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Mass spectrometric identification of proteins that interact through specific domains of the poly(A) binding protein

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Abstract

Poly(A) binding protein (PAB1) is involved in a number of RNA metabolic functions in eukaryotic cells and correspondingly is suggested to associate with a number of proteins. We have

used mass spectrometric analysis to identify 55 non-ribosomal proteins that specifically interact with PAB1 from *Saccharomyces cerevisiae*. Because many of these factors may associate only indirectly with PAB1 by being components of the PAB1-mRNP structure, we additionally conducted mass spectrometric analyses on seven metabolically defined PAB1 deletion derivatives to delimit the interactions between these proteins and PAB1. These latter analyses identified 13 proteins whose associations with PAB1 were reduced by deleting one or another of PAB1's defined domains. Included in this list of 13 proteins were the translation initiation factors eIF4G1 and eIF4G2, translation termination factor eRF3, and PBP2, all of whose previously known direct interactions with specific PAB1 domains were either confirmed, delimited, or extended. The remaining nine proteins that interacted through a specific PAB1 domain were CBF5, SLF1, UPF1, CBC1, SSD1, NOP77, yGR250c, NAB6, and GBP2. In further study, UPF1, involved in nonsense-mediated decay, was confirmed to interact with PAB1 through the RRM1 domain. We additionally established that while the RRM1 domain of PAB1 was required for UPF1-induced acceleration of deadenylation during nonsense-mediated decay, it was not required for the more critical step of acceleration of mRNA decapping. These results begin to identify the proteins most likely to interact with PAB1 and the domains of PAB1 through which these contacts are made.

Keywords

PAB1; Proteome; UPF1; Nonsense-mediated decay; Protein domain interactions

Introduction

The poly(A) binding protein (PAB1 from *Saccharomyces cerevisiae* and PABPC from humans) binds the poly(A) tail of mRNA and functions at a number of steps involving RNA metabolism (Kuhn and Wahle 2004; Mangus et al. 2003). mRNA polyadenylation, export, translation, and turnover have all been shown to be affected by PAB1 (Kuhn and Wahle 2004; Mangus et al. 2003; Hosoda et al. 2006). The fact that PAB1 plays a number of roles in both the nucleus and the cytoplasm indicates that it may be in contact with multiple proteins and complexes and that these interactions may dictate its functions. Most importantly, PAB1 protein interactions may be continually changing as it regulates RNA metabolism in the cell.

Several whole organism proteomic analyses have been conducted to identify all the protein complexes within a yeast cell or involved in a given process (Gavin et al. 2002; Ho et al. 2002; Krogan et al. 2004; Staub et al. 2006). A summary of mass spectrometric analyses involving purified TAP-tagged proteins has identified 41 significant nonribosomal protein interactions with PAB1 (Collins et al. 2007), and other studies have demonstrated additional putative PAB1 protein targets (SGD database). Yet, for the most part, the significance of these interactions has not been defined, nor has the domains of PAB1 important for these contacts been determined.

The PAB1 protein consists of six readily defined functional regions (Fig. 1). At its N-terminus are four RNA binding motifs (RRM domains). While RRM1 and RRM2 of PAB1 appear to bind most strongly to poly(A) (Kessler and Sachs 1998; Deardorff and Sachs 1997), RRM3 and RRM4 can also make critical contacts (Kessler and Sachs 1998; Deardorff and Sachs 1997; Deo et al. 1999) and may bind U-rich regions located adjacent to the poly(A) tail (Mullin et al. 2004; Sladic et al. 2004). Its C-terminal region comprises a penultimate proline-rich (P) domain and a terminal structured region (C), neither of which is critical for RNA binding (Kessler and Sachs 1998; Yao et al. 2007). Each RRM domain is comprised of four antiparallel α -strands (the RNA binding surface) that is backed by two β -helices (Deo et al. 1999).

A few of the protein contacts for each of these PAB1 domains have been defined through conventional biochemical analyses. For example, the C region of PAB1 binds the PAN2/3 deadenylase (Siddiqui et al. 2007; Mangus et al. 1998, 2004) that functions in poly(A) trimming. Whether this process takes place solely in the nucleus, upon export, or in the cytoplasm is still not clear (Brown et al. 1996; Brown and Sachs 1998; Kuhn and Wahle 2004). The translation termination factor eRF3 also contacts the C-terminus (Hosoda et al. 2003), as do other proteins in mammalian systems (Kuhn and Wahle 2004; Mangus et al. 1998, 2004). The P domain is responsible for PAB1 self-association (Yao et al. 2007; Melo et al. 2003; Kuhn and Pieler 1996). RRM2, with the aid of RRM1, contacts eIF4G (Tarun and Sachs 1996), important in forming the closed-loop structure of mRNA (Kuhn and Wahle 2004). Some of the key residues for this interaction in RRM2 are 180–182 and 184–187, whose alterations in vitro block PAB1-eIF4G contacts and translation (Otero et al. 1999). The RRM1 and the P domain are most important to PAB1 for deadenylation by the major deadenylase CCR4-NOT (Yao et al. 2007; Lee et al. 2010). The RRM1 domain but not the P domain was also found to be most critical for PUF3-induced deadenylation (Lee et al. 2010). Both of these regions of PAB1 were also shown to be critical for PAB1 self-association (Yao et al. 2007). In contrast, deleting the RRM3 domain accelerated deadenylation by some unknown mechanism (Yao et al. 2007). Also, deletion of RRM4, but not other domains, has been shown to reduce mRNA transport to the cytoplasm (K. Weis, personal communication) (Brune et al. 2005; Simon and Seraphin 2007). Finally, in vivo protein synthesis analysis indicates that RRM1 and RRM2 are more critical to this process (Yao et al. 2007; Ohn et al. 2007) with the other domains having no or limited effect on in vivo translation (see Table 1 for a summary of known PAB1 domain contacts).

To expand on previous studies that used mass spectrometric techniques to detect proteins interacting with PAB1, we have included two control mass spectrometric experiments in our studies so as to eliminate many nonspecific interactions that might have been detected. We also incorporated the analyses of PAB1 deletion derivatives to identify possible PAB1-specific domain interactions. We identified 55 proteins that associated with PAB1, the vast majority of which would be expected to be in the presence of PAB1 and mRNA due to their known roles in RNA metabolism. Using PAB1 deletion derivatives, we delimited this group of 55 proteins to 13 proteins that interacted with PAB1 dependent on one specific PAB1 domain or another. Four of the six previous known specific PAB1 domain interactors were found in this group of 13 proteins, confirming the validity of this approach. We extended this analysis by verifying that UPF1 did interact with PAB1 through the RRM1 domain. The RRM1 domain, in turn, while important for UPF1-induced deadenylation in nonsense-mediated decay (NMD), was not required for the more critical decapping step in NMD.

Materials and methods

Yeast strains and growth conditions

The parental yeast strain AS319/YC504 (*MAT⁺ ura3 leu2 trp1 his3 pab1::HIS3 (YC504-Flag-PAB1-TRP1)*) was generally used for Flag pull-downs and mass spectrometric analyses. Different PAB1 variants, as indicated in Fig. 1 and the text, were swapped into this strain using standard genetic methods (Yao et al. 2007). The strain carrying the *cdc33-1* allele (AS1881/YC504) was isogenic to this strain. For one series of mass spectrometric analyses with the seven different deletion derivatives of PAB1, strain 1773-10 was used whose genotype was the same as AS319/YC504 and is 75 % congenic with AS319/YC504. For the control experiments AS319/YC360 was used (isogenic to strain AS319/YC504 except for carrying plasmid *YC360-PAB1-URA3* instead of plasmid *YC504*) (Yao et al. 2007). For the UPF1-PAB1 protein analysis, strain AS319/YC504 (PAB1-wt) or YC505 (PAB1- RRM1) was transformed with plasmid pRS315 (*HA-UPF1-LEU2*) or pRP910 (*UPF1-LEU2*). Deadenylation assays were conducted in the *UPF1* background with strains AS319/YC504

and AS319/YC505 each transformed with plasmid pRP469 (*PGK1pG URA3*) or pRP1078 (*PGK1pG-319 URA3*) and in the *upf1* background with strains RR27-1 (*Mata ura3 leu2 trp1 his3 pab1::his3::Neo upf1 ::HIS3*) carrying the YC504, YC505, and *PGK1* plasmids as described for AS319.

Yeast strains were routinely grown on minimal medium supplemented with 2 % glucose and the appropriate amino acids (Yao et al. 2007). For the RNA pulse-chase experiments, the initial growth of cells was in medium containing 2 % sucrose.

Mass spectrometric analysis

Flag immunoprecipitated extracts were fractionated by SDS-PAGE prior to trypsin digestion of gel slices across the gel lane as described (Kristensen et al. 2008). Tandem mass spectrometry (LC MS/MS) was used for peptide and protein identification as previously described (Andersen et al. 2002; Gruhler et al. 2005).

Protein immunoprecipitations

Flag pull-down experiments conducted either for Western analysis or for mass spectrometric analysis were conducted as previously described (Liu et al. 1998; Yao et al. 2007). RNase A (0.1 mg/mL) treatment of extracts was conducted for 30 min prior to the treatment of the extracts with Flag beads. For Western analysis, 50 mL cultures were routinely used. For the mass spectrometric studies, 300 mL cultures were used. The HA immunoprecipitations were conducted in a manner similar to that described for the Flag immunoprecipitations.

RNA analysis

Pulse-chase analyses for the *GAL1-PGKpG* mRNA were conducted as previously described (Lee et al. 2010; Tucker et al. 2001; Cao and Parker 2003). Briefly, after growth of cells in non-inducing medium containing 2 % sucrose, the mRNA was induced for 10 min with 2 % galactose and the mRNA expression was shut off with 4 % glucose. At the time points indicated, the RNA was isolated and subjected to Northern analysis following polyacrylamide gel electrophoresis. The oligo(A) lengths were determined using the following standards: the length of the completely deadenylated *PGK1* poly(A) tail (dT sample), the length of the deadenylated *PGK1* fragment, and the length of the completely undeadenylated poly(A) tail at time zero based on other experiments using different *GAL1* poly(A) tail lengths as standards. All pulse-chase experiments were conducted at least in duplicate.

Results

Purification of proteins associating with PAB1 using Flag-PAB1 derivatives

Prior to conducting mass spectrometric studies on PAB1-associated proteins, we assessed whether proteins known to be associated with PAB1 could be co-purified using a PAB1 tagged at its N-terminus with the Flag peptide (Yao et al. 2007). Following purification of Flag-PAB1, both eIF4G1 and eIF4E were found to co-elute with PAB1 (Fig. 2a, lane 2). Removal of RRM2 from PAB1 diminished eIF4G1 co-elution and reduced eIF4E association (Fig. 2a, lane 4), as previously demonstrated using in vitro binding assays (Kessler and Sachs 1998). In addition, we showed that the PAB1-184 protein, carrying mutations in amino acids 184-186 of RRM2 that fails to bind eIF4G in vitro (Otero et al. 1999), resulted in reduced eIF4G1 binding to PAB1 but did not eliminate it (Fig. 2a, lane 10). Similar results, albeit not as dramatic as observed for PAB1-184, were obtained for PAB1-180, which mutated residues 180,181 (Otero et al. 1999) (Fig. 2b, lane 4). In contrast, other point mutations in PAB1 such as PAB1-134 that affects an unknown translation process of PAB1 (Otero et al. 1999; Ohn et al. 2007) and PAB1-F170V that affects PAB1

binding to poly(A) (Deardorff and Sachs 1997; Yao et al. 2007) did not have any effect on eIF4E or eIF4G1 binding (Fig. 2a, lanes 11 and 9, respectively). These results indicate that our purifications of Flag-PAB1 are capable of recapitulating PAB1-specific interactions that have been studied previously. However, our analysis of interactions present in crude extracts between PAB1 and eIF4G1 was found to be more robust than those observed in vitro, suggesting that in vivo the contact between PAB1 and eIF4G1 is not stabilized by a simple single interaction.

Isolation of Flag-PAB1 from a strain carrying the *cdc33-1* allele (encoding an eIF4E protein that is defective for cap binding at 37 °C but that is stably expressed) (Altmann et al. 1989), reduced both the eIF4E and eIF4G1 association with PAB1 (Fig. 2b, lane 2). Combining *cdc33-1* with either PAB1-184 or PAB1-180 (Fig. 2b, lanes 3 or 4) severely interfered with eIF4G1 and eIF4E binding to PAB1. It should be mentioned that eIF4A was not found to be present in our Flag-PAB1 immunoprecipitations as assessed by Western analysis (not shown). This result is expected, as eIF4A from yeast is known to be transiently associated with the eIF4F complex that contains eIF4G and eIF4E (Neff and Sachs 1999; Von der Haar and McCarthy 2002; Pause et al. 1994; Yoder-Hill et al. 1993).

Rationale for using mass spectrometric analysis to identify PAB1-mRNP protein contacts to specific PAB1 domains

Our rationale for identifying the most likely bona fide protein contacts either with PAB1 or within the context of the PAB1-mRNP structure was as follows. While a number of proteins are known to associate with PAB1 through previous mass spectrometric experiments (Gavin et al. 2002; Ho et al. 2002), the specificity of proteins interacting with PAB1 or its domains could not be determined. We sought to significantly bypass this limitation by delimiting contacts to specific domains of PAB1, thereby identifying the specificity of these interactions suggestive of their directness. This analysis would begin to approximate the bona fide PAB1 proteome. The identification of specific domains of PAB1 used in these contacts also would allow internal verification of the validity of the approach since a few proteins have been shown to bind to specific PAB1 domains (Table 1).

Two types of control experiments (done at least in duplicate) were conducted to eliminate contaminating proteins from the list of proteins interacting with PAB1. The first was to conduct mass spectrometric analysis on Flag bead purified material from a strain with PAB1 without the Flag tag. The second was to conduct mass spectrometric analysis on Flag bead purified material extracted from strains carrying the Flag-PAB1 following an extensive RNase A treatment. RNase A treatment eliminates PAB1 binding to the poly(A) tail, allowing us to identify only those proteins that associated with PAB1 within the context of the PAB1-mRNP structure (Yao et al. 2007). Each control experiment was conducted with strains carrying either wild-type PAB1 (without the Flag tag) or with Flag-PAB1 (RNase A treatment) and compared with Flag-PAB1 (no RNase A treatment). The number of unique peptides detected for each protein present following the Flag pull down experiment rather than the number of total peptides detected was compared between these samples. Significant bias can be introduced with the counting of the total peptides due to the fact that certain peptides are more readily detected by mass spectrometric analysis than other peptides (Fleischer et al. 2006). Proteins that were not present in the control samples and which were present in the arbitrary cut-off of greater than 40 % of the experimental samples with Flag-PAB1 were considered to be likely PAB1-associated proteins. Table 2 lists these 44 proteins, the average number of unique peptides observed in each case, their protein abundance factor (PAF), and the most likely function related to PAB1. A PAF value represents the average number of unique peptides observed divided by the molecular weight of the protein (10× kDa). The PAF value normalizes the number of unique peptides to the size of the protein, which, in turn, is proportional to the number of possible tryptic peptides

that could be observed in the experiment (Fleischer et al. 2006). An additional nine proteins interacted with PAB1 in 40 % or less of the mass spectrometric experiments (Table 3). However, the PAF score for these proteins was uniformly at the lower end when compared with the list presented in Table 2, supporting our limiting the most likely PAB1 interacting proteins to those in Table 2.

Comparison of our identified PAB1 interactors with known PAB1 protein contacts

We judged that our analysis was detecting and identifying specific PAB1-mRNP contacts by three means. First, the summary of two different TAP mass spec analyses of the yeast proteome (Collins et al. 2007; Ho et al. 2002; Gavin et al. 2002) have identified 41 significant non-ribosomal protein contacts to PAB1. Of the top 12 proteins on this list, we identified 8 of these (eIF4G1, eIF4G2, CBC1, NAB6, NAB3, SGN1, GBP2, and CBF5).

Second, the direct interactions of PAB1 with other proteins have been studied by other biochemical procedures. Translation initiation factors eIF4G1 and eIF4G2 are known to contact PAB1 through its domains RRM1 and RRM2 (Tarun and Sachs 1996; Otero et al. 1999), eRF3, involved in translation termination, is known to contact PAB1 through its C domain (Gorgoni and Gray 2004), and PBP2 is known to contact PAB1 through either the P or C domain (Mangus et al. 1998). All four of these proteins were found in our group of 44 proteins associating with the PAB1-mRNP structure.

Third, our list of 44 proteins contains 38 proteins that would be expected to associate with the PAB1-mRNP complex. There are nine proteins involved in translation, three in mRNA decay, seven in RNA binding, three in mRNA transport, one in splicing, and another fifteen proteins in nucleolar and/or ribosomal biogenesis, all processes known to include PAB1 (Table 2) (Peng et al. 2003; Brune et al. 2005; Yao et al. 2007). Only six other proteins were identified that play no obvious roles related to that of PAB1.

Identification of proteins that interacted with specific PAB1 domains

Because the above list of 44 possible and known PAB1-associated proteins may contain proteins that are part of the PAB1-mRNP structure but are not dependent on binding PAB1 through any of its domains, we wished to further delimit this group by identifying those proteins that interacted through a specific PAB1 domain. To identify specific protein contacts to different domains of PAB1, mass spectrometric analysis was conducted on all the proteins that co-purified with each of seven Flag-PAB1 derivatives (PAB1, – RRM1, – RRM2, – RRM3, – RRM4, – P, – C; see Fig. 1). Each strain carried only the Flag-PAB1 derivative as indicated, for the genomic *PAB1* gene that had been deleted (Yao et al. 2007). Prior to mass spectrometric analysis, the resultant immunoprecipitations were shown to contain equivalent amounts of each PAB1 derivative as detected by Western analysis (data not shown). Each of these derivatives have been extensively characterized for effects on poly(A) binding, mRNA export, translation, deadenylation, and decapping (unpublished observation) (Yao et al. 2007; Brune et al. 2005; Dunn et al. 2005; Kessler and Sachs 1998; Simon and Seraphin 2007), and they do not result in severe growth defects. All PAB1 derivatives were assayed for general effects on in vivo protein synthesis (Yao et al. 2007; Ohn et al. 2007) and for effects on ribosomal and polysomal abundance. No specific effects on 80S ribosomal and polysomal abundance were observed with any of the PAB1 deletions (data not shown). In terms of global protein synthesis, deleting either RRM1 or RRM2 had the most general effects: 28 % reduction by RRM1 and 15 % by RRM2, whereas the other deletions had insignificant effects. These effects by RRM1 and RRM2 deletions are, however, not overly severe, for in contrast, the *cdc33-1* (eIF4E) or *prt1-46* (eIF3b) alleles block translation by at least 70 % (Yao et al. 2007; Ohn et al. 2007). The RRM1 and P domains are known to be required for general and regulated deadenylation, and RRM3

restricts the dead-enylation process (Yao et al. 2007; Lee et al. 2010; Simon and Seraphin 2007). Deletion of the RRM4 domain, but not other domains, also appears to play some role in mRNA export from the nucleus (Brune et al. 2005; Simon and Seraphin 2007) (see Table 1 for a summary of these previously known interactions).

Following our mass spectrometric analysis done in duplicate for each PAB1 derivative, the number of unique peptides of proteins associated with a particular PAB1 variant was determined. Only those proteins (not present in the control experiments described above) which were found to be associated with at least 1 derivative in both duplicate analyses and which were present in greater than 40 % of the total of 14 PAB1 derivative mass spectrometric analyses were considered as likely PAB1 interacting proteins. This group of 43 proteins overlapped significantly with the proteins identified only in the wild-type PAB1 Flag pull downs described above: 32 were present in both sets (Table 4). The 11 new proteins found to be associated with the majority of PAB1 deletion derivatives included 1 RNA binding protein involved in translation and mRNA degradation (SBP1), 2 proteins in mRNA splicing (RAI1, and SMB1), 3 nucleolar/RNA biogenesis proteins (MAK21, GAR1, and NHP2), 2 mitochondrial mRNA splicing proteins (CBP2 and MSS116), and 3 other proteins (RMD11, MPD1, and an unknown protein). An additional eight proteins interacted in 40 % or less of the mass spectrometric experiments (Table 5) but were judged less likely to be associated with PAB1, as, again, their PAF scores were extremely low.

Of the 43 proteins that specifically co-immunoprecipitated with the various PAB1 deletion derivatives, the average number of unique peptides found for each PAB1 derivative was compared across the derivatives. Those PAB1-associated proteins that displayed twofold differences in peptide abundance (Table 6), as compared to wild-type PAB1, were initially judged as displaying possible specific contacts to one or another of the PAB1 domains. By this criterion, only 13 proteins were affected in their binding to one or another of the PAB1 domains (Table 6). Of these 13 proteins, we identified several proteins that previous biochemical studies have demonstrated or suggested to make specific contacts to one or another of the PAB1 domains. eIF4G1 and eIF4G2 are known to contact PAB1 through at least the RRM1 and RRM2 domains (Tarun and Sachs 1996; Otero et al. 1999) with RRM2 being most critical, which we have confirmed (Table 6). eRF3, involved in translation termination, is known to contact PAB1 through its C domain (Gorgoni and Gray 2004), an observation we confirmed and extended by indicating that the P region was also important to this contact. Finally, PBP2 is known to contact PAB1 through either the P or C domain (Mangus et al. 1998), and we established that it is the C region and not the P domain that was critical for this interaction. These correspondences and extensions indicated that the methodology we were using was consistent with published biochemical analyses. Importantly, these similarities indicate that the specific domain interactions we were observing for the other nine proteins (NAB6, UPF1, SSD1, CBC1, GBP2, CBF5, SLF1, NOP77, and yGR250c) were very likely to be valid. It should be noted that while NOP77 appears to interact through RRM1 of PAB1, there appears sufficient variability in all of its interactions with PAB1 deletion derivatives to place less confidence on this particular identification.

To further assess the importance of the differences we observed in the interactions of certain PAB1 deletion derivatives with the particular proteins listed in Table 6, we analyzed the intensities of specific peptide species that were co-immunoprecipitated with each PAB1 deletion derivative. Intensity refers to the number of times a particular peptide was detected in the mass spectrometric experiment. In this case, if a protein interacted through a particular PAB1 domain, then specific peptides of that protein should be decreased or absent for the mass spectrometric analysis conducted with that PAB1 deletion derivative when compared across all PAB1 deletion derivatives. By analyzing particular peptide species across the

mass spectrometric data for the PAB1 deletion derivative pull downs, we would not be creating a bias in terms of the ability of the mass spectrometer to detect a particular peptide species. This analysis was, however, limited to only those peptides for a particular protein that were identified amongst most, if not all, PAB1 deletion analyses. This analysis could not be confidently done for the following proteins because of the low level of unique peptides identified across most PAB1 deletion derivatives: CBC1, CBF5, SLF1, eRF3, NOP77, and yGR250c.

Table 7 lists the intensity averages for the peptides of each protein interacting with specific PAB1 deletion derivatives. For example, of the 14 peptides of eIF4G1 that were identified in this analysis, significantly less eIF4G1 peptide intensities were observed in the mass spectrometric studies with PAB1 derivatives deleted for RRM2 or RRM1 when compared to the experiments done with the other deletion derivatives. This result is consistent with the above identification of RRM1 and RRM2 as being important to the PAB1 contact made by eIF4G1 based on the average number of unique peptides identified in the mass spectrometric analysis (Table 6). Similarly, eIF4G2 displayed reduced peptide intensities in the pull-down experiments with the RRM1 and RRM2 deletion derivatives. Significantly, however, eIF4G2 did not display reduced intensities of particular peptides for the P domain deletion of PAB1, although eIF4G2 had a reduction in the average number of unique peptides identified in the P domain deletion derivative as compared to other domains deletions (Table 6). Therefore, the P domain of PAB1 is not likely to be a specific region through which eIF4G2 acts. As controls for these sets of analyses, proteins, such as XRN1, RRP5, RLR1, and yLR419w, which did not display differences in binding PAB1 deletion derivatives based on comparing unique peptides also showed no differences when the intensities of individual peptides associating with the PAB1 deletion derivatives were compared (see legend for Table 7).

The intensity analyses for the peptides of other proteins interacting with specific domains of PAB1 confirmed that SSD1 interacted through RRM3, GBP2 through RRM1, PBP2 through the C domain, and NAB6 through the RRM1 domain (Table 7). However, for NAB6 the data also indicate that the RRM2 domain may play some role in binding it. Overall, the analysis of the intensity differences for particular peptides supports the assignment of PAB1 domains for being important in interacting with specific proteins based on the number of unique peptides observed in the mass spectrometric analyses (Table 6).

UPF1 interaction with PAB1 requires the RRM1 domain but not the C-terminal region of PAB1

Based on our mass spectrometric studies, we chose to further investigate the putative UPF1 interactions with PAB1, as mammalian studies had indicated that UPF1 controls NMD in an interaction with termination factor eRF3 that, in turn, restricts binding of PABPC1 to eRF3 (Brognia and Wen 2009). Such an interaction suggests that it is the C-terminal domain of mammalian PABPC1 that would be important to UPF1 interactions, in contrast to the RRM1 domain as suggested by our studies for yeast PAB1. To examine UPF1 and PAB1 interactions further, we chose to study them in the reverse direction. By first using an HA-tagged UPF1 protein to purify UPF1 from yeast cells, we then queried whether PAB1 or PAB1- RRM1 could be co-immunoprecipitated. As displayed in Fig. 3, immunoprecipitation of HA-UPF1 was capable of immunoprecipitating Flag-PAB1 but was unable to co-immunoprecipitate Flag-PAB1-DRRM1 (compare lane 3–4). These results confirm the mass spectrometric analyses described above. Importantly, the truncated form of PAB1 (PAB1-T) (lane 3), which is missing both the P and C domains of PAB1 (mass spectrometric analysis of PAB1-T indicated that the truncation occurs between residues 496 and 506 of PAB1, unpublished observation), was quite able to interact with HA-UPF1. Therefore, PAB1 requires its RRM1 but not its P or C domain to interact with UPF1.

The RRM1 domain of PAB1 is required for UPF1-dependent NMD deadenylation but not decapping

If the interaction between PAB1 and UPF1 were to be physiologically important, we might expect that the RRM1 domain of PAB1 would play a role in UPF1-mediated NMD. However, a previous study indicated that deletion of PAB1 from yeast does not impair the NMD process (Mieux et al. 2008). As NMD consists of the acceleration of two separable steps in the degradation of mRNA, decapping and deadenylation (Cao and Parker 2003), it remains possible that PAB1 is not required for the major part of NMD (decapping) but plays a role in the secondary process of deadenylation. Previous results have also established that PAB1 deletion derivatives have no effect on general decapping (Yao et al. 2007) but that they are critical for deadenylation (Yao et al. 2007; Lee et al. 2010; Simon and Seraphin 2007).

We consequently used pulse-chase analysis to test the effect of deleting the RRM1 domain of PAB1 on nonsense-mediated decay processes. These analyses used *GAL1-PGK1* mRNA that was either wild-type or contained a nonsense mutation in residue 319 that subjects the mRNA to NMD (Cao and Parker 2003). Isogenic yeast strains carrying either Flag-PAB1 or Flag-PAB1- RRM1 with either *GAL1-PGK1pG* or *PGK1pG-319* were pregrown in non-galactose inducing medium and then subjected to a brief induction of the *GAL1* promoter with the addition of galactose (the pulse), prior to shutting off of gene expression with glucose (the chase). Following extraction of RNA at various times after the shutoff of transcription, northern analysis was utilized to identify the *PGK1* mRNA species present as a function of time. In Fig. 4a, using wild-type PAB1 and wild-type *PGK1pG* mRNA, *PGK1pG* mRNA was deadenylated slowly in a basically distributive manner represented by the bulk of the deadenylated species moving as a relatively tight band in which it became progressively deadenylated. At about 8–12 min the 10 A's oligo(A) species that represents nearly completely deadenylated species began to become abundant and the tightness of the band became reduced, as deadenylation shifted from a primarily distributive to a processive mode (see top densitometric scan). A decapped *PGK1* mRNA fragment that was deadenylated began to be visualized over background around 4–8 min and became quite abundant at later times at 20 min (see densitometric scans), indicative of extensive decapping once the oligo(A) species was formed. These results are very similar to those obtained previously for *PGK1* mRNA (Decker and Parker 1993; Muhlrads et al. 1994; Tucker et al. 2001; Cao and Parker 2003). However, it should be noted that in our experiments a small amount of decapped and nearly fully deadenylated fragment is present at the zero time point, although its abundance is very low relative to the abundance of the full-length *PGK1pG* mRNA at the same time.

As expected from previous studies (Cao and Parker 2003), in a wild-type PAB1 background the NMD target mRNA, *PGK1pG-319*, displayed much more rapid decapping and deadenylation, as evidenced in Fig. 4c. A significant abundance of deadenylated and decapped *PGK1pG-319* fragment appeared immediately and was in significant abundance as compared to that of the full-length mRNA (early time points). The increased ratio at early time points of decapped fragment to full-length mRNA for the *PGK1-319* mRNA in comparison to the ratio for the wild-type *PGK1* mRNA indicates much more rapid degradation of the *PGK1* mRNA containing the premature termination codon, as expected. Moreover, scrutiny of the early time points also indicates that a significant amount of the fragment displayed a large spread of poly(A) lengths from 70 A's to 10 A's, indicative of rapid decapping regardless of the poly(A) tail length that was present (see densitometric scans at early time points). In addition, it can be observed that the full-length *PGK1pG-319* mRNA did not uniformly decrease in poly(A) length as a tight band as it had for the *PGK1pG* mRNA (Fig. 4a). This is consistent with a switch to primarily processive

deadenylation in which full length poly(A) tails are present in the population along with completely deadenylated species.

In contrast to these results with wild-type PAB1, deleting the RRM1 domain of PAB1, blocked both normal deadenylation of *PGK1pG* and that of *PGK1pG-319* (Fig. 4b, d, respectively). In the wild-type *PGK1pG* mRNA situation, the RRM1 deletion blocked deadenylation in which no fragment accumulated (Fig. 4b). This result is consistent with deadenylation normally preceding decapping and being required for it (Decker and Parker 1993). For the *PGK1pG-319* mRNA, little apparent deadenylation of full-length *PGK1pG-319* occurred, yet the *PGK1* fragment appeared very rapidly, albeit immediately in the fully polyadenylated form. It should be noted that this fragment species did not represent the full-length mRNA species, for at later times it decreased noticeably to sizes smaller than the 0A form of the full-length mRNA version. Therefore, it corresponds to the *PGK1* fragment. In addition, both the full-length mRNA version and the fragment initially have more A's than are present in the wild-type PAB1 situation (compare Fig. 4b to that of a and Fig. 4d to that of 4c). This increased poly(A) tail length at initial times is due to the ability of the RRM1 deletion of PAB1 to block both CCR4 and PAN2 deadenylation (Yao et al. 2007) (data not shown). Blocking PAN2 deadenylation results in increased poly(A) tail lengths (Brown and Sachs 1998). These results indicate that RRM1 of PAB1 does not impair NMD-induced decapping, but it is required for the NMD acceleration of deadenylation.

Because RRM1 is required for all deadenylation processes that have been analyzed, including those that are constitutive (Yao et al. 2007) and regulated (Lee et al. 2010), it remains possible that the presumed contact of UPF1 to that of the RRM1 of PAB1 has nothing specifically to do with regulating NMD-enhanced deadenylation. To explore this possibility further, we tested the requirement for RRM1 on deadenylation in an *upf1* background. Using the same pulse-chase experimental strategy described above, we first analyzed the effect of the *upf1* on *PGK1* mRNA deadenylation. In this case, the mRNA became deadenylated at initial times nearly at the same rate as observed in the *UPF1* background, with the oligo(A) species becoming present at around 12 min after the transcriptional shutoff (Fig. 5a). However, it should be noted that the distribution of poly(A) tails is significantly different between the *UPF1* and the *upf1* backgrounds. The poly(A) tail distribution in the *upf1* background remained tighter at all time points, indicative of a primarily distributive deadenylation pattern (see densitometric scans for additional clarity). Importantly, there was no shift to the processive pattern that was apparent with the *UPF1* background at time points after 8 min as observed in Fig. 4a. This *upf1* effect on the spread of poly(A) tail lengths during deadenylation is the same as previously published, albeit unremarked upon at the time (Cao and Parker 2003). These data suggest that UPF1 may play a role in the switch from distributive to processive deadenylation, a process requiring PAB1 removal from the mRNA (Tucker et al. 2002; Viswanathan et al. 2003; Yao et al. 2007). In agreement with this observation, fewer deadenylated fragments were seen in the *upf1* background in Fig. 5a as compared to the *UPF1* background (Fig. 4a). This result is also the same as previously published (Cao and Parker 2003). This is consistent with fewer oligo(A) species being formed and subsequently decapped due to the reduction in processive deadenylation caused by the *upf1* mutant.

In the case of NMD in a *upf1* background, *PGK1-319* mRNA was deadenylated in a similar manner to that of the wild-type *PGK1* mRNA (compare Fig. 5c to a), as UPF1 is known to block NMD deadenylation. Little or no *PGK1* fragment was observed, consistent with distributive deadenylation and little or no oligo(A) species were formed due to reduction in processive deadenylation (in long exposures of Fig. 5c only a very little abundance of the fragment was detected).

In the strain background deleted for RRM1, wild-type *PGK1* mRNA did not appreciably deadenylate, as expected (Fig. 5b). Similarly, the RRM1 deletion significantly blocked *PGK1-319* mRNA deadenylation (Fig. 5d). No *PGK1* mRNA fragments were observed in either case. These data suggest that RRM1 is required for deadenylation independent of the presence of UPF1. Other roles for the UPF1–RRM1 interaction, as in the switch from distributive to processive deadenylation, remain possible (see “Discussion”).

Discussion

Mass spectrometric identification of proteins interacting through specific PAB1 domains

We have used mass spectrometric techniques to identify a total of 55 non-ribosomal proteins that associate with PAB1 (Tables 2, 4). All but 11 of these proteins are likely components of RNA complexes or processes that involve PAB1. Many of these proteins are RNA binding factors that would be expected to interact with the mRNA and therefore would be part of the PAB1-mRNP structure. A second large group of proteins that we found to be associating with PAB1 are nucleolar and/or involved in ribosomal biogenesis. Previous mass spectrometric studies on nucleolar components have identified PAB1, but it remains unclear as to the role that PAB1 plays in these processes (Mnaimneh et al. 2004; Staub et al. 2006; Krogan et al. 2004). There is some evidence, however, that PAB1 aids 60S assembly to form a competent translation complex (Searfoss et al. 2001). Therefore, the types of proteins we have identified as associating with PAB1 support the validity of mass spectrometric approaches in defining PAB1 interacting components. However, similar to previous mass spectrometric studies, our list of proteins may not be specific to PAB1 and may be found to associate with PAB1 through very indirect RNA interactions.

We have endeavored to surmount the above limitation attached to global mass spectrometric studies using seven different PAB1 deletion derivatives in our analyses. In this case, by comparing the effect of well-defined domain deletions in PAB1 to each other and to wild-type PAB1, we were able to significantly shorten this list of 55 proteins associating with PAB1 to 13 factors. Of these 13 proteins, each was affected in its interaction with PAB1 for at least one of the PAB1 deletion derivatives. In validation of this methodology, we were able to identify four of the six previously known proteins that interact through specific PAB1 domains: eIF4G1, eIF4G2, PBP2, and eRF3. Our results confirmed that the eIF4G proteins contact PAB1 through the RRM1 and RRM2 domains, extended the contact region of eRF3 to PAB1 to include the P domain, and delimited the PBP2 contact to PAB1 to just the C domain. Neither PBP1 nor PAN3, the other two proteins known to contact particular regions of PAB1 (Mangus et al. 1998; Hoshino et al. 1999), were found in any of our mass spectrometric analyses. In the case of PBP1, it has been shown recently to associate in PAB1-mRNP complexes following the stress of glucose deprivation in which particular stress granules are formed (Buchan et al. 2008). Unfortunately, our Flag-PAB1 immunoprecipitations do not detect PAB1 in yeast stress granules (unpublished observation).

For the other nine proteins found to associate with one or another of the PAB1 domains, five of them, CBC1, GBP2, NAB6, UPF1, and CBF5, have been previously found to associate with PAB1 by mass spectrometric studies (Collins et al. 2007). CBC1 is the nuclear mRNA cap-binding protein (Das et al. 2000). Because PAB1 is known to be present in the nucleus, it is possible that the nuclear mRNA configuration also involves a closed-loop structure similar to that found for cytoplasmic mRNA involving eIF4E, eIF4G, and PAB1 (Wells et al. 1998). In the case of CBC1, it may make a direct contact to the RRM4 and P domains of PAB1. This contact may not require an intermediary, as in the case of eIF4G bridging the circularization of the mRNA by eIF4E and PAB1. Because the RRM4 domain of PAB1 plays

a role in mRNA transport from the nucleus (Brune et al. 2005), CBC1 contact to this region of PAB1 may play an unknown role in this process.

GBP2 has been shown to be involved in mRNA transport, translation, and stress granules (Buchan et al. 2008; Windgassen et al. 2004), all processes involving PAB1. A role for RRM1, required for GBP2 interaction, in these processes has not been identified, although deleting RRM1 does have a significant, albeit not a large effect, on protein translation (Yao et al. 2007). Similarly, RRM1 is required for binding NAB6, an mRNA binding protein. NAB6 tends to bind mRNA involved in cell wall biogenesis (Hogan et al. 2008). Its RNA binding sites have, however, not been detected and the role of either PAB1 or its RRM1 domain in these processes is not clear. Similarly, the importance of the PAB1 RRM1 interaction with CBF5, a nucleolar protein involved in ribosomal biogenesis, is unknown. While the role of PAB1 in binding these various proteins remains to be elucidated, the observation that these proteins repeatedly are found to associate with PAB1 and to do so in a domain-specific manner, strongly suggests that they are important PAB1-associated factors.

The remaining four proteins, SSD1, SLF1, NOP77, and yGR250c, while not being previously shown to interact with PAB1, are known to be involved in translation (SSD1 and SLF1) (Sobel and Wolin 2006; Krogan et al. 2006), mRNA degradation (yGR250) (Windgassen et al. 2004), ribosomal biogenesis (NOP77) (Mnaimneh et al. 2004), or binding of eIF4E (SLF1) (Krogan et al. 2006), all pathways in which PAB1 has been directly linked. Several of these proteins deserve special comment. GBP2 and yGR250c have been suggested to be components of yeast stress granules formed upon the stress of glucose deprivation (Buchan et al. 2008). While we have not been able to detect stress granule complexes with our Flag-PAB1, it is possible that these two proteins also associate with mRNA translational complexes prior to stress granule formation. GBP2, in fact, is known to play roles in both mRNA export and translation (Windgassen et al. 2004). SSD1 is a known mRNA binding protein and possible RNase (Uesono et al. 1997), and its sites of binding to the mRNA are very close to the 5' end of mRNA (Hogan et al. 2008; Ohyama et al. 2010). Because this location is in the vicinity where eIF4E and eIF4G would interact to form the closed-loop mRNA structure with PAB1 (Wells et al. 1998), it is possible that SSD1 associates with PAB1 and the mRNA to form a closed-loop structure. Whether there are additional contacts to other translation factors such as eIF4G in this closed-loop configuration remains to be determined. SSD1 contact to PAB1 may therefore be a means to stabilizing its interactions with both the 5' and 3' ends of the mRNA. This may further advantage those particular cell wall encoding mRNAs that it may control (Kaeberlein and Guarente 2002; Moriya and Isono 1999; Hogan et al. 2008) for optimal translation. Alternatively, SSD1 may suppress the translation of certain mRNA, such as *CLN2*, by binding to the 5' end of the mRNA (Ohyama et al. 2010; Jansen et al. 2009).

While our list of proteins interacting indirectly or directly with PAB1 includes many of the processes in which PAB1 is known to be involved, several notable proteins are missing. For example, all components of the mRNA deadenylase complex (CCR4–NOT) were absent (Tucker et al. 2001; Chen et al. 2001, 2002; Cui et al. 2008). This is most likely due to the fact that most initial deadenylation of mRNA when PAB1 occupies two to three sites on the poly(A) tail takes place in a distributive manner (Yao et al. 2007; Decker and Parker 1993). In distributive deadenylation, CCR4 would not be stably bound to the PAB1-mRNP complex. In contrast, processive deadenylation of poly(A) tails by CCR4, in which it would be more stably associated with the mRNA, requires more expansive naked poly(A) tails when PAB1 would not be expected to be present (Tucker et al. 2002; Viswanathan et al. 2003). Proteins playing roles in mRNA decapping were also uniformly missing from our mass spectrometric analyses. In this case, decapping takes place in specialized P bodies (Sheth and Parker 2007), and previous studies have indicated that PAB1 can associate with

decapping proteins (Tharun and Parker 2001; Viela et al. 2000). Also, it has been reported that decapping can take place on translating ribosomes (Hu et al. 2009), in which case one would expect PAB1 to interact with decapping factors. Although PAB1 may be present in such complexes, we have no evidence that our Flag pull downs can detect PAB1 in these complexes. Other processes in which PAB1 is involved for which we did not identify PAB1-associated proteins include that of mRNA export (Brune et al. 2005; Dunn et al. 2005; Chekanova et al. 2001) and 3' end processing (Hosoda et al. 2006; Amrani et al. 1997). Therefore, one limitation in our mass spectrometric studies is the inability to use a single bait to identify all possible protein complexes in which a particular protein is present. Moreover, any differences that we observed between the proteins associating with PAB1 and proteins previously identified by mass spectrometric procedures may be due to the bait and conditions employed for obtaining protein complexes in the respective experiments.

Role of RRM1 of PAB1 in nonsense-mediated deadenylation and decapping

Because the RRM1 domain has been shown to play a critical role in mRNA deadenylation (Yao et al. 2007; Lee et al. 2010) and UPF1 is known to control mRNA degradation by accelerating both decapping and deadenylation of mRNA containing premature codons (Cao and Parker 2003), we subjected UPF1 to further study. Our reverse immunoprecipitation analysis using UPF1 as our bait established that RRM1 of PAB1 was required for PAB1 to bind to UPF1. Also, deletion of both the P and C domains of PAB1 did not interfere with UPF1 immunoprecipitating PAB1 and neither the P nor the C domain of PAB1 was required for the ability of Flag-PAB1 to bring down UPF1. Mammalian studies have indicated that the C-terminal domains of PABPC are important to compete presumably with UPF1 for binding to translation termination factor eRF3 (Brognia and Wen 2009). No such role is likely for the combined P and C domains of PAB1 in yeast given that deletion of these two regions of PAB1 did not affect the major part of NMD (Simon and Seraphin 2007).

We further established that the RRM1 domain of PAB1 blocked NMD deadenylation processes but had no effect on the more critical NMD-induced decapping process. This result is in agreement with recent results that indicate that PAB1 in yeast is not essential for NMD decapping (Mieux et al. 2008). Yet, because we have also shown that deleting the RRM1 domain blocks deadenylation for all processes that have been analyzed, as well as for deadenylation in an *upf1* background, we cannot necessarily conclude that the presumed RRM1–UPF1 interaction that we have identified has a specific role for UPF1 function in NMD.

However, one possible role for the RRM1–UPF1 interaction was suggested by our results to be in the transition from distributive to processive deadenylation by CCR4 that occurs in the process of shortening the poly(A) tail. This transition naturally occurs following PAB1 removal from the poly(A) tail, usually when the poly(A) tail shortens to about 25 A's (Yao et al. 2007; Ohn et al. 2007; Decker and Parker 1993), the minimal size to which PAB1 binds (Deo et al. 1999). This transition is also notably enhanced and can occur on even longer poly(A) tail lengths whenever deadenylation rates become accelerated, as for the rapid deadenylation of normal mRNA (e.g., *MFA2*), the PUF3 induction of increased deadenylation of *COX17* (Decker and Parker 1993; Olivas and Parker 2000; Lee et al. 2010), the general augmented rate of deadenylation caused by translation initiation defects (Schwartz and Parker 1999), and the NMD-induced acceleration of deadenylation mediated by UPF1 (Cao and Parker 2003).

Three observations support the RRM1–UPF1 interaction as possibly important to this distributive to processive transition. First, deleting *UPF1* resulted in a shift to distributive deadenylation for the *PGK1* mRNA. Second, removal of PAB1 is a prerequisite for this shift to processive deadenylation and deleting the RRM1 domain interferes with PAB1 being

removed from the mRNA (Yao et al. 2007). Third, under NMD, UPF1 is required for the very rapid processive deadenylation that occurs. These observations suggest the model that UPF1 accelerates deadenylation during NMD by particularly interacting with the RRM1 domain of PAB1 and hastening removal of PAB1 from the poly(A) tail. They also suggest that UPF1 plays a role in normal mRNA degradation, as previously reported (He and Jacobson 2001; Sheth and Parker 2006), by aiding this transition from distributive to processive deadenylation. Because the factors important to this transition have remained unknown, future studies will be required to resolve this particular process and the special role of UPF1 in this process.

Our identification of the likely domains of PAB1 to which a number of known and novel PAB1-interacting factors bind indicates that the PAB1 protein and its mRNP structure play diverse roles in the metabolism of mRNA. Clearly, it will require an in-depth analysis for each of these protein–PAB1 interactions to illuminate both the relevancy of the interaction and its biological role.

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Fig. 1.

PAB1 constructs as discussed in the manuscript. Residues for each domain are indicated at the *top*. PAB1-184 has residues 184DAL186 replaced with EKM, PAB1-180 has residues 180KE181 with ER (not shown in the figure), and PAB-134 has 134HPD136 replaced with DKS (Otero et al. 1999)

Fig. 2.

PAB1 regions involved in binding eIF4G and eIF4E. Cell extracts from strains carrying only the Flag-PAB1 variants as indicated were bound to Flag beads, eluted with Flag peptide, and detected by western analysis using the antibodies as indicated in the figure. eIF4G refers to eIF4G1, as the antibody does not recognize eIF4G2. The *asterisk in lane 3, left*, refers to the PAB1- RRM1 protein band. The major band above it is a non-specific species that binds to the Flag antibody. **a** All strains carried either a Flag-PAB1 derivative as indicated (*lanes 2–11*) or PAB1 alone (*lane 1*). **b** Flag-PAB1 derivatives were expressed in strains carrying either wild-type *CDC33* (*lanes 1 and 4*) or *cdc33-1* (*lanes 2, 3, and 5*)

Fig. 3.

UPF1 immunoprecipitates PAB1 through the PAB1 RRM1 domain. HA-UPF1 pull-downs were conducted as described in Fig. 2. Flag-PAB1 (*lanes 1 and 3*) and its RRM1 deletion derivative (*lanes 2 and 4*) were identified using antibody directed against the Flag peptide and antibody directed against the HA epitope was used to detect HA-UPF1. *Lanes 1, 2* crude extracts; *lanes 3, 4* HA immunoprecipitations

Fig. 4.

Transcriptional pulse-chase analyses on *PGK1* mRNA were conducted as previously described (Lee et al. 2010). Following induction of the *GAL1-PGK1* mRNA with galactose, transcription was shut off with glucose and, at the times (in min) indicated above the figure, RNA was extracted and northern analyses were conducted as described (Yao et al. 2007). dT refers to the RNA sample probed with oligo (dT) followed by RNase H digestion to remove

the poly (A) tail. Equivalent amounts of RNA were loaded into each lane across a panel, as determined by A_{260} spectrophotometric analysis. **a, c** Strain AS319/YC504 (wild type for PAB1); **b, d** strain AS319/YC505 (PAB1- RRM1). **a, b**. Strains were transformed with pRP469 carrying the *PGK1pG* wild-type gene; **c, d** strains were transformed with pRP1078 carrying the *PGK1pG-319* that has a nonsense mutation at amino acid 319 (Cao and Parker 2003). Densitometric scans of the data are presented to the right of the northern blots. **a** Both the full-length and fragment scans are presented and in separate figures to ease identification of the species. For the full-length scan in this case, the time points are given in reverse order to clarify visualization of the latter time points. **b** Only the full-length densitometric scans are presented, as there were no identifiable fragments. **c, d** Both full-length and fragment densitometric scans are presented, albeit in one continuous figure. For ease of interpretation the right y axis designations skip every other time points for clarity

Fig. 5. Effect of *upf1* on deadenylation. Pulse-chase experiments were conducted exactly as described in Fig. 4, except the following strain was used: RR27-1. **a** YC504/RP469 (*PGK1pG*); **b** YC505/RP469; **c** YC504/RP1068 (*PGK1pG-319*); YC505/RP1068. Densitometric scans are presented as described in Fig. 4

Table 1

Summary of the proteins identified by MS as linked to specific PAB1 domains

PAB1 domain	Known role	Previous protein contact	Our MS identified PAB1 contacts
RRM1	Translation, deadenylation	eIF4G1, eIF4G2, PAB1	eIF4G1, eIF4G2, UPF1, GBP2, NAB6, SLF1, NOP77
RRM2	Translation, CCR4–NOT complex stability	eIF4G1, eIF4G2	eIF4G1, eIF4G2
RRM3	Deadenylation		SSD1
RRM4	mRNA export		CBC1
P	Deadenylation	PAB1, PBP1, PBP2	eRF3, CBC1, yGR250c
C	Translation termination, deadenylation	eRF3, PBP1, PBP2, PAN3	eRF3, PBP2

Summary of known roles of PAB1 domains, previous known contacts, and proteins we identified as co-purifying with specific PAB1 domains

Table 2

Proteins that associated with wild-type PAB1

Protein	Mol. wt. (kDa)	Unique peptides (Avg.)	PAF score	Function related to PAB1
UTP20	288	13	0.45	Nucleolar
URB1	204	2.6	0.13	Nucleolar
RRP5	194	50	2.6	Nucleolar
RPA190	188	10	0.53	Transcription
RLR1	185	15	0.81	THO complex
XRN1	176	40	2.3	mRNA degradation
SKI3	165	8.6	0.52	mRNA degradation
yLR419w	164	10	0.61	RNA helicase
RSE1	155	3.2	0.21	Splicing
CLU1	145	9.8	0.68	Translation initiation
SSD1	140	19	1.4	RNA binding
NAB6	127	11	0.87	RNA binding
YEF3	117	6.0	0.51	Translation
RRP12	114	13	1.1	Nucleolar
UPF1	110	14	1.3	mRNA degradation
eIF4G1	107	31	2.9	Translation initiation
MIS1	107	12	1.1	Mitochondrial
eIF4G2	104	13	1.2	Translation initiation
UBP3	102	3.0	0.29	Nucleolar
CBC1	99.7	14	1.4	RNA binding
NAB3	90.5	2.6	0.29	RNA binding
yGR250c	89.7	7.0	0.78	Translation
ENP2	82.0	2.1	0.26	Nucleolar
NOP77	78.1	7.0	0.90	Nucleolar
eRF3	76.9	12	1.5	Translation
yGR054w	71.8	5.6	0.78	Translation initiation
yIL055c	70.8	4.0	0.56	Unknown
KRI1	68.6	3.6	0.52	Nucleolar
AEP2	68.1	7.4	1.1	Mitochondrial
NUG1	57.8	3.8	0.68	Nucleolar
CBF5	55.2	8.4	1.5	Nucleolar
SLF1	50.9	5.0	0.98	Translation
HRB1	49.3	4.0	0.81	mRNA export
GBP2	49.0	8.4	1.7	mRNA export
PUB1	48.0	4.6	0.96	RNA binding
TMA46	46.3	3.2	0.69	Translation
PBP2	45.6	5.7	1.2	RNA binding
KRR1	37.4	3.8	1.0	Nucleolar
RLP7	36.7	2.7	0.74	Nucleolar

Protein	Mol. wt. (kDa)	Unique peptides (Avg.)	PAF score	Function related to PAB1
BRX1	33.7	2.6	0.77	Nucleolar
LHP1	32.2	5.3	1.6	Nucleolar
SGN1	30.0	4.6	1.5	RNA binding
NOP6	25.2	3.3	0.13	Nucleolar
MNP1	20.6	1.4	0.68	Mitochondrial

Average number of unique peptides identified by mass spectrometric analysis across all wild-type PAB1 pull-downs for proteins not present in the control experiments. Proteins in the list were identified in greater than 40 % of the mass spectrometric experiments (5–7). The PAF score is the average number of unique peptides divided by MW (kDa) $\times$ 10. Protein names in bold were also present in greater than 40 % of the mass spectrometric pull-downs across all PAB1 deletion derivatives (Table 4). Nucleolar refers to proteins that are found in the nucleolus or play a role in rRNA biogenesis

Table 3

Proteins that associated with wild-type PAB1 in 40 % or less of the mass spectrometric experiments

Protein	Mol. wt. (kDa)	Unique peptides (Avg.)	PAF score	Function related to PAB1
RPB1	192	2.4	0.12	Transcription
TOP2	164	4.2	0.090	DNA metabolism
SKI2	147	2.2	0.15	mRNA degradation
RMD11	131	2.8	0.21	Unknown
MAK21	117	2.2	0.19	Nucleolar
PWP1	64	0.8	0.12	Nucleolar
AEP1	60	2.6	0.43	Unknown
PRP4	53	0.6	0.11	Splicing
NSR1	27	0.6	0.22	Nucleolar

Average number of unique peptides identified by mass spectrometric analysis across all wild-type PAB1 pull-downs for proteins not present in the control experiments. Proteins in the list were identified in 40 % or less of the mass spectrometric experiments. Other terms are defined in Table 2. Proteins yER138c, SOV1, and MRD1 were also identified in these mass spectrometric experiments, but since they were not identified in the wild-type pull-downs when the controls were conducted, they are not included in the list

Table 4

Proteins that interacted with PAB1 and its deletion derivatives

Protein	Mol. wt. (kDa)	Unique peptides (Avg.)	PAF score	Function related to PAB1
UTP20	288	5.7	0.2	Nucleolar
RRP5	194	41	2.1	Nucleolar
RPA190	188	4.1	0.22	Transcription
RLR1	185	12	0.68	THO complex
XRN1	176	46	2.6	mRNA degradation
SKI3	165	2.9	0.17	mRNA degradation
yLR419w	164	16	0.95	RNA helicase
RSE1	155	3.4	0.22	Splicing
SSD1	140	10	0.75	RNA binding
RRP12	138	5.3	0.38	Nucleolar
RMD11	131	2.4	0.18	Nitrogen regulation
NAB6	127	13	1.0	RNA binding
YEF3	117	1.8	0.15	Translation
MAK21	117	2.3	0.2	Nucleolar
UPF1	110	20	1.8	mRNA degradation
eIF4G1	107	38	3.6	Translation
MIS1	107	3.3	0.31	Mitochondrial
eIF4G2	104	17	1.7	Translation
CBC1	100	12	1.2	RNA binding
NAB3	90	2.1	0.23	RNA binding
yGR250c	90	6.4	0.72	Translation
NOP77	78	8.6	1.1	Nucleolar
eRF3	77	10	1.4	Translation
MSS116	77	3.6	0.48	Mitochondrial
CBP2	74	1.0	0.14	Mitochondrial splicing
yIL055c	71	5.3	0.75	Unknown
CBF5	55	3.4	0.62	Nucleolar
SILF1	51	4.7	0.93	Translation
GBP2	49	10	2.1	mRNA export

Protein	Mol. wt. (kDa)	Unique peptides (Avg.)	PAF score	Function related to PAB1
TMA46	46	1.3	0.28	Translation
PBP2	46	9.2	2.0	RNA binding
RAI1	45	1.3	0.29	Splicing
RLP7	37	2.1	0.56	Nucleolar
MPD1	37	1.2	0.33	Chaperone
BRX1	34	1.8	0.53	Nucleolar
SBP1	33	5.8	1.3	Translation
LHP1	32	5.9	1.8	Nucleolar
SGN1	29	6.4	2.2	RNA binding
NOP6	25	1.6	0.63	Nucleolar
SMB1	22	2.0	0.89	Splicing
GAR1	22	0.72	0.33	Nucleolar
NHP2	19	0.78	0.41	Nucleolar
Unknown	17	2.1	1.1	Unknown

Average number of unique peptides identified by mass spectrometric analysis across all PAB1 deletion derivative pull-downs for proteins not present in the control experiments. Proteins in the list were identified in greater than 40 % of the mass spectrometric experiments. The PAF score is the average number of unique peptides divided by MW (kDa) $\times$ 10. Protein names in bold were also present in greater than 40 % of the mass spectrometric pull-downs across all wild-type PAB1 analyses (Table 2). The Unknown protein has accession number 7537305. Six additional proteins, **BRR2**, **SNF2**, **PRP19**, **SOV1**, **MRD1**, and **NOP14**, were present in greater than 40 % of the PAB1 deletion derivative mass spectrometric analyses but could not be compared to controls, as they were not detected in PAB1 wild-type pull-downs when the control experiments were conducted. These six proteins were, therefore, not included as part of the 43 proteins that are listed in the table

Table 5

Proteins identified in 40 % or less of the mass spectrometric experiments conducted with the PAB1 deletion derivatives

Protein	Mol. wt. (kDa)	Unique peptides (Avg.)	PAF score	Function related to PAB1
GCN1	298	2.5	0.083	Translation
RPB1	192	0.36	0.018	Transcription
SKI2	147	0.64	0.044	mRNA degradation
RPC82	74	1.2	0.16	Transcription
UTP15	58	0.21	0.036	Nucleolar
RPC34	36	0.28	0.077	Transcription
RPB5	26	0.28	0.11	Transcription
SLX9	25	0.43	0.17	Unknown

The proteins PRP8, POL2, yER138c, TOP2, and RAT1 were detected in several of the mass spectrometric experiments but were not included in the list because they were not detected in the wild-type pull-downs when the control experiments were conducted

Table 6

Average number of unique peptides identified for proteins co-purifying with PAB1

Protein	PAB1-wt	RRM1	RRM2	RRM3	RRM4	P	C
PAB1	66	68	70	66	68	60	70
eIF4G1	50	20	16	46	46	42	42
eIF4G2	25	10	7	24	20	10	24
UPF1	26	8	22	21	22	18	24
CBC1	18	13	17	12	7	2	16
NAB6	14	6	10	12	20	18	17
eRF3	14	8	20	14	8	2	6
PBP2	12	14	10	11	11	7	0
SSD1	10	16	11	4	13	8	10
NOP77	10	5	13	8	6	9	10
GBP2	10	4	6	14	10	12	16
SLF1	8	2	5	4	3	3	8
CBF5	4	4	2	4	0.5	2	4
yGR250c	3	6	13	6	12	0.5	6

Mass spectrometric analysis on proteins co-purifying with individual Flag-PAB1 variants was conducted as described in the text. Only those proteins that are listed in Table 4 were compared. Values refer to the average number of unique peptides detected for each protein for the two series of experiments that were conducted. Values in bold displayed significant differences (at least two-fold differences in values as compared to the wild type)

Table 7

Average of individual peptide intensities for proteins associating with PAB1 deletion derivatives

Protein	PAB1	PAB1- RRM1	PAB1- RRM2	PAB1- RRM3	PAB1- RRM4	PAB1- P	PAB1- C
eIF4G1	100	7.4 (4.7)	3.9 (1.1)	120 (29)	110 (16)	140 (28)	160 (30)
eIF4G2	100	8.6 (4.7)	25 (13)	74 (26)	210 (25)	110 (50)	72 (30)
NAB6	100	3.8 (3.7)	23 (5.2)	62 (9.0)	130 (20)	100 (20)	94 (23)
PBP2	100	300 (160)	150 (31)	130 (9.0)	260 (21)	220 (60)	0(0)
SSD1	100	110 (45)	53 (20)	22 (3.5)	170 (42)	180 (44)	190 (90)
GBP2	100	0(0)	42 (13)	81 (28)	65 (19)	120 (18)	94 (24)

Mass spectrometric analysis was conducted as described in Table 6. The intensities for the peptides for each individual protein described in Table 6 were compared across the PAB1 deletion derivatives. Peptides that were represented in all or nearly all Flag-PAB1 deletion derivative pull-downs were considered "good" peptides for cross comparisons of peptide intensities. For several proteins no peptides were found in a particular Flag-PAB1 deletion derivative pull-down, but the peptide was retained in the "good" data set if this absence correlated with the domain that was suggested for interacting with the protein based on the results in Table 6. For eIF4G1, 14 peptides were identified as "good" peptides; for eIF4G2, there were 4 peptides; for NAB6, there were 4 peptides; for PBP2, there were 3 peptides; for SSD1, there were 4 peptides; and for GBP2, there were 3 peptides. One or no "good" peptides were identified for the other proteins listed in Table 6. The relative intensities were normalized to 100 for PAB1, wt, and the value given in the table is the average of these values for all of the peptides for each protein. Standard errors of the mean are given in parentheses where applicable. As additional controls, analysis of average intensities for "good" peptides for several proteins not listed in Table 6 but which were included in Table 4 as not associating with PAB1 through any particular domain demonstrated no differences in intensities across the various PAB1 deletion derivative pull-downs (RLR1, RRP5, yLR419w, and XRN1 were analyzed)