Species Specificity in Ribosome Biogenesis: a Nonconserved Phosphoprotein Is Required for Formation of the Large Ribosomal Subunit in Trypanosoma brucei

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In the protozoan parasite Trypanosoma brucei, the large rRNA, which is a single 3.4- to 5-kb species in most organisms, is further processed to form six distinct RNAs, two larger than 1 kb (LSU1 and LSU2) and four smaller than 220 bp. The small rRNA SR1 separates the two large RNAs, while the remaining small RNAs are clustered at the 3′ end of the precursor RNA. One would predict that T. brucei possesses specific components to carry out these added processing events. We show here that the trypanosomatid-specific nucleolar phosphoprotein NOPP44/46 is involved in this further processing. Cells depleted of NOPP44/46 by RNA interference had a severe growth defect and demonstrated a defect in large-ribosomal-subunit biogenesis. Concurrent with this defect, a significant decrease in processing intermediates, particularly for SR1, was seen. In addition, we saw an accumulation of aberrant processing intermediates caused by cleavage within either LSU1 or LSU2. Though it is required for large-subunit biogenesis, we show that NOPP44/46 is not incorporated into the nascent particle. Thus, NOPP44/46 is an unusual protein in that it is both nonconserved and required for ribosome biogenesis.

Ribosome biogenesis is a highly conserved process. The conservation is seen not only in the proteins and RNAs that make up the mature ribosome but also in the accessory proteins involved in its biogenesis. In eukaryotes, the initial steps take place in the nucleolus, where RNA polymerase I transcribes the rRNA precursor. The RNA is then processed in a characteristic pathway, while the ribosomal proteins and the 5S RNA are incorporated into the maturing ribosomal subunits. As biogenesis continues, the small subunit is exported to the cytoplasm, where maturation is completed to form the 40S subunit (14, 27). Meanwhile, the large-subunit precursor, in a ~66S particle, undergoes a number of processing events, first within the nucleolus and then within the nucleus, including the incorporation of 5S RNA. It is eventually exported to the cytoplasm, where it reaches maturation as the 60S subunit (12).

In the protozoan parasite Trypanosoma brucei, the overall framework of this pathway is conserved (10). This parasite, which causes African sleeping sickness in humans and wasting diseases in economically important domestic livestock, is related to other trypanosomatid pathogens, including Trypanosoma cruzi (the causative agent of Chagas' disease) and Leishmania species (causative agents of leishmaniasis). In T. brucei, as in other organisms, one of the earliest cleavage events after transcription is the separation of the small-subunit 18S rRNA precursor from the large-subunit precursor rRNAs. As in other eukaryotes, the large-subunit precursor rRNA is then processed to separate from the 5.8S rRNA. Putative orthologues of a number of ribosome biogenesis proteins have been annotated in the T. brucei genome sequence, and at least one biogenesis-specific protein (NOG1) has been shown to be functionally conserved (13, 19). Despite the conservation of these steps and proteins, there are key differences. The most notable difference is that the 25S (large-subunit) rRNA is further processed into six structural RNA species, including two large RNAs (1,500 to 1,900 nucleotides in size) and four small RNAs (77 to 215 nucleotides) (28).

Given the unusual processing of the large-subunit rRNA and the evolutionary distance between T. brucei and the other eukaryotes for which ribosome biogenesis has been studied (principally Saccharomyces cerevisiae and mammals), one would expect to find some novel components in the parasite. One potential novel component is the nucleolar phosphoprotein NOPP44/46. NOPP44/46 was initially identified as having developmentally regulated tyrosine phosphorylation. It is heavily tyrosine phosphorylated in the procyclic insect stage and in the nondividing mammalian intermediate and stumpy bloodstream forms but not in the proliferative mammalian slender bloodstream form (21). In addition, the abundance of the protein is also developmentally regulated, with three- to fourfold-higher levels in stumpy and procyclic forms than in the slender bloodstream form (21). Yeast two-hybrid analysis and coimmunoprecipitation from trypanosome lysates demonstrated that NOPP44/46 associates with NOG1 and the protein kinase CK2 (previously known as casein kinase II) (18).

The NOPP44/46 protein consists of a series of four domains. The first domain (U) consists of the first 96 amino acids and has modest homology to domains found in other nucleolar proteins, including nucleolar histone deacetylase and...
FK506 binding protein. The next domain (I) consists of 43% aspartic acid and glutamic acid residues, while the third domain (A) consists almost exclusively (78%) of acidic residues.

The final domain (R) contains a series of Arg-Gly-Gly (RGG) repeats that enable the protein to bind nucleic acids (5, 6). RGG repeats are found in certain proteins required for ribosome biogenesis, such as fibrillarin (NOP1) and GAR1 (2, 8). Multiple NOPP44/46 isoforms are visible upon immunoblotting specific monoclonal antibodies, resulting from differing numbers of RGG repeats (6) and possibly variations in the number of acidic residues in the A region.

Here we report that NOPP44/46 is a trypanosomatid-specific protein involved in ribosome biogenesis. Knockdown of the endogenous mRNA by RNA interference (RNAi) led to growth arrest accompanied by a severe large-ribosomal-subunit biogenesis defect.

**MATERIALS AND METHODS**

**Strains and media.** All of the work described uses the procyclic *T. brucei* strain 29-13 (29), which expresses both T7 RNA polymerase and the tetracycline (TET) repressor, allowing for TET-regulated expression of introduced genes. Cells were grown in SDM-79 (JRH Biosciences) plus 15% fetal calf serum and also containing 15 μg of G418/ml and 50 μg of hygromycin/ml to maintain the T7 RNA polymerase and TET repressor constructs. Transfections were performed as described previously (1), and stable transfectants were selected by using 2.5 μg of hygromycin/ml. Clonal isolates A6 and C11 were derived by limiting dilution of 29-13 cells stably transfected with pZJM-NOPP44/46. TET-regulated constructs were induced with 1 to 2 μg of TET/ml.

**Plasmids.** To construct the pZJM-NOPP44/46, plasmid DNA from pTbmyc2-UJA (5) was used as a PCR template to amplify the U and J regions with a 5’sense primer (AAGCTT AATAAATATTTCCATGCGCAGCAATGG) and a 3’sense primer (ATGCCTCAGGATTACCCGTTAACATTCCATGT). The PCR fragment was ligated into the pGEM-T Easy vector (Promega). The HindIII-Xhol fragment (sites italicized in the primer sequences) was excised and ligated into the vector pZJM (17).

A portion of the coding region corresponding to the Tb08.12O16.320 gene, a NOPP44/46-related gene, was PCR amplified from 29-13 genomic DNA with a 5’sense primer (AAGCTTATAAATATTCCATGCGCAGCAATGG) and a 3’sense primer (ATGCCTCAGGATTACCCGTTAACATTCCATGT). The PCR fragment was ligated into the pGEM-T Easy vector (Promega). The HindIII-Xhol fragment (sites italicized in the primer sequences) was excised and ligated into the vector pZJM (17).

**RNA isolation and Northern analysis.** Total RNA was isolated with TRIzol (Invitrogen). For Northern analysis, 5 μg of total RNA was loaded per lane on a formaldehyde agarose gel and transferred to Nytran nylon membranes (Schleicher and Schuell). Membranes were hybridized with either an RNA probe (NOPP44/46, Tb08.12O16.320, international transcribed spacer 1 [ITS1], and Tb08.12O16.320 gene, which encodes a predicted protein with U- and R-related domains (but not J and A domains). The first 333 nucleotides of this gene show a significant level of identity (75%) to the NOPP44/46 gene. We therefore tested whether the induction of NOPP44/46 RNAi affected the expression of this related mRNA. Hybridization of RNA from induced and uninduced day 7 NOPP44/46 RNAi cells demonstrated that the abundance of Tb08.12O16.320 mRNA was unaffected (data not shown). In the hope of generating a stronger phenotype, we cloned the transfectant line by limiting dilution. Two clonal lines that had a much more significant reduction in NOPP44/46 mRNA upon induction of RNAi (many other lines did not show a strong effect) were isolated. Northern analysis of one of the clones demonstrated that the NOPP44/46 mRNA had decreased more than 10-fold after 2 days and that this reduction was maintained in samples taken after 7 days (Fig. 1A).

Analysis of the recently completed genome sequence for *T. brucei* revealed a NOPP44/46-related gene, the Tb08.12O16.320 gene, which encodes a predicted protein with U- and R-related domains (but not J and A domains). The first 333 nucleotides of this gene show a significant level of identity (75%) to the NOPP44/46 gene. We therefore tested whether the induction of NOPP44/46 RNAi affected the expression of this related mRNA. Hybridization of RNA from induced and uninduced day 7 NOPP44/46 RNAi cells demonstrated that the abundance of Tb08.12O16.320 mRNA was unaffected (Fig. 1B).

To determine the effect of RNAi on protein expression, cell lysates were prepared 7 days after induction. NOPP44/46 levels dropped approximately sevenfold compared to those of uninduced lysates (Fig. 1C). At the same time, the level of the NOPP44/46-associated protein NOG1 decreased slightly. Since NOG1 levels decreased as cells enter stationary phase (A. Randall, unpublished data), it is not clear if NOG1 levels are directly affected by the decrease in NOPP44/46 or are responding to a change in growth rate (Fig. 1D). Another NOPP44/46-associated protein, p34/p37, and the two isoforms of the glycolytic enzyme PGK were unchanged upon NOPP44/46 depletion.

The growth of cells depleted of NOPP44/46 was monitored. Commensurate with the drops in RNA and protein, the pro-
liferation of the induced strains dropped dramatically at day 5 (Fig. 1D). Despite the lack of proliferation, the cells appeared to be relatively healthy throughout this period. These data demonstrate that NOPP44/46 is required for cell growth.

**NOPP44/46 RNAi leads to a defect in large-subunit biogenesis.** To determine the effect of depletion of NOPP44/46 on ribosome biogenesis, the ribosomal content of the cells was assessed by analytical sucrose gradient analysis. Cells were treated with 100 μg of cycloheximide/ml to freeze the translating ribosomes on the RNA. The cells were then lysed, and the lysate was layered onto sucrose gradients to resolve the various ribosomal particles. In the uninduced cells, a small but definite 40S peak and a distinct 60S peak were observed (Fig. 2A). The polysomes were well defined with increasing numbers of ribosomes. In contrast, the cells induced for NOPP44/46 RNAi had a prominent 40S peak and no visible 60S peak. In addition, the polysome peaks were detected as doublets or half-mers (Fig. 2A). Half-mers result from 40S subunits stalled at the start codon, waiting for recruitment of a 60S subunit. They have been seen in cells that have a 60S biogenesis defect, including yeast (for examples, see references 24 and 30 for yeast and 13 for T. brucei). These data indicate a requirement for NOPP44/46 in 60S biogenesis.

The nucleolar GTP-binding protein NOG1 associates with a 60S precursor containing rRNA intermediates, peaking slightly after the mature subunit on sucrose gradients (13). To assess whether NOG1 could still be incorporated in the absence of NOPP44/46, fractions were collected from the induced gradient and analyzed for the presence of NOG1. Even though NOG1 was not consistently seen to accumulate and may rather reflect differences in the quality of the RNA preparations. However, there was a consistent increase in the relative abundance of the 5.9-kb precursor compared to that of intermediates later in the pathway, with the concomitant appearance of several atypical...
NOPP44/46 is required for large-ribosomal-subunit biogenesis. NOPP44/46 is one such component, being both unique to trypanosomatids and required for large-subunit biogenesis. Analysis of genomic sequences from T. cruzi and L. major show the presence of putative orthologues of NOPP44/46, with each of the four domains that characterize the T. brucei protein. No recognizable homolog outside of the trypanosomatids has been found in repeated searches of GenBank. Perhaps most similar are nucleolin and related molecules (e.g., GAR2), which contain acidic regions and RGG repeats. However, the predominant phenotype when these molecules are depleted is an accumulation of the precursors for 40S rRNA and a depletion of the 40S (66S) region of the gradient. Some of the most unusual rRNAs occur in protozoa and algae, such as the highly fragmented rRNAs in Plasmodium mitochondria and large-subunit RNAs in Euglena (7, 26). The large subunit RNA of trypanosomatids (including T. brucei, Leishmania major [16], T. cruzi [4, 11], and Crithidia fasciculata) is fragmented into six molecules corresponding to the 25S rRNA species found in most eukaryotes. This more-complicated processing should require additional novel components.

DISCUSSION

Ribosome biogenesis is a highly conserved process, although differences in rRNA processing are evident in many organisms. Some of the most unusual rRNAs occur in protozoa and algae, such as the highly fragmented rRNAs in Plasmodium mitochondria and large-subunit RNAs in Euglena (7, 26). The large subunit RNA of trypanosomatids (including T. brucei, Leishmania major [16], T. cruzi [4, 11], and Crithidia fasciculata) is fragmented into six molecules corresponding to the 25S rRNA species found in most eukaryotes. This more-complicated processing should require additional novel components.

Species. The 610-bp 5.8S precursor RNA revealed by an ITS2 probe decreased ninefold in the induced sample in comparison to RNA from the uninduced cells. The small precursor bands at 550 and 340 bp detected by the ITS3 probe were essentially absent in the RNA isolated from the induced cells. In contrast, the small precursor bands at 530 and 340 bp that are detected by the ITS7 probe appeared to be less strongly affected, decreasing at most fourfold in the induced samples. An unusual 2.6-kb product, previously detected by ITS2 probes when NOG1 was depleted, also increased when NOPP44/46 was depleted. The ITS3 and ITS7 probes showed an accumulation of two novel species, represented by a faint 4.5-kb band and a darker 3.8-kb band. No cleavage event within any of the ITS sequences could generate either of these two bands, indicating that at least one of the ends of these molecules must result from an aberrant cleavage within the sequence of the structural RNAs (most likely LSU1). Finally, a probe directed against ITS1, which detects small-subunit precursors, failed to detect any significant differences between the induced and uninduced RNA (data not shown).

**NOPP44/46 is not incorporated in the 60S precursor.** Since depletion of NOPP44/46 blocks large-subunit biogenesis, we wanted to determine whether NOPP44/46 associates with the ribosomal precursor particle. The localization of NOPP44/46 in fractions from the uninduced polysome gradient shown in Fig. 2A was examined. The majority of NOPP44/46 protein was found mainly in the initial fractions, one through seven, from the top of the gradient where soluble proteins are found, with protein levels tapering off in additional fractions (Fig. 4). A similar pattern was seen with the soluble protein PGK. In contrast, the NOG1 protein, a component of the precursor (13), had a distinct peak of protein in fractions 8 through 11 (Fig. 2A), corresponding to the 60S (66S) region of the gradient. This finding demonstrates that, though required for 60S biogenesis, NOPP44/46 is not stably incorporated into the nascent particle.

**FIG. 2.** NOPP44/46 is required for large-ribosomal-subunit biogenesis. (A) Polysome analysis of NOPP44/46 RNAi. Lysates from clone A6 cells, either uninduced or induced (day 8), were layered onto 10 to 40% sucrose gradients. The top of the gradient is on the left. The peaks corresponding to 40S, 60S, and 80S are marked. A horizontal line indicates the location of fractions 8 through 11. (B) Western analysis of NOPP44/46 RNAi polysomes. Fractions 1 to 15 from the induced gradient were loaded onto an SDS-PAGE gel, and the resulting blots were probed with anti-NOG1. The band representing the full-length protein is marked with an asterisk. The dashed lines connect fractions 8 to 11, which contained the bulk of the full-length NOG1 protein, to the same fractions on the gradient.
would suggest a direct role in processing (such as RNase or helicase domains), it does have a series of RGG repeats that allow NOPP44/46 to bind single-stranded DNA and RNA (5). This binding activity could allow NOPP44/46 to recruit processing factors to the site of processing by acting as a bridge between proteins and RNAs. The lack of stable association with the precursor particle does not preclude transient interactions that could be important in such activities.

NOPP44/46 has been shown to interact with a number of the proteins. One interacting protein, NOG1, is a GTP-binding protein that is a component of the 60S precursor. Another set of proteins is p34/p37, a family of nuclear proteins that have been shown to bind 5S RNA (22, 23). NOPP44/46 also associates with protein kinases, one of which is CK2 (18). Still, none of these proteins themselves contain domains or activities consistent with a direct role in RNA processing.

The developmental regulation of NOPP44/46 abundance and phosphorylation suggests the possibility that some aspects of ribosome biogenesis differ between the two life stages of the parasite. Analysis of the recently completed T. brucei genome reveals a family of NOPP44/46-related proteins. Two related genes were found closely linked to the NOPP44/46 gene. These genes (given the systematic names Tb08.12O16.300 and Tb08.12O16.320) both contain a U-like domain, but they lack either the acidic domain or the RGG repeats. An additional U region fragment (Tb08.12O16.310) is evident in the present genomic sequence but awaits verification due to ambiguities in assembly. We have shown that at least one of these genes, the most similar to the NOPP44/46 gene (Tb08.12O16.320), is expressed at the RNA level. Given that NOPP44/46 has its strongest effect on the processing involving the small RNA SR1, it is intriguing to speculate that the other NOPP44/46-related proteins could be involved in some of the other unique processing events found in T. brucei.

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