Evidence for core exosome independent function of the nuclear exoribonuclease Rrp6p

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ABSTRACT

The RNA exosome processes and degrades RNAs in archaeal and eukaryotic cells. Exosomes from yeast and humans contain two active exoribonuclease components, Rrp6p and Dis3p/Rrp44p. Rrp6p is concentrated in the nucleus and the dependence of its function on the nine-subunit core exosome and Dis3p remains unclear. We found that cells lacking Rrp6p accumulate poly(A)+ rRNA degradation intermediates distinct from those found in cells depleted of Dis3p, or the core exosome component Rrp43p. Depletion of Dis3p in the absence of Rrp6p causes a synergistic increase in the levels of degradation substrates common to the core exosome and Rrp6p, but has no effect on Rrp6p-specific substrates. Rrp6p lacking a portion of its C-terminal domain no longer co-purifies with the core exosome and Rrp6p, but retains its function on the nine-subunit core exosome and Dis3p/Rrp44p. Rrp6p interaction results in the inability of the cell to efficiently degrade certain poly(A)+ rRNA processing products that require the combined activities of Dis3p and Rrp6p. These findings indicate that Rrp6p may carry out some of its critical functions without physical association with the core exosome.

INTRODUCTION

The process of transcription in eukaryotic cells produces a significant amount of RNA that is destroyed during maturation into functional transcripts (1). Most eukaryotic RNAs are synthesized as precursor-RNAs (pre-RNAs) that are cleaved, spliced and end-trimmed to produce mature, functional molecules. The RNA fragments removed by these internal cleavages are degraded in the nucleus and the cytoplasm by 5'-3' and 3'-5' exoribonucleases. The major 5'-3' exoribonucleases include the nuclear Rat1p/Xrn2p and the cytoplasmic Xrn1p/Kem1p. Rat1p plays roles in rRNA 5'-end formation, mRNA transcription termination and the degradation of unprocessed pre-mRNAs and pre-rRNAs (2–7). Xrn1p functions in the major mRNA decay pathway and the nonsense-mediated mRNA decay pathway in yeast, as well as a pathway responsible for the degradation of some unspliced mRNAs and those targeted by siRNAs within the RISC complex (8–11).

The RNA processing exosome appears to provide the major 3'-5' exoribonucleolytic activity in eukaryotes (12,13). The core exosome contains nine proteins with six of these arranged in a ring-like structure capped on the top by the remaining three subunits (14–16). The cap proteins contain putative RNA binding domains thought to interact with RNP substrates, thereby enhancing their insertion into the central core of the six-member ring. In archael exosomes, the central core contains the active sites for phosphorylolytic cleavage of the RNA. Interestingly, recent evidence indicates that, despite considerable sequence similarity between the eukaryotic ring proteins and bacterial exoribonucleases, all of the human and yeast ring proteins have lost the active site residues required to carry out RNA cleavage (15,17). Nevertheless, two hydrolytic 3'-5' exoribonucleases interact with the core exosome in eukaryotes and provide the essential RNA degradation activities of the complex. Dis3p/Rrp44p co-purifies with the core exosome from yeast and resides in the nucleus, nucleolus and the cytoplasm (18,19). Rrp6p also co-purifies with the core exosome and appears confined to the nucleus and nucleolus in yeast, but may have a cytoplasmic role in higher eukaryotes (20–22).

The core exosome plays an important role in the 3'-5' mRNA degradation pathway in the cytoplasm. The complex is recruited to unstable adenosine-rich element (ARE) containing mRNAs by RNA binding proteins such as tristetraprolin and catalyzes their deadenylation and decay (23–27). Similarly, the Ski complex composed of Ski2p, Ski3p and Ski8p interacts via Ski7p with the core

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and core exosome components and found evidence for
Rrp6p-specific degradation substrates. Consistent with
these findings, loss of the C-terminal domain of Rrp6p
allows normal RNA 3'-end processing, but inhibits the
degradation rRNA substrates that require Rrp6p and
the core exosome for degradation.

MATERIALS AND METHODS

Northern blot analysis
Total RNA or poly(A)+ RNA was isolated from yeast
strains grown to an A600 of 0.5–2.0 in YPD media as
described (52). Depletion of the indicated cells was
carried out by the addition of doxycyclin (10μg/ml) for 5h,
before cells were collected. Northern blot analysis was
carried out as previously described in Briggs et al. (33).
The 5' 32P oligonucleotide probes OSB138 and OSB267
were described in Phillips and Butler (34), OSB156 in
Briggs et al. (33) and OSB157 in Fang et al. (54).
Northern blots were analyzed by Phosphorimaging and
quantitated using ImageQuant software (GE Healthcare,
Piscataway, NJ).

Plasmid construction

Yeast centromere plasmids expressing each of the RRP6
mutants fused to green fluorescent protein (GFP) were
constructed by gap repair of pGFP-RRP6H as previously
described (34) or by direct cloning into the same vector.
Plasmid pGFP-RRP6Δ210 (deletes amino acids 1–210)
was constructed by inserting a PCR fragment ampli-
ified using OSB 620 and OSB431, into pGFP-RRP6H
cut with XbaI and BglII. Plasmid pGFP-RRP6Δ343
(deletes amino acids 176–210) was constructed by inserting
a PCR fragment amplified using OSB 709 and OSB41 to
amplify hybridized templates produced by PCR with
OSB707 and OSB709, and OSB708 and OSB41 into
pGFP-RRP6H cut with XbaI and BglIII. Plasmid pGFP-RRP6Δ343
was constructed by inserting a PCR fragment gener-
ated using OSB41 and OSB42 to amplify hybridized templates
produced by PCR with OSB 695 and OSB41, and OSB696
and OSB42 into pGFP-RRP6H cut with BglII. Plasmid
pGFP-RRP6ΔC1+2 (delete amino acids 441–733) was
constructed by inserting a PCR fragment generated
using OSB706 and OSB726 to amplify hybridized templates
produced by PCR with OSB697 and OSB706, and
OSB698 and OSB726 into pGFP-RRP6ΔHRDC1 (34)
with ClaI and XhoI. Plasmid pGFP-RRP6ΔC2 (deletes
amino acids 523–733) was constructed by inserting a
PCR fragment (OSB733 and OSB734) into pGFP-
RRP6ΔC1+2 cut with ClaI.

Physical association of proteins

Core exosomes was purified from 2L of an RRP46-TAP
rrp6Δ strain constructed in a W303 derivative by the
method of Rigaut et al. (53) (YSB232, MATα ade2-1
his3-11,5 leu2-3,112 trp1-1 upr3-1 pep4::HIS3 RRP46-
TAP rrp6Δ::LEU2) carrying derivatives of pGFP-RRP6H
[CEN6 URA3 MET17-GFP-RRP6; (34)] grown to an
OD600 of 2.0–3.0 in SCD–MET–URA at 30°C. Cells

exosome in the cytoplasm of yeast leading to the degrada-
tion of LA viral RNAs and aberrant mRNAs containing
nonsense codons or lacking a stop codon (10,22,28–31).
In the nucleus, Rrp6p and the core exosome form the
mature 3'-ends of 5.8S rRNA and many sn/sno-RNAs in
a two-step process featuring initial 3'–5' trimming by the
core and Dis3p, followed by removal of the remaining
nucleotides by Rrp6p (20,32–35). The nuclear exosome
also acts in a RNA surveillance pathway that degrades
incompletely processed RNAs. Thus, mRNAs that fail
to complete some splicing or 3'-end processing steps
accumulate in cells with defects in components of the
nuclear exosome (21,36–38). Likewise, rRNA processing
intermediates accumulate in exosome-deficient cells, as do
noncoding RNAs and hypomodified pre-tRNAi
intermediates accumulate in exosome-deficient cells, as do
nuclear exosome (21,36–38). Likewise, rRNA processing
intermediates, which carry poly(A) tails synthesized by the poly(A)-polymerases
Trf4p and Trf5p, which function in complexes termed
TRAMP4 and TRAMP5, respectively, that also contain
the RNA helicase Mtr4p and the zinc-knuckle proteins
Air1p and Air2p (39–42). Recognition and polyadenyla-
dation of these RNAs by TRAMP is thought to facilitate
interaction of the transcripts with the exosome, thereby
enhancing the rate of RNA degradation.

Rrp6p also plays an important role in DRN (degrada-
tion of RNA in the nucleus), a surveillance pathway that
requires the nuclear mRNA cap-binding complex (43).
Loss of function of Rrp6p or Cbp1p, the large subunit of
the cap-binding complex, stabilizes mRNAs trapped
in the nucleus, as well as certain transcripts thought to
exit more slowly from the nucleus than bulk mRNA
(43,44). However, loss of Cbc1p activity does not lead
to a general accumulation of polyadenylated rRNAs,
suggesting that it does not function in concert with
TRAMP to facilitate the degradation of this class of
RNAs (45).

Rrp6p plays a critical role in RNA 3'-end formation
and degradation pathways in the nucleus, yet no experi-
ments have addressed the important question of whether
the activities of Rrp6p require its physical association
with the core exosome. The current model for RNA processing
and surveillance by the nuclear exosome implies that
Rrp6p activity acts in concert with the core exosome com-
plex, either through physical association or close coupling
(13,46,47). Indeed, affinity purifications of core exosome
subunits from yeast co-purify all of the other core poly-
peptides, as well as Dis3p/Rrp44p and Rrp6p, and affinity
purification of Rrp6p co-purifies all of the core subunits
and Dis3p/Rrp44p (48,49). Rrp6p also co-purifies with the
human and Trypanosoma brucei core exosomes (25,50).
Moreover, experiments in vitro that first characterized
TRAMP activity did so in the context of its ability to
enhance the activity of the nuclear exosome, or core
exosome preparations likely containing Rrp6p (41,51).
These considerations and the fact that Rrp6p and the
core exosome appear to carry out distinct steps in RNA
3' end formation prompted us to evaluate the relative
contributions of Rrp6p and the core exosome to RNA
degradation in vivo. We investigated the RNA processing
and degradation phenotypes of strains depleted of Rrp6p
and core exosome components and found evidence for
were collected by centrifugation at 7500g and suspended in 10 M K-HEPES pH 7.9, 10 M KCl, 1.5 M MgCl2, 0.5 M DTT, 2 mM benzamidine, 0.5 mM PMSF, 1 mM leupeptin, 2 mM pepstatin, 4 mM chymostatin and 2.6 mM aprotinin, and disrupted by two passes through a French pressure cell at 850 psi. The cell lysate was cleared by centrifugation at 34 000g for 22 min and then at 75 000g for 60 min. The supernatant was dialyzed against 20 M K-HEPES pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% DTT, 2 mM benzamidine and 0.5 mM PMSF and 2 mM benzamidine for 3.5 h at 4°C. Five microliter of lysate was bound to 120 μl IgG beads (Amersham Biosciences, Piscataway, NJ) for 2 h at 4°C with gentle rocking. The IgG beads were pelleted by centrifugation and the supernatant was collected and labeled the ‘Free’ fraction. The beads were then washed with 50 ml of 10 mM Tris–Cl pH 8.0, 150 mM NaCl and 0.1% NP40 at 4°C. Core exosome complexes were released from the beads by incubation in 500 μl of 10 mM Tris–Cl pH 8.0, 150 mM NaCl and 0.1% NP40, 0.5 mM EDTA, 1 mM DTT with 70 U AcTEV enzyme (Invitrogen, Carlsbad, CA) for 3.5 h at 14°C with gentle rocking. The IgG beads were pelleted by centrifugation and 150 μl of the supernatant was precipitated with trichloroacetic acid, resuspended in SDS–PAGE sample buffer and called the ‘Bound’ fraction. Western blot analysis was performed as previously described (21) with monoclonal anti-GFP (1:1000; Roche), anti-Rrp6p (1:1000; Dr Phillip Mitchell), anti-Rrp4p (1:1000; Dr David Tollervey).

RESULTS

Identification of Rrp6p-specific RNA degradation intermediates

To determine the relative contribution of Rrp6p and the core exosome to RNA degradation, we analyzed the levels of poly(A)+ rRNAs in cells lacking Rrp6p, or after depletion of the core exosome component Rrp43p, the core exoribonuclease Dis3p/Rrp44p or the TRAMP component Mtr4p. Depletions of the essential Rrp43p, Dis3p or Mtr4p were accomplished using strains in which their genes are fused to the doxycycline repressible tetO7 promoter. Total RNA was isolated from these strains after 5 h of doxycycline treatment, the duration we previously determined to be the shortest necessary for maximal accumulation of RNA processing intermediates. The RNA samples were then separated into poly(A)- and poly(A)+ fractions using oligo(dT)-cellulose, separated by gel electrophoresis and rRNA processing intermediates detected by northern blot analysis. In this last step, we used radiolabeled oligonucleotides complementary to the external and internal transcribed spacers of the pre-rRNA transcript containing the mature 18S, 5.8S and 25S rRNAs (Figure 1). Thus, only pre-rRNA processing intermediates are detected. Previous studies showed that this method detects those intermediates that become polyadenylated by TRAMP, but fail to be degraded in the absence of Rrp6p (39,45,54). To control for differences in gel loading and the efficiency of oligo(dT)-cellulose fractionation, we also analyzed the level of ACT1 mRNA in each sample (Figure 2). Note that ACT1 mRNA pools contain significant levels of poly(A)-deficient transcripts that do not bind to oligo(dT)-cellulose (Figures 2 and 3) (52,55). Analysis of the effects of these depletions on normal pre-rRNA processing intermediates, which do not bind oligo(dT)-cellulose, reveals that depletion of Rrp43p, Dis3p and Mtr4p results in the enhanced accumulation of 35S and 23S pre-rRNAs, consistent with an indirect inhibition of pre-rRNA processing, as previously shown (Figure 2A) (32). These depletions also cause the expected accumulation of 7S pre-rRNA and the accumulation of the 5′ETS, an rRNA processing byproduct degraded by the combined actions of the core exosome, Mtr4p and Rrp6p (Figure 2A). Other smaller changes in pre-rRNA levels seen in Figure 2 were not consistent from experiment to experiment, and are of questionable significance. We conclude that the depletions of Rrp43p, Dis3p and Mtr4p result in the expected defects in pre-rRNA metabolism.

Analysis of poly(A)+ RNA allows identification of transcripts targeted for degradation by TRAMP specific polyadenylation (39,41). The results from the depleted strains revealed varying levels of poly(A)+ pre-rRNA accumulation (Figure 2B). In these experiments, pre-rRNAs designated 23S and 23S*, 21S and 21S* migrate together, respectively. They are distinguished from one another by the fact that 23S* and 21S* hybridize to an oligonucleotide targeted between cleavage sites D and A2 (OSB154), but not to one targeted between cleavage sites A3 and A3 (OSB183), while 23S and 21S hybridize to both oligonucleotides (Figures 1A and 2B). The 23S* and 21S*, along with 17S (see below), pre-rRNAs were previously identified by Allmang et al. (32), who concluded that they accumulate specifically in exosome mutants. Depletion of Rrp43p and Dis3p showed similar patterns of poly(A)+ pre-rRNA accumulation, consistent with their function together in the core exosome complex. These depletions each cause the accumulation of poly(A)+ forms of 23S*, 21S* and 27S pre-rRNAs (Figure 2B). Depletion of Mtr4p also results in the accumulation of poly(A)+ 7S and 27S pre-rRNAs, consistent with its role as a co-factor in core exosome function (56,57). The rrp6Δ strain differs from the Rrp43p and Dis3p depletions in that it accumulates polyadenylated forms of 27SA2, 23S, 21S, 17S, pre-rRNAs, an apparently novel poly(A)+ RNA designated D-B1L, and a previously identified species called 5′-S whose 5′-end probably corresponds to the 5′-end of the pre-rRNA transcript (58) (Figure 2B). Some of these Rrp6p-specific RNAs, notably 23S, 21S and D-B1L, also accumulate in the Mtr4p depletion suggesting that Mtr4p and Rrp6p may act together to degrade them.

Next, we tested the effects of depletion of Dis3p in the absence of Rrp6p to determine if synergistic effects on poly(A)+ rRNA processing occur. The intensity of the northern blot bands corresponding to the poly(A)+ 23S+23S*, 21S+21S* and 5′ ETS RNAs increased significantly after depletion of Dis3p in the tetO7→DIS3 rrp6Δ strain compared to the rrp6Δ and tetO7→DIS3 strains (Figure 3A and B). In contrast, the 27SA2, 23S, 21S, 17S, 5′-S and D-B1L RNAs showed no significant increase in the tetO7→DIS3 rrp6Δ strain compared to
the rrp6-Δ and tetO7-DIS3 strains (Figure 3C). These findings support the conclusion that Rrp6p plays a core exosome-independent role in the degradation of some of these poly(A)+ rRNAs.

Rrp6p-specific degradation of unadenylated rRNAs

We asked if the specificity of Rrp6p RNA degradation is confined to poly(A)+ rRNAs. We analyzed 5.8S rRNA degradation products on northern blots of total RNA from various strains (Figure 4A). We included RNA from an rrp6-Δ strain, and strains depleted of Dis3p, Mtr4p and the 5′–3′ exoribonuclease Rat1p. The blots were probed with OSB156, which hybridizes to the mature portion of 5.8S rRNA, and OSB157, which hybridizes to ITS2 just downstream of 5.8S rRNA (Figure 1). The absence of Rrp6p causes the accumulation of the 3′-extended 5.8S+27 pre-rRNA (Figure 4A, lanes 2, 4 and 7). Depletion of Dis3p and Mtr4p cause the accumulation of intermediates migrating faster than 7S pre-rRNA (Figure 4). Depletion of Rat1p is confirmed by the increase in the ratio of the long to short forms of 5.8S rRNA (Figure 4, lanes 6 and 7). Prolonged exposure of the blot probed with OSB156 revealed truncated 5.8S RNAs that accumulate in the rrp6-Δ and Rat1p depleted strains, but not in the Dis3p or Mtr4p depleted strains (Figure 4). These short RNAs do not bind to oligo(dT), suggesting that they do not carry poly(A) tails (data not shown). These findings suggest that these unadenylated 5.8S rRNA fragments are degraded independently of the core exosome by the combined action of Rrp6p and Rat1p.

Rrp6p requires its C-terminal domain for interaction with the core exosome

We constructed deletions of Rrp6p with the goal of identifying domains necessary for its interaction with the core exosome. The crystal structures of a fragment of yeast Rrp6p and the Escherichia coli Rrp6p homologue RNaseD, and amino acid sequence similarity analysis
formed the basis for these deletions (33,59,60). Rrp6p has an N-terminal region found in eukaryotic, but not in bacterial homologs. This region is required for interaction of Rrp6p with its co-factor Lrp1p/Rrp47p (61). In addition, the Rrp6p and RNaseD structures indicated three independently folded domains. The first contains three motifs (Figure 5A, ExoI, II, II) that we and others previously showed to be necessary for the exoribonuclease activity of Rrp6p (34,59). The second contains conserved amino acids found in the HRDC domains of enzymes involved in the metabolism of nucleic acids (62). The third domain shows no significant sequence similarity to other RNaseD family members. Nevertheless, in RNaseD this domain folds into a structure very similar to the adjacent, conserved HRDC domain (60). Following the convention of Zuo et al., we refer to these two domains as HRDC1 and HRDC2. Finally, Rrp6p contains two sequences similar to nuclear localization signals (NLS) that we showed previously to play a modest role in localization of Rrp6p to the nucleus, but are unnecessary for other functions of Rrp6p (34). We constructed deletions of these domains, as well as several within the N-terminal region, and expressed them as N-terminal GFP fusions from low copy plasmids in yeast (Figure 5A). We showed previously that GFP-Rrp6p fusions expressed in this manner complemented a deletion of the RRP6 gene (34). We analyzed the functions of Rrp6p and RNaseD indicated three independent domains. The first contains three motifs that we and others previously showed to be necessary for the exoribonuclease activity of Rrp6p (34,59). The second contains conserved amino acids found in the HRDC domains of enzymes involved in the metabolism of nucleic acids (62). The third domain shows no significant sequence similarity to other RNaseD family members. Nevertheless, in RNaseD this domain folds into a structure very similar to the adjacent, conserved HRDC domain (60). Following the convention of Zuo et al., we refer to these two domains as HRDC1 and HRDC2. Finally, Rrp6p contains two sequences similar to nuclear localization signals (NLS) that we showed previously to play a modest role in localization of Rrp6p to the nucleus, but are unnecessary for other functions of Rrp6p (34). We constructed deletions of these domains, as well as several within the N-terminal region, and expressed them as N-terminal GFP fusions from low copy plasmids in yeast (Figure 5A). We showed previously that GFP-Rrp6p fusions expressed in this manner complemented a deletion of the RRP6 gene (34). We analyzed the function of the mutant Rrp6p proteins in a strain carrying a deletion of RRP6 and a C-terminal fusion of Rrp46p to the TAP affinity tag.
Western blot analysis showed that all of the proteins were expressed at levels similar to wild type GFP-Rrp6p (Figure 5 and data not shown). The core exosome was purified from lysates of these cells by adsorption of Rrp46-TAP to IgG-Sepharose beads, followed by release of the core exosome complexes with the TEV protease. Western blot analysis of the released core exosomes using antibodies to the core component Rrp4p and to Rrp6p allowed us to determine if Rrp6p deletion derivatives bind the core exosome. One advantage of fusing Rrp6p-deletions to GFP is that it allowed us to use anti-GFP antibody to detect the N-terminal deletion Rrp6p-C1+2 and Rrp6p-C2 (Figure 5B and C). In some experiments, Rrp6p bands appear as doublets, apparently due to proteolysis. In the cases of the Rrp6p-C1+2 and Rrp6p-C2 mutants, Rrp6p was not detected in the bound fractions, despite significant enrichment of the core component Rrp4p (Figure 5C).

We conclude that removal of the Rrp6p C-terminal domain causes a significant decrease in its ability to interact with the core exosome.

Previous studies showed that deletion of RRP6 results in a temperature sensitive growth phenotype (21). Interestingly, mutations in the exonuclease motifs that inactivate the RNA processing and degradation activities...
of Rrp6p still allow growth of the mutant strains at high temperature suggesting that Rrp6p possesses some uncharacterized activity necessary for growth at high temperature (34). We tested some of the deletion mutants for their ability to complement this growth defect and found that the Δ210 and ΔHRDC1 proteins allow significantly more growth at 37°C than the GFP negative control (Supplementary Figure S1). The ΔC1 + 2 mutation grows no better than the negative control suggesting the absence of some critical activity and/or that the protein may be misfolded and completely inactive. The ΔC2 mutant complements the temperature sensitivity quite well. This finding suggests that the temperature sensitive phenotype of an rrp6-Δ strain does not result from the lack of Rrp6p–core exosome interaction.

RNA 3’-end formation does not require Rrp6p interaction with the core exosome

Next, we examined RNA processing (3’-end formation) associated with defects in Rrp6p. Previous studies showed that deletion of RRP6, or mutations inactivating its exoribonuclease activity, resulted in defects in the 3’-end processing of 5.8S rRNA and a variety of sn/-snoRNAs (34,35,57,64). We analyzed 5.8S rRNA and the snoRNAs snR72 and U24 in rrp6-Δ cells expressing each of the deletion derivatives of Rrp6p (Figure 6A, E and F). We included the previously characterized ΔNLS mutant as a positive control since it behaves normally for all tested RNA phenotypes, despite partial cytoplasmic localization (34). The results showed the expected 3’ extensions on 5.8S rRNA, snR72 and U24 in the strain expressing GFP only, and GFP-Rrp6p complements these defects (Figure 6B, E and F, compare lanes 1 and 2). All of the Rrp6p domain deletions except ΔC2 and ΔNLS show defects in 5.8S rRNA 3’-end processing similar to that observed in the absence of Rrp6p. These two deletion mutants also show normal 3’-end processing of snR72 and U24 RNAs. In contrast, all of the other deletion mutants, except ΔHRDC1, show defects in snR72 and U24 processing. The lack of an effect of the ΔHRDC1 mutation on snRNA 3’-end processing agrees with previous studies showing that a mutation in this region (rrp6-13; D457A) caused snRNA 3’-end formation defects for some, but not all snoRNAs tested (34,59). Importantly, the ΔC2 mutation, which disrupts binding of Rrp6p to the core exosome, shows normal 3’-end formation for 5.8S rRNA and snoRNAs. These findings indicate that deletion of the C-terminal domain does not inhibit the exoribonuclease activity of Rrp6p. We conclude that disruption of Rrp6p–core exosome interaction does not significantly inhibit the RNA 3’-end formation activity of Rrp6p.

Loss of the Rrp6p C-terminal domain inhibits the degradation of some rRNA processing products

Rrp6p also plays a role in the degradation of rRNA processing byproducts and intermediates (7,32,39). One by-product is the 5’-ETS released after cleavage of the rRNA precursor at site A0 (Figure 1). This RNA is normally degraded by the action of the core exosome and Rrp6p (Figures 2 and 3) (32). Analysis of the levels of 5’-ETS in the Rrp6p deletion mutants shows that all of the mutations, except ΔNLS, elevate 5’-ETS levels (Figure 6A). The fact that the ΔC2 mutation results in the accumulation of 5’-ETS RNA suggests that disruption of Rrp6p interaction with the core exosome hampers the ability of Rrp6p to efficiently degrade this by-product of rRNA processing.

Kadaba et al. (40) showed that defects in Rrp6p and the core exosome component Dss3p result in the accumulation of a 3’ truncated form of 5S rRNA. This 5S rRNA fragment appears to be an intermediate in the exosome degradation pathway since it accumulates in the absence of Tr4p, which activates RNAs for degradation by the core exosome and Rrp6p. We observe this 5S intermediate in all of the Rrp6p deletion mutants except the ΔNLS (Figure 6C and D; note that 5d is a longer exposure than 5c). In the case of the ΔC2 domain, we also observe a unique RNA fragment migrating faster than 7S pre-rRNA (Figure 6A; lane 8). Primer extension analysis of this fragment does not reveal new products compared to wild-type or other deletion mutants suggesting that its is shorter at its 3’- rather than its 5’-end (Supplementary Figure S2). The fact that the ΔC2 mutant fails to degrade these RNAs suggests that their disposal requires normal affinity of Rrp6p for the core exosome, or that recognition
of these intermediates requires some other function of the C-terminal domain.

Previous studies showed that a fraction of 3' truncated 5S and 5'S-ETS RNAs accumulate in exosome mutants as polyadenylated species reflecting activation of their degradation by the TRAMP complex (39,40). Our analysis shows that poly(A)+ 5'S-ETS RNA does not accumulate in the ΔC2 mutant to the same degree seen in the absence of Rrp6p, suggesting that efficient removal of the poly(A) tail does not require the interaction of Rrp6p with the core exosome (Figure 7). In contrast, the poly(A)- form of 5'S-ETS accumulates in the ΔC2 mutant to the same extent as that seen in the absence of Rrp6p (Figure 7).

**Loss of the Rrp6p C-terminal domain does not affect degradation of Rrp6p-specific RNAs**

Next, we asked whether the degradation of 5.8S fragments requires the interaction of Rrp6p with the core exosome. The fragments appear in all of the Rrp6p deletion mutants except for the ΔC2 and ΔNLS (Figure 8). Their absence in the ΔC2 mutant supports the conclusion that Rrp6p degrades them, despite its reduced affinity for the core exosome.

Finally, we investigated whether disruption of Rrp6p–core exosome interaction interferes with the degradation of poly(A)+ rRNAs that accumulate in the absence of Rrp6p (Figure 2B). Northern blot analysis of oligo(dT)-selected rRNAs shows that these transcripts accumulate in strains lacking the N-terminus of Rrp6p, its HRDC1 or C1 + 2 domains (Figure 9) In contrast, they do not accumulate in the absence of C-2. These findings support the conclusion drawn from the analysis of the effects of depleting Dis3p in absence of Rrp6p (Figure 2B), that the degradation of these poly(A)+ RNAs by Rrp6p occurs without significant influence by the core exosome.

**DISCUSSION**

The experimental results reported here shed light on the question of whether the activity of the exoribonuclease Rrp6p requires physical association with the core exosome. Our findings indicate that Rrp6p functions
in a core exosome-independent manner for some activities \textit{in vivo}. Previous studies that analyzed polypeptides associated with affinity purified core exosomes showed that Rrp6p co-purifies with these complexes (48,49,65,66). Likewise, affinity purification of yeast Rrp6p in some of these studies co-purified the core exosome components (25,50). However, these studies did not address the relative stoichiometries of the proteins or the possibility that some of these proteins might exist in sub-complexes or free pools of individual subunits. More recent analyses in human cells, plants and flies suggested that functionally important sub-complexes of core exosome subunits may exist (67–69). Initial evidence for functional independence of Rrp6p from the core exosome comes from the fact that the core exosome functions prior to Rrp6p in the 3'-end processing of 5.8S rRNA and many snRNAs and snoRNAs (20,33,35,57,70). Indeed, our results show that deletion of the C-terminal domain of Rrp6p significantly impairs it ability to interact with the core exosome, but has no effect on these 3'-end formation activities. Moreover, Dis3p, the active component of the core exosome, and Rrp6p exhibit significant differences in their exoribonuclease activities. Dis3p appears to degrade RNA processively, while Rrp6p uses a more distributive mode (15,17,21). Rrp6p also showed better activity than Dis3p in the removal of poly(A) tails from model substrates \textit{in vivo} (15). Interestingly, the interaction of Dis3p with the rest of the core proteins significantly inhibited its activity relative to free Dis3p, while Rrp6p showed similar activity in the presence or absence of the core exosome.

The findings reported here suggest that Rrp6p may function \textit{in vivo} without direct interaction with the core exosome. Deletion of the C-terminal domain of Rrp6p disrupts its ability to co-purify with the core exosome. Nevertheless, the mutant enzyme retains its exoribonuclease activity as shown by the fact that it carries out Rrp6p-specific 3'-end processing of 5.8S rRNA and snoRNAs. In contrast, the \( \Delta \)C2 mutant fails to efficiently degrade some rRNA intermediates that appear to require the action of Rrp6p and the core exosome. For example, the 5'-ETS by-product of rRNA processing accumulates in \textit{rrp6}-mutants and in strains depleted of core exosome components, and combination of these defects causes a synergistic increase in 5'-ETS levels (Figures 2, 3 and 5) (20,34). The \( \Delta \)C2 mutant fails to degrade this RNA suggesting that efficient degradation requires physical interaction between Rrp6p and the core exosome, or that the C-terminal domain plays a specific role in the recognition of this RNA. The 5'-ETS RNAs also accumulate as poly(A)+ forms in Rrp6p- and core exosome-deficient cells implying that the TRAMP complex polyadenylates them prior to degradation by Rrp6p and the core exosome [Figures 2 and 7, (39)]. However, poly(A)+ forms of 5'-ETS do not accumulate to a significant extent in the \( \Delta \)C2 mutant, suggesting that Rrp6p removes their poly(A) tails in an exosome-independent manner (Figure 7, lane 4). We observed similar results for the 3' truncated form of 5S rRNA, shown previously to require Rrp6p, TRAMP and the core exosome for its degradation (data not shown) (40). Thus, in the cases where Rrp6p and the core exosome co-operate to degrade an RNA, it appears that Rrp6p may remove the poly(A) tails in an exosome-independent manner and then participate with the core exosome in the degradation of the body of the transcript.

Our experiments also identified rRNA processing intermediates that appear to be degraded by Rrp6p in a core exosome-independent manner. These poly(A)+ RNAs (27S\( _A_2 \), 23S, 21S, 17S, 5S and D-\( _{B1} \)) accumulate in the absence of Rrp6p and in certain Rrp6p deletion mutants, but not upon depletion of core exosome components (Figures 2, 3 and 8). Moreover, while the depletion of Dis3p in an \textit{rrp6}-A strain results in a synergistic enhancement of the accumulation of some poly(A)+ RNAs, these Rrp6p-specific transcripts are unaffected (Figure 3). Thus, unlike the 5'-ETS and 3' truncated 5S rRNAs, these intermediates seem to require only Rrp6p for their degradation. Indeed, they do not accumulate in the \( \Delta \)C2 mutant suggesting that their degradation does not require the participation of the core exosome. However, the detection of Rrp6p-core exosome interaction does not disrupt the mechanism, the fact that these RNAs do not accumulate in the \( \Delta \)C2 mutant indicates that disruption of Rrp6p-core exosome interaction does not affect their degradation.

Analysis of unadenylated degradation intermediates of 5.8S rRNA also showed that Rrp6p degrades these RNAs in a core exosome-independent manner. The hydrolysis of these RNAs requires Rrp6p, but not the core exosome or the TRAMP complex (Figure 7A). The fact that these intermediates do not appear in the \( \Delta \)C2 mutant indicates that their degradation may not require the interaction of Rrp6p with the core exosome. Notably, these RNAs do not appear in poly(A)+ fractions of RNAs fractionated by oligo(dT) indicating that they do not carry significant poly(A) tails (data not shown). Thus, unlike the RNAs discussed above, they may never have entered, or they may have already exited, the TRAMP-dependent RNA degradation pathway.

The conclusion that the C-terminal portion of Rrp6p plays a critical role in its interaction with the core exosome agrees with experiments on the structure of the human exosome. Two-hybrid system studies of the interaction of human Rrp6p (PMScl-100) with other core exosome components indicated that the C-terminal 276 amino acids of hRrp6p interact with hRrp43 (Oip2) (71). This fragment includes a region corresponding to the \textit{E. coli} HRDC2 domain and shows significant similarity between the yeast and human proteins (33,60). Thus, the human interaction data support our conclusion that the C-terminal domain plays an important role in the interaction of Rrp6p with the core exosome.

In summary, the findings reported here provide evidence that Rrp6p may act independently of the core exosome during the degradation of some rRNA processing products. Structure-function analysis of Rrp6p revealed that it requires its C-terminal domain for interaction with the core exosome. When this interaction is disrupted,
Rrp6p continues to carry out critical nuclear RNA 3'-end processing and degradation steps, yet fails to degrade RNAs that appear to require the combined action of Rrp6p and the core exosome. These results suggest a functional sub-specialization of core exosome and Rrp6p functions in the nucleus.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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