A Pre-Ribosome with a Tadpole-like Structure Functions in ATP-Dependent Maturation of 60S Subunits

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Summary

Analyses of isolated pre-ribosomes yielded biochemical “snapshots” of the dynamic, nascent 60S and 40S subunits during their path from the nucleolus to the cytoplasm. Here, we present the structure of a pre-60S ribosomal intermediate located in the nucleoplasm. A huge dynactin-related AAA-type ATPase (Rea1) and the Rix1 complex (Rix1-Ipi1-Ipi3) are components of an extended (~45 nm long) pre-60S particle. Antibody cross-linking in combination with electron microscopy revealed that the Rea1 localizes to the “tail” region and ribosomal proteins to the “head” region of the elongated “tadpole-like” structure. Furthermore, in vitro treatment with ATP induces dissociation of Rea1 from the pre-60S subunits. Rea1 and the Rix1 complex could mediate ATP-dependent remodeling of 60S subunits and subsequent export from the nucleoplasm to the cytoplasm.

Introduction

Ribosome biogenesis is a complicated process that begins with transcription of ribosomal RNA by RNA polymerase I (25S, 18S, and 5.8S rRNA) and RNA polymerase III (5S rRNA) in the nucleolus (Warner, 1990) and ends with final export of 60S and 40S subunits to the cytoplasm. During transcription, many RNA processing enzymes and non-ribosomal factors are recruited, creating the “terminal knob” or the small subunit (SSU) processing. Some on the nascent 35S pre-rRNA (Dragon et al., 2004). As a result of these complicated assembly steps, a 90S pre-ribosomal particle is first formed that contains predominantly small subunit ribosomal proteins and ~35 non-ribosomal factors, including many U3 snORF components and factors required for 18S rRNA synthesis. Thus, 90S pre-ribosomes are generated through the early binding of pre-40S factors onto the 35S pre-rRNA before the majority of the pre-60S factors are recruited (Grandi et al., 2002).

After cleavage of the pre-rRNA at sites A1 and A2, the small and large subunits’ pathways diverge (Venema and Tollervey, 1999). Both pre-60S biogenesis and pre-40S biogenesis have been followed by biochemical purification of intermediate particles (Bassler et al., 2001; Hampicharchai et al., 2001; Savea et al., 2001; Fatica et al., 2002; Nissan et al., 2002; Schafeler et al., 2002). A large number of non-ribosomal proteins that have been identified in pre-60S particles have no assigned function in RNA metabolism. However, many of these factors have intriguing motifs, such as putative GTPases and AAA-type ATPases (Tschochner and Hurt, 2003). The number of non-ribosomal proteins associated with pre-60S particles decreases during the maturation process, from more than 30 components initially present on early particles to a handful of proteins associated with late particles that are close to nuclear export.

The rix1-1 mutation was identified in a screen for temperature-sensitive (ts) strains with defects in nuclear export of 60S subunits (Bassler et al., 2001; Gadal et al., 2004). Rix1 is an essential nucleoplasmic protein associated with late pre-60S ribosomes. The rix1-1 mutant showed no clear pre-rRNA processing defect, but accumulated a large subunit reporter Rpl25-GFP throughout the nucleoplasm at the restrictive temperature, indicating a relatively late block in the export pathway. Tandem affinity purification (TAP) of Rix1 revealed its association with three uncharacterized ORFs, Yhr085w (Ipi1), Ynl182c (Ipi3), and Rea1, which at 560 kDa is the largest protein identified in the yeast genome (Nissan et al., 2002). Rea1 possesses six N-terminal AAA ATPase repeats and a C-terminal MIDAS (metal ion dependent adhesion site) motif. Furthermore, sequence analysis indicated a relatedness to dynein (Garbarino and Gibbons, 2002).

Affinity purification of Rix1 and Ipi1 after centrifugation to pellet ribosomes and pre-ribosomes resulted in a complex of three proteins composed of Rix1, Ipi1, and Ipi3 (Krogan et al., 2004). The Rix1 complex members were shown to be specifically required for the 3’ processing of the 5.8S rRNA, a late pre-rRNA processing step (Krogan et al., 2004). Defects in this step have also been reported for other late acting factors responsible for the export of the ribosome such as Nmd3, Gsp1, and Rrp12 (Ho and Johnson, 1999; Suzuki et al., 2001; Oeffinger et al., 2004).

In this study we report the EM structure of the Rix1-containing pre-60S particle, which is predominantly associated with Rea1 and the Rix1 complex (Rix1-Ipi1-Ipi3). EM images reveal a striking elongated structure with a tadpole-like morphology. We demonstrate the ATP-dependent release of Rea1 along with the Nug2 GTPase from the pre-ribosomal particle. Rea1 and the Rix1 complex may play an essential role in ATP-dependent maturation and nuclear export of nascent 60S subunits from the nucleoplasm to the cytoplasm.

Results

In recent studies, different ribosomal precursors were isolated by employing TAP purification of tagged pro-

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Figure 1. A Late pre-60S Ribosome Particle Containing Rix1, Ipi1, Ipi3, and Rea1

(A) The indicated proteins were isolated from yeast lysates by the TAP method and analyzed on a Coomassie stained SDS 4%-12% polyacrylamide gradient gel. The position of the tagged bait proteins is indicated by a star. The indicated copurifying proteins were identified by mass spectrometry. The major copurifying non-ribosomal bands (Rea1, Rix1, Ipi3, Ipi1) are indicated on the right and further copurifying bands (Sda1, Nog1, Nug2, Rpl3) on the left. The Nog1 band also contained Nap1 and Ycr072, which were found as second and third hits, respectively, by MS. A molecular weight marker is also shown on the left.

(B) ATP-dependent release of Rea1 and Nug2 from the Rix1 particle. Rix1-TAP was affinity purified under standard conditions, with the exception that all buffers contained an additional 10 mM MgCl2 to avoid nucleotides complexing magnesium ions. ATP, AMP-PNP, ADP, and GTP were added after binding of Rix1-TAP to IgG-Sepharose and were present in subsequent steps. Shown are the final EGTA eluates of otherwise identical Rix1-TAP purifications performed in the absence of NTPs (lane 1), or in the presence of 2 mM ATP, AMP-PNP, ADP (lanes 2–4, respectively), or 2 mM GTP (lane 5).

bands, other common bands were also significantly present in the Rix1, Ipi1, and Ipi3-TAP preparations, including Sda1 (particularly enriched in the Ipi3-TAP), Nog1, and Nug2 (Figure 1A). However, these factors were also found in other TAP purifications of pre-60S particles (Nissan et al., 2002). Taken together, biochemical analyses defined a cluster of common non-ribosomal proteins, Rix1, Ipi1, Ipi3, and Rea1, which are specifically enriched in a late nucleoplasmic pre-60S particle. Based on their association with pre-export nascent 60S ribosomes, Rix1, Ipi1, Ipi3, and Rea1 could function in late nucleoplasmic maturation of pre-60S subunits (see also Krogan et al., 2004) and/or their export to the cytoplasm (Baßler et al., 2001; Gadal et al., 2001).

Rea1, which is specifically enriched in the Rix1, Ipi1, and Ipi3 particles, is an AAA-type ATPase (Figure 1A). AAA-domain containing proteins form hexa- or heptameric “doughnut”-shaped rings, which change their conformation depending on the bound nucleotide (Vale, 2000). Thus, ATP-dependent conformational changes in AAA-ATPases can induce dissociation of protein-protein interactions. We wished to analyze whether ATP regulates the interaction of Rea1 with pre-60S ribosomes; therefore, we performed in vitro studies. Rix1-
A Tadpole-like Pre-60S Ribosome

Figure 2. Structure and Classification of the Rix1 Pre-60S Ribosomal Particle

(A) Structure of the pre-60S ribosomal particle with associated Rea1 and Rix1 complex. TAP-purified Rix1, Ipi1, and Ipi3 particles, as well as isolated 60S ribosomal subunits, were negatively stained and viewed in the electron microscope. Shown are overview electron micrographs (upper panel) and a gallery of selected particles (lower panel). Scale bars, 50 nm.

(B–K) Classification of Rix1 particles (B–G) and mature 60 subunits (H–K). Galleries of selected Rix1 particles divided into different classes and corresponding final averages (C, E, and G). (B and C) Class 1 particles represent ~50% from the selected 478 particles and show a typical tadpole-like structure. (D and E) Class 2 particles (~13%) exhibit a shorter tail. (F and G) Class 3 particles (~37%) lack the tail, which could be due to dissociation of Rea1 and/or the Rix1-complex from the 60S moiety. Mature 60S subunits were separated into two different classes (H and J) and corresponding final averages (I and K). Class 1 (H and I) represents ~47% and class 2 (J and K) ~45% from all selected 635 particles. Crosscorrelation factors as compared to the selected reference are indicated in the upper left corner of the image averages: 0.8 (C), 0.7 (E and G), and 0.9 (I and K). The final projection averages are shown as an average (on the left) as well as an electron density contour map (on the right). Scale bars, 10 nm.

TAP was affinity purified in the presence of 2 mM ATP and as controls with 2 mM ADP, AMP-PNP, and GTP as well as without the presence of additional nucleotides. As shown in Figure 1B, TAP-purified Rix1 in the absence of ATP (lane 1) or in the presence of AMP-PNP, ADP, or GTP (lanes 3–5) exhibits the typical pattern of copurifying bands including Rea1, Ipi1, Ipi3, and ribosomal L proteins. In contrast, when Rix1-TAP was affinity purified in the presence of ATP, Rea1 was significantly dissociated (Figure 1B, lane 2). Moreover, the amount of Nug2 protein was also considerably reduced. These data show that in vitro ATP, but not a non-hydrolyzable ATP analog, ADP or GTP, can mediate dissociation of Rea1 and other proteins from the pre-60S subunits.

The finding that it is possible to isolate pre-ribosomal particles in a biochemically stable form promoted us to investigate whether we could determine the structure of these intermediates by electron microscopy (EM). In the past, EM has convincingly revealed the structures of 60S and 40S subunits and 80S ribosomes (Verschoor et al., 1996). We expected the purified pre-60S ribosomes to exhibit a structure distinct from the mature 60S subunit due to the presence of the huge 560 kDa Rea1 protein and the Rix1 complex. Negatively stained pre-60S particles that were affinity purified via the TAP-tagged Rix1-, Ipi1-, and Ipi3 bait proteins (see Figure 1A) each exhibited a predominant morphology in EM, which is strikingly different from mature 60S subunits (Figure 2A). Most particles exhibited a tadpole-like structure, with an overall length of 48.2 ± 5.5 nm.

Crosscorrelation and multivariate statistical analysis was performed for the Rix1 particles, yielding three major classes (Figures 2B–2G). Most abundant was the class 1 (~50% from all selected particles), which exhibits the typical tadpole-like structure. The final average of this class shows flexibility and a non-uniform length of the tail domain. The second class (~13%) is similar...
to class I particles, except that it exhibits a shorter tail.
Class 3 particles (~37%) exhibit only the head and lack
the tail domain structure (15.0 ± 1.8 nm in diameter).
Multivariate statistical analysis was also performed for
isolated 60S subunits, which were separated into two
different classes with the typical overall structure (20.4 ±
2.7 nm in diameter). The particles could be classified
into two major classes, with class 1 (Figures 2H and 2I)
representing ~47% and class 2 (Figures 2J and 2K)
representing ~45% of all particles. Notably, class 2 par-
ticles exhibit most clearly the well-known structure of
the 60S subunit. Thus, electron microscopy of negatively
stained pre-60S ribosomes revealed a structure that sig-
nificantly differs from mature 60S subunits.

To address the issue of specific location of Rea1 and
ribosomal proteins within Rix1 pre-ribosome structure,
we performed immunoelectron microscopy. To cross-
link Rea1 molecules between two adjacent tadpole-like
particles, we have purified Rix1-TAP that contained
N-terminal HA-tagged Rea1 and incubated with anti-
HA antibodies. Western blot analysis revealed that this
antibody was specifically bound to the Rix1 particle that
contains HA-Rea1, but not to a control Rix1 particle with
untagged Rea1 (see Supplemental Figure S1 at http://
www.molecule.org/cgi/content/full/15/2/295/DC1). Specific
dimer formation of the tadpole-like particles induced by antibody crosslinking was then revealed by
electron microscopy and quantitative evaluation of this
data (Figure 3). In the case of Rix1 particles with HA-
Rea1, anti-HA antibodies generated ~30% tadpole-like
dimers with a high preference of tail-to-tail contact (Fig-
ures 3A and 3C). Notably, many of the tail-to-tail interac-
tions specifically crossed in the middle region of the tail
suggesting the N terminus of Rea1 lies in this area.
Control Rix1 particles with untagged Rea1 did not ex-
hibit a significant dimer formation upon incubation with
anti-HA antibodies (Figure 3C). To crosslink ribosomal
proteins between Rix1 particles, we used anti-Rpl3 or
anti-Rp10 antibodies. In each case, we observed pre-
dominant head-to-head crosslinks between the tad-
pole-like particles (Figures 3B and 3C). Thus, specific
dimer formation between the tail domains is observed,
when the antibodies against HA-Rea1 were used and
head-to-head dimerization in the case of anti-L3 and
L10 antibodies. These data suggest that Rea1 localizes
to the “tail” region and ribosomal proteins to the “head”
region of the tadpole-like Rix1 particle.

Discussion

More than 150 non-ribosomal factors have been re-
ported to be transiently associated with the developing
pre-ribosomes during their path from the nucleolus to
the cytoplasm. In this study, we have analyzed a late
precursor to the 60S subunit, which represents a nucleo-
plasmic intermediate on its way from the nucleolus to
the cytoplasm. This pre-60S intermediate carries the 560
kDa AAA-type ATPase Rea1, which is distinctly related
to the motor protein dynein, and the Rix1 complex that
is composed of Rix1, Ipi1, and Ipi3 subunits (Krogan et
al., 2004). Reverse tagging of Ipi1 and Ipi3 demonstrate
that their protein composition is similar. Moreover, all
three particles exhibit a similar structure. However, they
differ markedly from mature 60S subunits, presumably
due to the additional presence of Rea1 and the Rix1 complex.

To our knowledge this is the first pre-ribosomal parti-
cle whose structure has been examined by electron mi-
croscopy. The structure resembles a tadpole or sperm,
with a globular head domain (~15 nm in diameter) and a
highly elongated tail domain (~25–30 nm in length).
This structure is suggestive that the head domain is the
60S moiety and the tail contains the Rea1 ATPase. To
address this issue, we performed immunoelectron mi-
croscopy. Formation of dimers, which were induced by
crosslinking antibodies, indicated that the N terminus
of Rea1 is localized to the tail. We assume that also the
Rix1 complex as well as additional factors (e.g., Nug2,
Nog1; see below) are part of the tail. In contrast, ribo-
somal proteins Rpl3 and Rpl10 are present in the head
domain, indicating that this is the 60S moiety. The di-
ensions of the 60S region appear to be less than the
full size of a mature 60S subunit. Suggestively, this could
be the result of a different conformation than the mature
60S subunit because of structural rearrangement before
export through the NPC. Alternately, it may lack some
of the ribosomal proteins present in the mature subunit.
3-D reconstruction of the structure of the pre-60S parti-
bles should provide more details.

Rea1 is a very large, 560 kDa protein, which belongs
to the family of AAA-type ATPases. Rea1 was reported
to be distantly related to dynein (Garbarino and Gibbons,
2002), an AAA-type ATPase with a microtubule motor
activity involved in diverse processes such as vesicular
transport, nuclear migration, and chromosome separa-
tion (Burgess et al., 2003, and references therein). Elec-
tron microscopy has revealed that dynein heavy chain
folds to form a head globular domain with six AAA-type
protomers, from which two elongated structures—the
stalk and stem—emerge. It has been proposed that due
to ATP-induced conformational changes within the AAA
head domain, the position of the stem and stalk changes
relative to the head, which could generate a power
stroke (Burgess et al., 2003, and references therein).
Rea1 also consists of an amino-terminal domain with
six AAA-type protomers, followed by a long middle
domain and a short C-terminal MIDAS domain (Garbarino
and Gibbons, 2002). The MIDAS domain is known to be
a protein-protein interaction motif. Thus, it is possible
that Rea1 forms a globular head domain with the six
AAA protomers arranged in a hexamer ring fashion,
from which a long stalk emerges (formed by the middle
domain) that terminates in the C-terminal MIDAS interac-
tion domain. If Rea1 performs a function related to dy-
nein, it could be involved in intranuclear movement or
export of pre-60S particles from the nucleoplasm to the
cytoplasm. However, the putative tracks upon which
Rea1 might move are not known.

Rea1 could also have a role in remodeling the nascent
pre-60S subunit prior to nuclear export. Proteins of the
AAA-type family are involved in many cellular pro-
cesses including proteolysis, membrane trafficking,
organellar biogenesis, microtubule organization, DNA repi-
lation, intracellular motility, and ribosome biogenesis
The AAA module, which includes Walker A and B motifs,
is involved in ATP binding and hydrolysis. Structural
Figure 3. Antibody-Induced Crosslinking Dimer Formation between Tadpole-like Rix1 Particles

(A) HA-Rea1 containing Rix1-TAP particles were incubated with anti-HA antibodies, further purified, negatively stained, and viewed in the electron microscope. Electron micrographs are shown in overview (upper panel) and as a gallery of selected dimeric complexes (lower panel) with specific tail-to-tail contacts. Scale bars, 50 nm.

(B) Rix1-TAP particles were incubated with anti-Rpl3 antibodies, further purified, negatively stained, and viewed in the electron microscope. Electron micrographs are shown in overview (upper panel) and as a gallery of specific dimeric complexes (lower panel) with specific head-to-head contacts. Scale bars, 50 nm.

(C) Quantitative analysis of antibody-induced dimer formation between tadpole-like Rix1 particles. The bar graph shows both the percentage of overall particles observed and the ratio of tail-tail, tail-60S, and 60S-60S crosslinks. In the case of the HA-Rea1 containing Rix1 particle, two samples were collected (n = 1015 and 870), in which 22% (± 0.8) of all particles were tail-tail interactions, 3.2% (± 1.5) tail-60S, and 2.4% (± 0.8) 60S-60S contacts. For the control with Rix1-TAP and anti-HA (n = 986 and 1316), 1.2% (± 1.2) were tail-tail, 0.73% (± 0.04) tail-60S, and 0.5% (± 0.15) 60S-60S contacts. Incubation of anti-Rpl3 against Rix1-TAP generated 2.3% tail-tail contacts, 6.8% tail-60S, and 26% 60S-60S (n = 1720). The Rix1-TAP anti-Rpl10 treatment showed 2.2% tail-tail, 4.4% tail-60S, and 21% 60S-60S (n = 999).

analysis revealed that the AAA domain forms hexameric or heptameric rings that change conformation depending on the bound nucleotide. This nucleotide-dependent conformational switch applies tension to the bound substrate, causing unfolding of proteins, dissociation of protein-protein interactions, or generation of unidirectional movement. Rea1 may have one or more of these functions. Rea1 may act as a molecular motor or as a chaperone to trigger ATP-dependent remodeling of pre-60S subunits.

Our in vitro studies have shown that ATP induces dissociation of Rea1 and Nug2 from the pre-60S subunits. Taken together, these findings suggest that Rea1 could function in concert with other factors (e.g., Nug2) to remodel the nascent pre-60S ribosome in the nucleoplasm (e.g., to dissociate non-ribosomal factors). This
maturation step could be pre-requisite for successive "downstream" steps that eventually lead to nuclear export of 60S subunits. Consistent with this model, the nuclear export adaptor Nmd3 is not significantly present in the Rix1 particle, but associates only with a subsequent pre-60S particle that can be purified via the Arxl-bait protein (Nissan et al., 2002). Since Nug2 is found associated with the Rea1 and is highly enriched in the Rix1 particle, it is suggestive of a regulatory interaction. This could even be a more general principle, in which pre-ribosomal associated GTPases in concert with AAA-type ATPases coordinate pre-60S biogenesis and nuclear export to the cytoplasm.

Experimental Procedures

Yeast Strains and Plasmids
Genomic integration of TAP (TRP1-marker), which served as C-terminal tags, into yeast strains to create fusion proteins of lpl1/YHR085w (strain DS1-2b, MATa, ura3, trp1, his3, leu2) and Ip3/YNL182c (strain JBa, MATa trp1 ura3 ade2 ade3 leu2 his3) was performed as described (Grandi et al., 2002). For construction of GAL1::HA-REA1 and GAL1::HA-REA1-TAP strains, the GAL1::HA cassette containing the HIS3MX6 marker was integrated 5'-upstream of the ATG start codon of REA1 in the RS453 or RIX1-TAP strains (Longtine et al., 1998).

Affinity Purification and Nucleotide Treatment
Affinity purification of TAP-tagged proteins was performed as described (Grandi et al., 2002). Mass spectrometry using tryptic digests from Coomassie stained bands was performed as described (Baßler et al., 2001). For purification of TAP-tagged bait proteins for electron microscopy, the standard purification protocol was followed except that detergent was omitted after lysis, CaCl2 was omitted from the buffer after binding to Calmodulin-beads, and elution from Calmodulin-beads was with 1.5 mM MgCl2, 50 mM NaCl, 10 mM Tris [pH 8], and 1 mM EGTA. For ATP-dependent release of Rea1 from pre-60S subunits, TAP purification of the Rix1-particle was performed with the addition of 10 mM MgCl2, plus 2 mM ATP, AMP-PNP, ADP, GTP, or without additional nucleotides.

Electron Microscopy and Image Processing
5 μl droplets of TAP-purified Rxl, Ip1, and Ip3 particles, respectively, or purified 60S subunits (isolated by high-speed [50000 × g] sucrose gradient centrifugation) were applied on a glow discharged, carbon-parlodion-coated 200-mesh copper EM grid. After 1 min incubation excess suspension was removed by blotting with filter paper, washed 3 times on clean water droplets, and negatively stained with 2% aqueous uranyl acetate (Fluka, Switzerland) for 45 s. Images were recorded at 50,000 × magnification on an EM912 Omega EFTEM (LEO Electron microscope, Oberkochen, Germany) operated at 120 kV of acceleration voltage equipped with a slow-scan CCD camera with a 2 MHz read out, 16 bit depth, 1024 × 1024 pixel CCD chip (pixel size 19 μm), and a P43 phosphorus scintillator (Proscan, Scheuring, Germany). 2D averaging was carried out using the SEMPER image processing system run on a DEC VAX workstation. 4000-6000 478 boxed particles from TAP-purified Rix1 and 635 Ho, J.H., and Johnson, A.W. (1999). NMD3 encodes an essential Saccharomyces cerevisiae cytoplasmic protein required for stable 60S ribosomal subunits in the nucleolus until export to the cytoplasm. EMBO J. 18, 2389–2399.

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