPI was added. Microscopy, all of the primary and secondary antibodies, except the mouse anti-myc serum, were diluted 1:50 in PBS. Endogenous Pan2 was detected using rabbit anti-Pan2 labeled with Zenon 555 rabbit IgG labeling reagent. Endogenous Dcp1α, Xrn1, or Pan3 was detected using rabbit anti-Dcp1α, anti-Xrn1, or anti-Pan3 labeled with Zenon 488 rabbit IgG labeling reagent. HA-tagged Pan3, Ccr4, or Caf1 was detected using rat anti-HA monoclonal antibody and Alexa Fluor 350 goat anti-rat IgG. HA-Pan2 or V5-tagged proteins were detected using rat monoclonal anti-HA or mouse monoclonal anti-V5 and Alexa Fluor 555 goat anti-rat IgG. Rabbit anti-Xrn1 antibody was a gift from J. Lykke-Andersen (University of Colorado at Boulder, Boulder, CO), human anti-GW182 antisera was a gift from M.J. Fritzler (University of Calgary, Calgary, Canada), rabbit anti-G3BP1 was a gift from R. Lloyd, and rabbit anti-Dcp1α antibody was a gift from S. Ohno. The mouse anti-myc monoclonal antibody from culture medium collected from anti-myc transfected monkey COS7 cells. Online supplemental material is available at http://jcb.org/cgi/content/full/jcb.200801196/DC1.

References


