Determinants of Rbp1p Localization in Specific Cytoplasmic mRNA-processing Foci, P-bodies

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Rbp1p, a yeast RNA-binding protein, decreases the level of mitochondrial porin mRNA by enhancing its degradation, but the intracellular location of the Rbp1p-mediated degradation complex remains unknown. We show here that Rbp1p in xrn1Δ mutant yeast localizes in specific cytoplasmic foci that are known as P-bodies. The N-terminal and RNA recognition motif (RRM) 1 domains of Rbp1p are necessary but not sufficient for its localization in P-bodies. Rbp1p forms oligomers through its C-terminal domain in vivo; N-terminal-delete, or RRM1-mutated Rbp1p can be more efficiently recruited to P-bodies in an xrn1Δ strain, expressing a full-length Rbp1p. Although POR1 mRNA is localized to P bodies in an xrn1Δ strain, this localization does not depend on Rbp1p. Decapping activator Dhh1p directly interacts with Rbp1p. However, the recruitment of Rbp1p to P bodies does not require Dhh1p or Ccr4p. In wild-type cells, Rbp1p can localize to P bodies under glucose deprivation or treatment with KCl. In addition, Rbp1p-mediated porin mRNA decay is elicited by Xrn1p, a 5’ to 3’ exonuclease. These results provide new insight into the mechanism of Rbp1p function.

Cellular mRNA is associated with proteins from the time of its synthesis until its degradation. In eukaryotic cells, a multitude of RNA-binding proteins play roles in posttranscriptional regulation of gene expression (1). The proper regulation of gene expression is critical for all biological processes. In addition, mRNA turnover plays an important role in mRNA biogenesis and the degradation of aberrant transcripts (2). The process of mRNA turnover is important in numerous aspects of eukaryotic mRNA physiology. For example, mRNA turnover participates in controlling gene expression both by setting the basal level of gene expression and as a site of regulatory responses (3). Control of mRNA stability provides a means by which a cell can rapidly change its mRNA abundance and, hence, gene expression (for review, see Refs. 4 and 5). The process of mRNA degradation includes removal or inactivation of the poly(A) tail, exit from active translation, assembly of a decapping complex on the mRNA, and sequestration of the mRNA into discrete cytoplasmic foci where decapping and 5’ to 3’ degradation occurs (3). A major pathway of mRNA degradation in yeast occurs by deadenylation of the mRNA, which leads to a decapping reaction, thereby exposing the mRNA to rapid 5’ to 3’ exonuclease degradation (6). Some mRNAs also can be degraded in the opposite direction by the exosome, which is a large complex of 3’ to 5’ exonucleases functioning in RNA degradation and processing events. The balance between these two pathways varies among mRNAs and organisms. In yeast, mRNA decaying followed by 5’ to 3’ exonuclease decay occurs in specific cytoplasmic foci, P-bodies (7). These P-bodies contain decapping enzymes (Dcp1p and Dcp2p), decapping activators (Dhh1p, a complex of Sm-like proteins (Lsm1–7p), and Pat1p), the 5’ to 3’ mRNA exonuclease (Xrn1p), and proteins that bind to the mRNA after deadenylation by a major cytoplasmic deadenylase (Ccr4p). The number of P-bodies is remarkably low, i.e. two or three per cell (8). A recent study showed that P-bodies require RNA for assembly and contain nontranslating mRNAs, suggesting additional biological roles of P-bodies in addition to being sites of mRNA degradation (9).

We have identified an RNA-binding protein gene (RBP1) in Saccharomyces cerevisiae (10). Rbp1p contains three copies of an RNA recognition motif (RRM), two glutamine stretches, and an asparagine/methionine/proline-rich (NMP) region. RBP1 is not an essential gene, but yeast yield a slow growth phenotype with overexpression of Rbp1p. Rbp1p was also reported to be a negative regulator of meiosis and spore formation (11). Recently, we found that overexpression of Rbp1p impairs mitochondrial function and affects mitochondrial porin expression. rbp1Δ mutants showed increased stability of porin mRNA compared with wild-type cells, whereas cells overexpressing Rbp1p, but not N-terminal-deleted, or RRM1-, RRM2-, or RRM3-mutated Rbp1p, showed a decrease in the stability of porin mRNA (12). Rbp1p binds to the 3’-UTR of porin mRNA in vitro, and the three RRMs of Rbp1p are each involved in destabilizing porin mRNA.

To understand how Rbp1p regulates porin mRNA decay, we set out to examine the location and mechanisms of mRNA degradation that involve Rbp1p. Here, we show that Rbp1p loca-

2 The abbreviations used are: RRM, RNA recognition motif; NMP, asparagine/methionine/proline; RFP, red fluorescence protein; UTR, untranslated region; YPD, yeast extract/peptone/dextrose; HA, hemagglutinin; GST, glutathione S-transferase; GFP, green fluorescent protein; RT, reverse transcription; NLS, nuclear localization signal; TIA-1, T-cell internal antigen-1; TIA, TIA-1-related protein; CT, C terminus.

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Table 1

| Strains | Genotype | Source |
|---------|----------|--------|
| YPH499 | MATa ura3-52 lys2-801 ade2-101 trp1::TRP1 rps15a-1 leu2-3,112 his3-11,15 ura3-1 GAL + | Tien-Hsien Chang |
| WC303a | MATa ade2-1 trp1::TRP1 can1-100 leu2-3,112 his3-11,15 ura3-1 GAL + | Michael F. Henry |
| YTC345 | MATa rpb1-1 ura3 leu2 | This study |

Experiements

Experimental Procedures

Strains, Media, and Microbiological Techniques—Table 1 lists the yeast strains used in this study. Yeast culture media were prepared as described by Sherman et al. (13).YPD contained 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose. SD contained 0.067% Difco yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% ammonium sulfate, and 2% glucose. Nutrients essential for auxotrophic strains included 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose. SD contained 0.17% Difco yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% ammonium sulfate, 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose. SD contained 0.17% Difco yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% ammonium sulfate, 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose.

Peroxidase activity in the presence of H2O2 was assayed in a 40°C incubation mixture containing 100 mM TrisCl, pH 8.0, 25 μg/ml myoglobin, 0.75 mM H2O2, and 100 mg/ml NADH. The reaction was initiated by the addition of 0.25 units/ml of horseradish peroxidase (Sigma Diagnostics, St. Louis, MO). All experiments, unless otherwise noted, were performed in duplicate and repeated three times. The results were calculated as percent inhibition of control activity.

The efficiency of porin mRNA decay in yeast overexpressing Rbp1p requires Xrn1p. Although Rbp1p directly interacts with Dhh1p, the recruitment of Rbp1p to P-bodies in an xrn1 Δ strain does not require Dhh1p. Together, our results show that self-interaction and unidentified signals in the N-terminal domain play an important role in recruitment of Rbp1p to dynamic P-bodies in the absence of Xrn1p.

Indirect Immunofluorescence—Cells were prepared for indirect immunofluorescence staining as described previously (18). Anti-HA (Berkeley Antibody Co., Inc., Richmond, CA, 1:1000) and anti-GST (Cell Signaling, Beverly, MA, 1:1000) antibodies were used for detection of HA-Rbp1p and GST-Rbp1p, respectively. Anti-GFP antibody (Molecular Probes) was diluted 1:200. Alexa 488 goat anti-rabbit IgG and Alexa 594 goat antimouse IgG (Molecular Probes) were used as secondary antibodies at 1:1000 and 1:2000 dilutions, respectively. Nuclei were visualized by staining with H33258 (2 μg/ml), which was included in the mounting solution.

Pull-down Assay and RNA Detection by RT-PCR—Cells in which the RBP1 gene was deleted were transformed with plasmids carrying GST-tagged RBP1 and GST-tagged-deleted or -mutated RBP1. Cells were harvested by centrifugation, washed twice with HB buffer (50 mm Tris, pH 7.5, 15 mm EGTA, 100 mm NaCl, 0.1% Triton X-100, protease inhibitor mixture, 0.4 units/μl RNase inhibitor), and dispersed in HB buffer. Cells were broken by vortex mixing with glass beads, and debris was removed by centrifugation (4000 rpm for 5 min). Each supernatant was centrifuged for 10 min at 10,000 rpm to yield mitochondria-rich pellets (P10). Each P10 pellet was suspended in HB buffer and mixed with glutathione-Sepharose beads (rotation for 5 h at 4°C). The GST-Rbp1p-RNA complex that was bound to beads was recovered by centrifuging at 400 × g for 2 min and then washed 3 times with buffer (HB containing 0.02% Triton X-100). The beads were suspended in HB buffer containing 2 units of DNase I. After rotating at room temperature, the glutathione-Sepharose beads were collected by centrifugation. RNA was extracted with acid phenol/chloroform and precipitated with ethanol.

Table 2

| Plasmid | Characteristic | Source |
|---------|----------------|--------|
| pVT101U | 2 μm URA3 Pneo | Verent et al. (34) |
| pEG(KT) | Ura3 gene Leu2-2 pGal1 | Mitchell et al. (35) |
| pHAR | HA-RBP1 in pVT101U | This study |
| pHARn | HA-RBP1-nD in pVT101U | This study |
| pHARr1 | HA-RBP1-rm1 in pVT101U | This study |
| pHARrr1 | HA-RBP1-rm2 in pVT101U | This study |
| pHARr3 | HA-RBP1-rm3 in pVT101U | This study |
| pHARc | HA-RBP1-dc in pVT101U | This study |
| pHARCT | HA-RBP1-CT in pVT101U | This study |
| pGSTR | GST-RBP1 in pVT101U | This study |
| pGST-HARn | GST-HA-RBP1-nD in pVT101U | This study |
| pGST-HARr1 | GST-HA-RBP1-rm1 in pVT101U | This study |
| pGST-HARr2 | GST-HA-RBP1-rm2 in pVT101U | This study |
| pGST-HARr3 | GST-HA-RBP1-rm3 in pVT101U | This study |
| pGST-HARc | GST-RBP1-dc in pVT101U | This study |
| pER | RBP1 in pEG(KT) | This study |
| pER | HA-RPB1-nD in pEG(KT) | This study |
bromphenol blue), heated for 10 min at 95 °C, and then subjected to SDS-PAGE and Western blot analysis.

**In Vitro Binding Assay**—GST–Rbp1p, GST, His–Rbp1p, and His–Hrp1p were expressed in *Escherichia coli* and purified as described (18). Purified GST or GST–Rbp1p bound to glutathione-Sepharose beads was incubated with purified recombinant His–Rbp1p or His–Hrp1p in 800 μl of binding buffer (1% bovine serum albumin, 50 mM KCl, 100 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 5 mM dithiothreitol, 50 mM Tris, pH 7.5, and protease inhibitors) overnight at 4 °C. After incubation, the beads were washed 5 times with binding buffer, 0.02% Tween 20. Bound proteins were eluted with 30 μl of elution buffer (10 mM glutathione, 50 mM Tris, pH 8.0). Samples were analyzed by Western blot using antibodies against the His or GST tag.

**In Vivo mRNA Labeling**—The MS2 RNA labeling system was constructed as described by Bertrand *et al.* (19). Plasmid pG14-MS2-GFP (a gift of R.H. Singer) contains a fusion between the MS2 coat protein and GFP. Plasmid pG14-MS2-mRFP contains a fusion between the MS2 coat protein and mono red fluorescence protein (RFP). The POR1–6/MS2bs–3′-UTR plasmid contains the PgalI promoter controlling the expression of the POR1 gene with 6 copies of an MS2 binding site inserted between the stop codon and 3′-UTR. Yeast strains were transformed with various combinations of the episomal vectors and selected on the appropriate selection media to maintain the plasmids. Transformed cells were then grown to mid-log phase in synthetic media containing 2% raffinose. Cells were subsequently induced with 2% galactose for 3 h to induce expression of the reporter mRNA. Because of the variable expression levels of the two plasmids, some cells have particles without much GFP nuclear signal, and some cells have strong GFP nuclear signal without visible particles.

**RNA Blot and RNA Turnover Analysis**—Yeast total RNA was isolated by the hot acid phenol method (20). RNA blot analysis was carried out as described previously (10). We used a yeast strain YTC345 carrying a temperature-sensitive RNA polymerase II allele (*rpb1-1*) (21) to study the RNA turnover rate. YTC345 and YTC345 mutants (*xrn1Δ*, *lsm1Δ*, *dhh1Δ*, and *upf1Δ*) were grown in synthetic medium containing 2% glucose with shaking overnight at 25 °C. An equal volume of 50 °C sterile double-distilled H₂O was then added, and the culture was placed in a 37 °C water bath. Samples of cells were withdrawn at the indicated times for RNA isolation and Northern blot analysis. The mRNA decay level was determined by densitometry of Northern blots using an AlphaImager documentation and analysis system (AlphaImager 1220, Version 5.5). The data were processed with SigmaPlot 2000 software for the slope plot.
Rbp1p Localizes to P-bodies in xrn1/Δ Strains—Recent reports have indicated that certain mRNAs and mRNA processing molecules are localized in distinct cytoplasmic foci. Xrn1p is predominantly localized to the cytoplasm and is excluded from the vacuole and nucleus (22). The xrn1/Δ deletion increased the size and number of foci; that is, clogging the pipeline seems to obstruct passage through the mRNA decay system (7, 8). A previous study showed that Rbp1p is predominantly localized in the cytoplasm, with higher concentration in the perinuclear region and some punctuates (12). Because P-bodies are subcellular sites of mRNA decay, we reasoned that Rbp1p, involved in POR1 mRNA decay, might be transiently associated with these sites just before or during degradation.

HA-tagged Rbp1p was overexpressed in wild-type, xrn1/Δ, lsm1/Δ, dhh1/Δ, upf1/Δ, and ccr4/Δ mutant yeast. Indirect immunofluorescence analysis shows that HA-Rbp1p is localized to large cytoplasmic foci similar to the P-bodies in an xrn1/Δ mutant (supplemental Fig. S1). Dhh1p, a DEAD-box protein, interacts with both the decapping complex and components of the deadenylase and acts as an activator for efficient decapping of mRNAs after deadenylation (23). Therefore, we used Dhh1p as a marker for P-bodies as indicated in a previous study (7). To examine whether endogenous Rbp1p is localized to P-bodies in an xrn1/Δ mutant, we generated a yeast strain by integrating 3HA and GFP tags to chromosomal RBP1 and DHH1 genes, respectively. Consistent with a previous report (7), we observed large cytoplasmic foci containing Dhh1p-GFP, which are similar to the P-bodies in an xrn1/Δ mutant (Fig. 1).
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HA-tagged Rbp1p was also seen in discrete foci in the xrn1Δ mutant and partly co-localized with Dhh1p, suggesting that the Rbp1p-mediated POR1 mRNA degradation possibly takes place at P-bodies.

The N Terminus and RRM1 Are Necessary but Not Sufficient for the Recruitment of Rbp1p to P-bodies in xrn1Δ Mutants—To delineate the molecular mechanism of xrn1Δ-dependent translocation, we next determined which domain(s) is responsible for recruiting Rbp1p to P-bodies in an xrn1Δ mutant. Two deletion mutants of Rbp1p were generated lacking either the N-terminal (Rbp1p-dN) or C-terminal region (Rbp1p-dC) (Fig. 2B). Rbp1p-dN and Rbp1p-dC were expressed in an xrn1Δ strain. Fig. 2A shows a loss of signal in P-bodies with Rbp1p-dN and most of Rbp1p-dC. We also examined the C-terminal domain of Rbp1p (Rbp1p-CT) and found that it lost localization to P-bodies. These results indicated that the N-terminus of Rbp1p is necessary but not sufficient for its recruitment to P-bodies in the xrn1Δ mutant.

The RRM domain of RNA-binding proteins is generally believed to have a functional role in directing RNA binding. To examine whether RRM1, RRM2, or RRM3 is essential for the recruitment of Rbp1p to P-bodies in the xrn1Δ mutant, we next determined the localization of HA-tagged Rbp1p-rrm1, -rrm2, and -rrm3 mutants in an xrn1Δ strain. Fig. 2A shows that HA-Rbp1p-rrm2 and HA-Rbp1p-rrm3 were mostly co-localized with Dhh1p at P-bodies, but a much lower quantity of HA-Rbp1p-rrm1 was co-localized with Dhh1p. These results suggest that RRM2 and RRM3 domains are not essential for Rbp1p localization in P-bodies. The expression of each mutated of truncated form of Rbp1p was verified by Western blot analysis (Fig. 2C). Unexpectedly, when we expressed Rbp1p-dN or Rbp1p-rrm1 in the xrn1Δ strain, which contained endogenous Rbp1p, Rbp1p-dN or Rbp1p-rrm1 were more efficiently recruited to P-bodies than that in the xrn1Δ strain (Fig. 2D). This result suggests that endogenous Rbp1p may have a role in their recruitment to P-bodies. Moreover, Rbp1p, Rbp1p-dN, Rbp1p-dC, and mutants Rbp1p-rrm1, -rrm2, and -rrm3 did not appear to localize at P-bodies in the presence of Xrn1p (supplemental Fig. S2).

FIGURE 3. Rbp1p interacts with itself and Rbp1p-dN in an RNA-independent manner. A, Rbp1p interacts with itself and Rbp1p-dN in vivo. Rbp1p-ADH1-GST-RBP1 yeast were transformed with vector expressing HA-tagged-Rbp1p, -Rbp1p-dN, -Rbp1p-dC, -Rbp1p-CT, or -Vps74p. HA-tagged Vps74p was used as a control. Cell lysates were prepared and incubated with glutathione-Sepharose beads in the absence (−) or presence (+) of RNase A as described under “Materials and Methods.” After pulldown, bound proteins were analyzed by Western blotting (WB) using various antibodies as indicated. B, Rbp1p directly interacts with itself in vitro. Purified (1 μg) His-Rbp1p or His-Hrp1p (as a positive control) was added into reaction tubes containing either bead-bound GST (as a negative control) or GST-Rbp1p. His-Rbp1p or His-Hrp1p interacted with GST, or GST-Rbp1p was detected with a monoclonal anti-His antibody. Polyclonal anti-GST antibody was used to detect the GST and GST-Rbp1p fusion protein. Input, 10% of the proteins used in the binding assays.

Overexpression of Full-length Rbp1p Can Recruit Rbp1p-dN and Rbp1p-rrm1 to P-bodies—Rbp1p-dN, Rbp1p-rrm1, and most of Rbp1p-dC lost their signals in P-bodies in an xrn1Δ double mutant. In addition, in the presence of endogenous Rbp1p, Rbp1p-dN and Rbp1p-rrm1 were more efficiently recruited to the P-bodies in the xrn1Δ strain. Thus, we next examined whether overexpression of full-length Rbp1p in an xrn1Δ strain could recruit Rbp1p-dN, Rbp1p-dC, Rbp1p-rrm1, and Rbp1p-CT to P-bodies. We generated an engineered yeast strain xrn1ΔΔ-ΔDH11-GFP-GST-RBP1, which is an xrn1ΔΔ double mutant containing a chromosomal GFP-tagged DHH1 and GST-tagged RBP1 gene under the control of the ADH1 promoter. Both Rbp1p and Dhh1p in this strain were co-localized in P-bodies (supplemental Fig. S3A). The engineered yeast

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**FIGURE 3.** Rbp1p interacts with itself and Rbp1p-dN in an RNA-independent manner. A, Rbp1p interacts with itself and Rbp1p-dN in vivo. Rbp1p-ADH1-GST-RBP1 yeast were transformed with vector expressing HA-tagged-Rbp1p, -Rbp1p-dN, -Rbp1p-dC, -Rbp1p-CT, or -Vps74p. HA-tagged Vps74p was used as a control. Cell lysates were prepared and incubated with glutathione-Sepharose beads in the absence (−) or presence (+) of RNase A as described under “Materials and Methods.” After pulldown, bound proteins were analyzed by Western blotting (WB) using various antibodies as indicated. B, Rbp1p directly interacts with itself in vitro. Purified (1 μg) His-Rbp1p or His-Hrp1p (as a positive control) was added into reaction tubes containing either bead-bound GST (as a negative control) or GST-Rbp1p. His-Rbp1p or His-Hrp1p interacted with GST, or GST-Rbp1p was detected with a monoclonal anti-His antibody. Polyclonal anti-GST antibody was used to detect the GST and GST-Rbp1p fusion protein. Input, 10% of the proteins used in the binding assays.
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was then transformed with a pVT101U vector expressing HA-Rbp1p, HA-Rbp1p-dN, HA-Rbp1p-dC, or HA-Rbp1p-rrm1. We show that HA-Rbp1p-dN, HA-Rbp1p-rrm1, and Rbp1p-CT were more efficiently recruited to P-bodies in the xrn1Δrbp1Δ strain when full-length Rbp1p was overexpressed (supplemental Fig. S3B). The expression of each mutated or truncated form of HA-Rbp1p and GST-Rbp1p was verified by Western blot analysis (supplemental Fig. S3C). Although HA-Rbp1p-dC was mostly dispersed in the cytoplasm in the xrn1Δrbp1Δ strain (Fig. 2A), only part of it was recruited to P-bodies when full-length Rbp1p was overexpressed. These results suggest that possible self-interaction of Rbp1p involving at least one copy of full-length Rbp1p may be important for its translocation to P-bodies.

Intermolecular Interaction of Rbp1p Requires the C-terminal Domain and Occurs in an RNA-independent Manner—We next addressed whether Rbp1p can interact with itself directly or through association with mRNA or another protein(s) in the Rbp1p-mRNP complex. GST pull-down assays were performed to evaluate the binding of GST-Rbp1p to HA-Rbp1p, HA-Rbp1p-dN, or HA-Rbp1p-dC fusion proteins in vivo. HA-Vps74p, a Golgi-associated protein, served as a control. As shown in Fig. 3A, with or without RNase A treatment Rbp1p exhibited interactions with itself and Rbp1p-dN but not with Rbp1p-dC. Rbp1p had no interaction with the control protein Vps74p. These results suggested that interactions between Rbp1p molecules required its C-terminal domain and occurred in an RNA-independent manner. Intermolecular interaction of Rbp1p was also confirmed by yeast two-hybrid interaction analysis (data not shown).

We next generated an HA-Rbp1p-CT fusion protein to determine whether the C-terminal domain is sufficient for this intermolecular interaction. Fig. 3A shows that HA-Rbp1p-CT interacted with GST-Rbp1p, indicating that the C-terminal domain of Rbp1p is necessary and sufficient for intermolecular interaction with Rbp1p. To further substantiate the above findings, purified recombinant His-tagged Rbp1p and His-tagged Hrp1p fusion proteins from E. coli were applied to glutathione-Sepharose beads in the absence and presence of RNase A. Although His-Rbp1p was retained with GST-Rbp1p on the column (Fig. 3B), Western blot analysis performed and confirmed the identities of the fusion proteins. These experiments demonstrated that Rbp1p displayed direct interaction with itself in vitro.

Rbp1p Interacts with Dhh1p in an RNA-independent Manner—Knowing that Rbp1p and Dhh1p associate with P-bodies in the xrn1Δ mutant, we next examined whether Rbp1p-mRNP interacts with Dhh1p via direct Rbp1p-Dhh1p interaction or through association with mRNA or another protein(s) in a multiprotein complex. GST pull-down assays were performed to evaluate the binding of Dhh1p-GFP to GST-Rbp1p or GST-Rbp1p-dN fusion proteins in vivo in an xrn1Δrbp1Δ mutant. As shown in Fig. 4, Dhh1p interacted with Rbp1p and Rbp1p-dN, but not with GST, even in the presence of RNase A, suggesting a direct interaction between Rbp1p and Dhh1p in an RNA-independent manner. This result also indicates that the N terminus of Rbp1p is not required for interaction with Dhh1p. We also noticed that GST-Rbp1p-dN pulled down more Dhh1p than GST-Rbp1p, suggesting that the conformation of GST-Rbp1p-dN could be changed, resulting in a higher affinity to interact with the Dhh1p than GST-Rbp1p. Because only a portion of Dhh1p co-localized with Rbp1p in the wild-type strain (Fig. 1), we next examined whether Rbp1p and Dhh1p could interact with each other in an rbp1Δ mutant strain, using similar pull-down assays. The result also indicates that Rbp1p interacts with Dhh1p in an RNA-independent manner and in the absence of major P-bodies (supplemental Fig. S4). Yeast two-hybrid analysis further supported the above findings that Rbp1p interacts with Dhh1p (data not shown).

Recruitment of Rbp1p to P-bodies Does Not Require Dhh1p or Ccr4p—Knowing that Rbp1p can interact with Dhh1p in vivo, we next examined whether Dhh1p recruits Rbp1p to P-bodies in an xrn1Δ strain. HA-Rbp1p was overexpressed in rbp1Δxrn1Δ or rbp1Δxrn1Δdhh1Δ mutant cells. Fig. 5A shows that HA-Rbp1p formed cytoplasmic foci in the absence of Dhh1p, suggesting that recruitment of Rbp1p to P-bodies in the xrn1Δ strain is not dependent on Dhh1p. To determine whether Ccr4p is required for recruitment of Rbp1p to P-bodies in the xrn1Δ strain, we overexpressed HA-Rbp1p in rbp1Δxrn1Δ or rbp1Δxrn1Δccr4Δ mutant cells. Fig. 5B shows that HA-Rbp1p formed cytoplasmic foci in rbp1Δxrn1Δccr4Δ cells, suggesting that proteins involved in deadenylation are not required for recruitment of Rbp1p to P-bodies.

Ability of Rbp1p and Its Deletion or Mutation Constructs to Bind to mRNA in Vivo—The size of P-bodies is proportional to the flux of mRNAs undergoing the decapping step in turnover.

\[\text{Figure 4. Rbp1p interacts with Dhh1p in an RNA-independent manner.} \]

\[\text{rbp1Δxrn1Δ} \quad \text{rpb1Δxrn1Δdhh1Δ} \]

\[\text{yeast were transformed with vector expressing GST alone or expressing GST-Rbp1p or GST-Rbp1p-dN. Cells lysates were prepared and incubated with glutathione-Sepharose beads in the absence (−) or presence (+) of RNase A as described under "Materials and Methods." After pull-down, bound proteins were analyzed by Western blotting (WB) using antibodies as indicated. The input panel shows 10% of the input lysate. Similar amounts of recombinant GST, GST-Rbp1p, and GST-Rbp1p-dN were used in each case for GST pull-down assays (bottom).}\]

\[\text{input panel} \quad \text{GST} \quad \text{GST-Rbp1p} \quad \text{GST-Rbp1p-dN} \]

\[\text{Dhh1p-GFP} \quad \text{RNase A} \]

\[\text{anti-GFP} \quad \text{anti-GST} \]

\[\text{N. T. Jang, L.-C. Chang and F.-J. S. Lee, unpublished data.}\]
In addition, mRNA decay intermediates trapped in the process of degradation are also localized to P-bodies. We next asked whether the recruitment of Rbp1p to P-bodies is dependent on its mRNA binding ability. GST fusion constructs of Rbp1p mutants or fragments were expressed under the control of the ADH1 promoter in an rbp1 mutant. With the notion that P-bodies could be enriched by sedimentation centrifugation in the P10 fraction (9), we first examined whether GST-Rbp1p is enriched in the P10 fraction. The cytoplasmic extracts of yeast rbp1/H9004-DHH1-GFP-LEU2:ADH1p-GST-RBP1 and rbp1/H9004-DCP2-GFP-LEU2:ADH1p-GST-RBP1 were separated into P10 and S10 fractions by sedimentation centrifugation, and Rbp1p, Dhh1p, Dcp2p, Pma1p (plasma membrane marker), Por1p (mitochondrial marker), and Pgk1p (cytoplasmic marker) were identified in each fraction by Western blot analysis (supplemental Fig. S5). Our data showed that GST-Rbp1p, like P-body markers Dhh1p-GFP and Dcp2p-GFP, appeared in the P10 fraction. P-bodies-rich pellets (P10) obtained from cytoplasmic extracts of different cells were then subjected to pulldown with glutathione-Sepharose beads. mRNAs from P10 extracts (used as input control) or released from pull-down Rbp1pmRNP complexes were analyzed by RT-PCR. Fig. 6 shows that in vivo, POR1 mRNA is associated with Rbp1p, Rbp1p-dN, Rbp1p-rrm1, Rbp1p-rrm2, Rbp1p-rrm3, and Rbp1p-dC. This result suggests that mRNA binding ability is not sufficient for recruitment of Rbp1p to P-bodies. A lower quantity of PGK1 mRNA was also detected, suggesting that Rbp1p may interact with PGK1 mRNA. Nevertheless, neither COX2 nor COX3 mRNA, two mitochondrial DNA encoded mRNAs, associated with Rbp1p or its derivatives.

Rbp1p Is Not Required for the Recruitment of POR1 mRNA to P-bodies—The presence of Rbp1p within P-bodies suggests that Rbp1p-mediated POR1 mRNA decay might result in the targeting of Rbp1p-POR1 mRNA complexes to P-bodies. To test this hypothesis, we first asked whether POR1 mRNA targeted by Rbp1p would accumulate with P-bodies. We generated a POR1 mRNA expression construct containing six binding sites for the MS2 coat protein in the 3′-UTR as described by...
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Rbp1p localizes to P-bodies under glucose deprivation or treatment with KCl—Glucose deprivation and osmotic stress are known to inhibit translation initiation, leading to dramatic increase in P-bodies (9). To examine whether endogenous Rbp1p localizes to P-bodies under these stress conditions, a yeast strain was generated by integrating DsRed and GFP tags for Xrn1p, Dhh1p, and Rbp1p with PCR-mediated gene disruption (24). Single mutant (rbp1Δ, dhh1Δ, xrn1Δ), double mutant (rbp1Δdhh1Δ, rbp1Δxrn1Δ, dhh1Δxrn1Δ), and triple mutant (rbp1Δdhh1Δxrn1Δ) strains were spotted onto plates and incubated at 30 °C to assess growth viability (supplemental Fig. S7). Rbp1p, Dhh1p, and Xrn1p are nonessential genes, and their mutants (rbp1Δ, dhh1Δ, and xrn1Δ) showed no significant growth defects. Although the dhh1Δxrn1Δ and rbp1Δdhh1Δxrn1Δ had considerable growth defects, neither of the mutants rbp1Δdhh1Δ or rbp1Δxrn1Δ showed a growth defect, suggesting that Rbp1p is not directly involved in general bulk mRNA decay.

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Rbp1p Requires Xrn1p to Elicit Porin mRNA Decay—From prior observation, we found that Rbp1p decreased the mRNA level of mitochondrial porin by enhancing its mRNA turnover (12). To further evaluate the role Rbp1p plays in regulating POR1 mRNA turnover in S. cerevisiae, we analyzed whether the mRNA decay machinery can specifically affect Rbp1p-mediated POR1 mRNA decay. We took advantage of an rpb1Δ-1 temperature-sensitive mutant to generate RNA degradation mutants and identified the mRNA turnover rate in the presence of overexpressed Rbp1p. We deleted the genomic loci coding for Xrn1p with PCR-mediated gene disruption (24) and over-

Bertrand et al. (19). These MS2 binding sites allowed us to follow the localization of POR1 mRNA by co-expression of an MS2-GFP-NLS fusion protein, which contains MS2-binding protein. In cells that lack the POR1 mRNA MS2-reporter, the fusion protein remains localized to the nucleus, reducing background cytoplasmic fluorescence. However, in the presence of a POR1 mRNA MS2-reporter, a fraction of the fusion protein is carried into the cytoplasm where its localization reports the location of target mRNA. To observe the localization of Rbp1p in vivo, we first constructed and expressed DsRed-tagged Rbp1p in an rbp1Δ mutant strain. The DsRed-Rbp1p is observed as multiple small foci distributed in the cytoplasm (Fig. 7A, upper panel), similar in appearance to the pattern observed by indirect immunofluorescence staining. Co-expression of the POR1 mRNA reporter, MS2-GFP-NLS fusion protein, and DsRed-Rbp1p in rbp1Δ mutant shows that DsRed-Rbp1p is evidently co-localized with the POR1 mRNA reporter (Fig. 7A, middle panel), suggesting that Rbp1p can be recruited to the overexpressed POR1 reporter. When DsRed-Rbp1p, the POR1 mRNA reporter, and MS2-GFP-NLS fusion protein were co-expressed in an rbp1Δxrn1Δ mutant, we observed discrete cytoplasmic foci of the MS2-GFP-NLS fusion protein that also co-localized with DsRed-Rbp1p, suggesting that POR1 mRNA can localize to P-bodies. A DsRed-tagged small GTPase Arl3p was used as a control, and unlike DsRed-Rbp1p, the DsRed-Arl3p did not co-localize with the POR1 mRNA reporter in xrn1Δ or rbp1Δxrn1Δ mutants (Fig. 7, A and B, lower panel). More-
expressed HA-tagged Rbp1p in the \textit{xrn1} mutant. The half-life of \textit{POR1} mRNA in these cells was measured by Northern analysis. Fig. 8 shows that the half-life of \textit{POR1} mRNA is prolonged in the \textit{xrn1} mutant ($\approx 60$ min). More importantly, overexpression of HA-Rbp1p in \textit{xrn1} mutant cells had no effect on the half-life of \textit{POR1} mRNA. Xrn1p is known to be a 5'$'$ to 3'$'$ exonuclease; hence, suggesting that the major RNA degradation pathway in yeast is required for Rbp1p-mediated porin mRNA decay.

The Lsm1–7p complex and Dhh1p are involved in the efficient decapping process of most mRNAs; however, they are not absolutely required for decapping \textit{per se}. Another group of mRNA-specific regulators is Upf1p, Upf2p, and Upf3p, which are primarily involved in nonsense-mediated decay (3). We also examined Lsm1p, Upf1p, and Dhh1p, and the results showed that Rbp1p-mediated \textit{POR1} mRNA decay was not significantly altered in \textit{upf1}Δ, \textit{lsm1}Δ, and \textit{dhh1}Δ mutants ($\approx 35$–$40$ min, data not shown). Because there might be a redundant function of these RNA decay proteins, we cannot rule out the possibility that Lsm1p, Upf1p, and Dhh1p are involved in Rbp1p-mediated \textit{POR1} mRNA decay using individual deletions of these mRNA-specific regulators. Expression of Rbp1p in different transformed yeast was also determined by Western blot analysis (data not shown).

**DISCUSSION**

Sheth and Parker (7) found that mRNA decapping enzymes, activators of decapping, and an mRNA nuclease all clustered in two to three P-bodies per budding yeast cell. Recently we have shown that the RNA-binding protein Rbp1p, which appears in small punctate foci in the cytoplasm, is involved in \textit{POR1} mRNA degradation (12). Here we report that Rbp1p is found in the P-bodies in an \textit{xrn1} mutant and that this localization is dependent on integrity of an Rbp1p complex through C-terminal self-interaction and unidentified signals at its N terminus. We also show that there is no clear correlation between the ability of Rbp1p to bind RNA and its localization to P-bodies and that Rbp1p is not required for \textit{POR1} mRNA localization to P-bodies in an \textit{xrn1} strain. Because Dhh1p can also localize to P-bodies, we show that \textit{in vivo} Rbp1p and Dhh1p can interact, albeit in the absence of P-bodies, and that this interaction does not affect Rbp1p recruitment to P-bodies. Furthermore, Rbp1p can be recruited to P-bodies under glucose deprivation or treatment with hyper-osmotic stress.

Knowing that P-bodies are subcellular sites of mRNA decay, we reasoned that Rbp1p should be transiently associated with these sites just before or during degradation. Localization of Rbp1p and its mutants, as shown in Fig. 2 and supplemental Fig. S3, indicated that both the N terminus and RRM1 domain are necessary but not sufficient for the recruitment of Rbp1p to P-bodies. We found that expressing a full-length Rbp1p in an \textit{xrn1} mutant enhanced recruitment of Rbp1p-dN and Rbp1p-fusion protein without (upper panel) or with (middle panel) the chimeric \textit{POR1} reporter RNA. DsRed-ARL3 was used as a control (lower panel). C, YPH499\textit{xrn1}Δ, \textit{DHH1-GFP} (upper) and YPH499\textit{rbp1}Δ\textit{xrn1}Δ, \textit{DHH1-GFP} (lower) were transformed with \textit{POR1} reporter RNA and the mRFP-MS2 fusion protein. Cells were analyzed by fluorescence microscopy. All images are representative of at least three independent observations.
rrm1 to P-bodies; however, there was a large fraction of the Rbp1p-dC protein dispersed in the cytoplasm (supplemental Fig. S3). Consistent with this notion, the GST pull-down data shown in Fig. 4 indicated that Rbp1p could interact with itself through the C-terminal NMP-rich region. Deletion of this C-terminal region resulted in a less punctate pattern of Rbp1p in the cytoplasm (Fig. 2A). This is consistent with the observation that Rbp1p-dC has much less ability to interact with full-length Rbp1p (Fig. 3). Two-dimensional electrophoresis analysis showing nine spots of Rbp1p with different isoelectric points suggested that functional Rbp1p may be regulated post-translationally. Whether this post-translational modification can mediate protein-protein interaction or recruitment to P-bodies is still an open question. Our data suggest that the N terminus and RRM1 domain of Rbp1p can interact with an unidentified molecule or receive signaling from posttranslational modification, which can recruit or activate the Rbp1p complex to P-bodies in the xrn1/H9004 strain.

The RRM domain of RNA-binding proteins is generally believed to imply a functional role in direct RNA binding. A previous study showed that the RRM1 and RRM2, but not RRM3, domains of Rbp1p are necessary for binding to the 3'-UTR of porin mRNA in vitro (12). Nevertheless, the RNA turnover assay revealed that all three Rbp1p-rrm mutants lost their destabilization activity on porin mRNA (12). However, only RRM1, but not RRM2 or RRM3, is involved in recruitment of Rbp1p to P-bodies, suggesting that functional Rbp1p is not required for this recruitment. Using GST pulldown followed by RT-PCR analysis, we demonstrated that Rbp1p, Rbp1p-dN, Rbp1p-rrm1, Rbp1p-rrm2, Rbp1p-rrm3, and Rbp1p-dC interacted with porin mRNA in vivo. There is no obvious correlation between Rbp1p and its deletion or mutated forms with regard to localization to P-bodies and porin mRNA binding ability. Our preliminary data showed that Rbp1p mutants that block Rbp1p association with P-bodies also lost their effects on POR1 mRNA stability. Moreover, our data indicated that porin mRNA binding ability of Rbp1p in vivo is not dependent on any single RRM. Thus, we infer that the RNA binding ability of Rbp1p may not be necessary for Rbp1p localization to P-bodies. Consistent with this notion, our data also showed that porin mRNA could localize to P-bodies in the absence of Rbp1p (Fig. 7). Our data showed that RRM1 is necessary for the recruitment of Rbp1p to P-bodies in an xrn1Δ strain. It is possible that the RRM1 of Rbp1p is not directly involved in RNA binding but functions in RNA metabolism by interaction with other proteins, or RRM1
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may provide an auxiliary function to assist in stabilizing the protein–RNA complex in a tertiary structure. Therefore, the biological activity of Rbp1p is not only dependent on its RNA binding capacity but is also involved in the interaction between molecules to form an Rbp1p-mRNP complex. Our analysis indicated that the recruitment of Rbp1p to P-bodies is mediated by a direct interaction between Rbp1p molecules and pointed out that the N-terminal RRM1 domain of the protein is a major determinant of this interaction. This possibility, however, needs to be examined directly in future studies.

Dhh1p is a member of the DEAD-box helicase protein family and is also required for efficient decapping (23, 25). Dhh1p physically binds to both the decapping enzyme and the deadenylylase, possibly mediating the communication between these two events (23). When cell cultures reached mid-log, late-log, and stationary phases, larger Dhh1p-GFP-containing P-body granules were detected (26). Larger Dhh1p-GFP-containing granules were not obviously observed in our wild-type yeast strains that were used. However, we detect Rbp1p co-localized with Dhh1p in P-bodies in an xrn1Δ strain and that Rbp1p directly interacted with Dhh1p in vivo in an RNA-independent manner (Fig. 4). Although Rbp1p partially co-localized with Dhh1p, there were a greater number of foci containing either protein (Fig. 1), suggesting that different forms of P-bodies exist. Moreover, depletion of RBP1 did not exacerbate growth when combined with either dhh1Δ or xrn1Δ mutations (supplemental Fig. S7), suggesting that Rbp1p did not play an essential role in the decapping/mRNA degradation pathway. The decapping/5′-3′ pathway is the predominant mRNA decay pathway in yeast (27). Rbp1p requires Xrn1p to elicit POR1 mRNA decay, and Lsm1p, Upf1p, and Dhh1p may partially participate in Rbp1p-mediated POR1 mRNA decay (Fig. 9). This result suggests that the deadenylation-dependent decapping/5′-3′ pathway plays the major role in Rbp1p-mediated mRNA decay.

Rbp1p exists as a >500-kDa RNP complex and displayed punctate or granule signals that were partly concentrated in the perinuclear region or scattered in the cytoplasm (12). Biochemical characterization of Rbp1p revealed that Rbp1p is associated with heavy sedimenting structures (P10 and P100 fractions). Rbp1p has a domain organization in amino acid sequence similar to two related multifunctional RNA-binding proteins, T-cell internal antigen-1 (TIA-1) and TIAR (TIA-1-related protein), which are important translational regulators in both development and the stress response (28–31). P-bodies show some remarkable similarities to another form of mRNA containing cytoplasmic particles, referred to as stress granules. Stress granules form in response to decreased translation initiation and contain poly(A)+ mRNA, translation initiation factors, specific RNA-binding proteins TIA and TIAR, and 40 S ribosomal subunits (for review, see Ref. 31). After the stress-induced phosphorylation of translation initiation factor eIF-2, TIA-1 and TIAR recruit most cytoplasmic mRNAs to discrete foci of stress granules (29), suggesting that the TIA-1/TIAR-dependent sequestration of these mRNAs prevents their translational initiation. Stress granules and P-bodies are similar in their dynamics, because both are increased by blocking translation initiation, and both decline when mRNAs are driven into polysomes (30). At this time, however, stress granules and P-bodies appear to have distinct protein compositions and to differ physically. Hilleren and Parker (2) imply that the relative translational efficiency of a transcript is a major determinant of mRNA half-life. Not all mRNAs are routed into stress granules, indicating that the RNA composition of stress granules is selective. In other words, any event that results in inefficient translation initiation may promote mRNA turnover. Interestingly, we also observed that Rbp1p could be recruited to P-bodies under translation-repression conditions such as glucose deprivation and treatment with hyper-osmotic stress (Fig. 8). Although the stability of yeast porin mRNA is affected by Rbp1p, at present we cannot rule out that Rbp1p may be regulated by post-translational modification and function as a translational silencer, similar to TIA-1/TIAR, and therefore, interferes with or causes a lag in the association of ribosomal subunits with selective mRNAs resulting in inefficient translation and/or acceleration of mRNA decay.

In summary, we conclude that recruitment of Rbp1p to P-bodies in an xrn1Δ mutant requires integrity of the Rbp1p complex but not its mRNA binding ability. Based on the properties of cytoplasmic mRNA degradation, a rearrangement in mRNP organization will be critical for the cytoplasmic transition from a translating mRNA to a transcript targeted for degradation (32, 33). Although the molecular mechanisms by which Rbp1p regulates the degradation of POR1 mRNA are still elusive, our data showed that Rbp1p interacted with Dhh1p, POR1 mRNA, and Xrn1p, suggesting involvement of Rbp1p in mRNP reorganizations in P-bodies. Is the activity of Rbp1p limited only to mRNA decay, or might it have other functions? Further exploration into the mechanistic details of Rbp1p granule formation and function should provide insights into the regulation of mRNA translation, storage, and decay.

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