Trypanosoma brucei RNA Binding Proteins p34 and p37 Mediate NOPP44/46 Cellular Localization via the Exportin 1 Nuclear Export Pathway

Kristina Hellman,† Kimberly Prohaska,† and Noreen Williams*

Department of Microbiology and Immunology and The Witebsky Center for Microbial Pathogenesis and Immunology, 253 Biomedical Research Building, University at Buffalo, Buffalo, New York 14214

Received 16 May 2007/Accepted 21 September 2007

African trypanosomes are the causative agents of African sleeping sickness in humans and nagana in cattle (22). This parasite continues to pose a serious threat to human health and to cause devastating economic losses (25). African trypanosomiasis is a reemerging infectious disease with estimates ranging between 300,000 and 500,000 new cases each year (34). This rise in infection rates is due, in part, to an increase in parasite drug resistance and vector pesticide resistance.

In previous work from our laboratory, two Trypanosoma brucei RNA binding proteins, p34 and p37, were identified and characterized (39, 40). The only major difference between them is an 18-amino-acid insert located within the amino terminus of p37 that is absent in p34. These proteins contain two RNA binding domains (RBD) within the central coding region, and they have been shown to interact with 5S rRNA (31). In addition to the interaction with 5S rRNA, p34 and p37 have been found to interact with a family of nucleolar phosphoproteins, NOPP44/46 (30). These abundant proteins have been identified and characterized by M. Parsons and colleagues (9, 10, 27, 29). An interaction(s) between p34 and p37 and NOPP44/46 was mapped through yeast two-hybrid analysis and protein affinity chromatography and was shown to be mediated via the RNA binding domain of p34 and p37 (30). Thus, the RBD region of p34 and p37 serves to mediate a protein-RNA interaction with 5S rRNA as well as the protein-protein interaction with the NOPP44/46 proteins. At this time, we do not know whether these interactions are cooperative or competitive.

Here, we employ a T. brucei cell line expressing RNA interference (RNAi) that specifically targets both p34 and p37 (19) to further examine the cellular role of the interaction between p34 and p37 and the NOPP44/46 proteins.

MATERIALS AND METHODS

Cell culture. Procyclic-form T. brucei brucei strain 427 was grown in Cunningham's medium supplemented with 10% fetal calf serum (8). Clonal p34/p37 RNAi procyclic cells have been previously described (19). Induction of p34/p37 double-stranded RNA (dsRNA) was initiated with the addition of tetracycline (1.0 μg/ml; Sigma) at a cell concentration of 1 × 10⁶ cells/ml. Additionally, 10 μg/ml rifampicin and 10 μg/ml puromycin were added to maintain the RNAi cell line expressing RNA interference (RNAi).

Antibodies. Antibodies utilized were the following: p34/p37 polyclonal antiserum (39), NOPP44/46 monoclonal antiserum (29), and NOPP44/46 peptide antibody (raised to the peptide CEDMESYGVPPKRGGKS; Bethyl Laboratories); antiserum to T. brucei phosphoglycerate kinase (28); polyclonal antiserum to the T. brucei RNA polymerase II C-terminal domain (9); anti-Xpo1 (6), polyclonal antiserum to the T. brucei poly(A) binding protein (PABP); and β-tubulin monoclonal antibody (Chemicon). Nuclear extract preparation and sucrose gradient analysis. Nuclear extracts were prepared (32) from wild-type cells and p34/p37 clonal RNAi cells at 2 days postinduction of dsRNA expression. Nuclear extracts (either 0.1 or 1.0 mg total protein as determined by the Bradford method) were applied to a continuous sucrose gradient (10 to 30%) and sedimented (31). Total protein from gradient...
fractions was precipitated with trichloroacetic acid (TCA), resolved on a 12%/ sodium dodecyl sulfate (SDS)-polyacrylamide gel, and transferred to a nitrocellulose membrane (Schleicher & Schuell). Western blot analysis was performed with antibodies directed against p34 and p37 or the NOPP44/46 proteins.

**Isolation of trypanosome nuclei.** Nuclei were isolated from wild-type cells and p34/p37 RNAi cells as described elsewhere (33). Briefly, 2.5 x 10^7 procyclic cells were pelleted and resuspended in 5% polyvinylpyrrolidone (PVP; Sigma) containing 0.05% Triton X-100 (Sigma), 5 mM dithiothreitol (DTT), mammalian protease inhibitor mixture (Sigma), and solution P (100 mg phenylmethylsulfonlfuoride, 2 mg pepstatin A in 5 ml of ethanol). Cells were homogenized and passed through a 25-gauge needle. The lysate was underlaid with 0.3 M sucrose in 5% PVP, solution P, 1 M DTT, and protease inhibitor mixture and sedimented at 11,000 g. The resulting top layer containing the cytoplasmic material was stored at -80°C. The pellet containing the crude nuclear extract was resuspended in 8 ml of 2.1 M sucrose in 8% PVP, DTT, protease inhibitor cocktail, and solution P (final volume containing approximately 2 x 10^7 cells equivalents). A 12-ml aliquot of this mixture was applied to a discontinuous gradient in an SW28 tube (bottom to top, 8 ml 2.3 M sucrose-PVP, 8 ml 2.1 M sucrose-PVP, 8 ml 2.01 M sucrose-PVP) and sedimented at 110,000 g for 2 h at 4°C. Nuclei were recovered from the 2.1/2.3 interface. The protein concentration of the cytoplasmic and nuclear isolates was determined using the Bio-Rad DC protein assay.

**Analysis of protein steady-state levels and cellular distribution.** Total cellular protein was prepared from an equivalent number (5 x 10^7) of wild-type cells and cells expressing p34/p37 dsRNA by resuspension in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (16) and boiling for 5 minutes. Half of each sample was electrophoresed in two separate 12% SDS-PAGE gels, followed by transfer to nitrocellulose membranes and Western blot analysis (39). Each pair was analyzed using NOPP44/46 monoclonal antisera or p34/p37 polyclonal antisera for one blot and β-tubulin antibody (Chemicon) for the other blot as an internal loading control. Densitometric analysis was performed using a GS-700 imaging densitometer in combination with the Multi-Analyte software (Bio-Rad) with the corresponding wild type as the reference.

For analysis of protein cellular distribution, 25 μg of cytoplasmic and purified nuclear extracts from wild-type and p34/p37 RNAi cells was analyzed by Western blot analysis as described above.

**Analysis of mRNA steady-state and stability assays.** Total RNA was isolated from 5 x 10^7 wild-type or p34/p37 RNAi cells using TRIzol reagent (Invitrogen). Serial dilutions of total RNA were resolved by electrophoresis through a 2% agarose-formaldehyde gel and transferred to a nylon membrane (Schleicher & Schuell). Northern blot analysis was performed using oligonucleotide probes as previously described (31, 39), and the blots were probed for subsequent analysis (3). Oligonucleotides directed against small subunit (SSU; 18S) and large subunit (LSU; 28S) rRNA were used as loading controls. The oligonucleotide probe used to detect the presence of NOPP44/46 mRNA was the U-3 primer 5'-CCGCATCATTCTTCTTTGCTC-3' (10).

For analysis of mRNA stability, actinomycin D (10 μg/ml; Sigma) was added to 5 x 10^6 wild-type or p34/p37 RNAi cells, after which total RNA was isolated at the indicated time points. For each sample, 5 μg of total RNA was resolved through a 2% agarose-formaldehyde gel and examined by Northern blot analysis as above. Densitometric analysis for both steady-state and stability assays was performed as described above.

**Cellular fractionation for immune capture and LMB experiments.** Wild-type and p34/p37 RNAi cells were grown to the appropriate density (1 x 10^6 cells/ml) and subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis probing for NOPP44/46.

**LMB experiments.** A total of 2 x 10^7 wild-type procyclic cells were treated with LMB (a gift from D. Campbell, UCLA) at a final concentration of 0.001 ng/cell, and aliquots of 1 x 10^6 cells were taken at 0, 12, 24, and 48 h. Following treatment, cells were fractionated into nuclear and cytoplasmic extracts as described above. The equivalent of 1 x 10^6 cells for each sample was subjected to SDS-PAGE using 5 to 20% gradient gels for 16 h at 150 V. The gel was cut in half at the 60-kDa marker band, and each half was transferred to a nitrocellulose membrane. Western blot analysis to detect the presence of NOPP44/46 (lower half of the gel) in each sample was performed as described above. For a loading control, the upper half of the gel was used for Western blot analysis against the RNA polymerase II C-terminal domain. Densitometric analysis was performed as described above.

**RESULTS**

Loss of p34 and p37 leads to an increase in nuclear levels of NOPP44/46 protein. We have previously shown that p34 and p37 interact with the NOPP44/46 proteins in both stages of the trypanosome life cycle, suggesting this protein-protein interaction serves a conserved role within the parasite (30). RNA interference of the p34 and p37 proteins was employed in order to further characterize this interaction. We have previously established a clonal cell line which, upon addition of tetracycline, induces production of dsRNA specific to p34 and p37 (19). This leads to degradation of p34 and p37 mRNA and a phenotypic absence of these proteins, which were shown to be essential for survival of the parasite. Since p34 and p37 and the NOPP44/46 proteins are predominantly localized to the nucleus and nucleolus, respectively, we first investigated this interaction in nuclear extracts, which contain both nuclear and nucleolar material. To determine whether loss of p34 and p37 disrupts NOPP44/46-containing complexes, we examined nuclear extracts that were sedimented through a continuous 10 to 30% sucrose gradient. Figure 1 is representative of five separate experiments. Two overlapping peaks that contain NOPP44/46 were consistently detected in sucrose gradient fractions of nuclear extracts from wild-type procyclic cells (Fig. 1A, bottom panel). The first of these peaks comigrates with p34 and p37 (fractions 18 to 22), while the second peak does not (fractions 24 and 26). In the p34/p37 RNAi cell extracts, the first peak of NOPP44/46, which previously migrated with p34 and p37, was lost (Fig. 1B, lower panel, fractions 18 and 20). The second peak of NOPP44/46 proteins, which did not comigrate with p34 and p37, was still present. This suggests that, in wild-type cells, p34 and p37 and the NOPP44/46 proteins are part of a higher-molecular-weight complex(es) and that loss of p34 and p37 leads to disruption of alterations within this complex(es). We also observed that the abundance of the NOPP44/46 proteins in p34/p37 RNAi nuclear extracts was significantly increased. For comparison pur-
poses, in the bottom panel of Fig. 1B, 0.1 mg (compared to 1.0 mg in Fig. 1A, bottom panel) of p34/p37 RNAi nuclear extracts had to be used for sedimentation analysis to allow for comparable exposures of these blots. Hence, these experiments demonstrate that an increase in nuclear NOPP44/46 protein levels occurred in the absence of p34 and p37.

To further explore this phenomenon, we examined the level of NOPP44/46 proteins by using an equivalent amount of nuclear extract from wild-type and p34/p37 RNAi cells. Using exposures from each experiment in the linear range, Western blot analysis from five separate experiments demonstrated a 12-fold increase in the levels of NOPP44/46 in the p34/p37 RNAi nuclear extracts (Fig. 2, compare NOPP44/46 wild type versus RNAi). These results indicate that p34 and p37 may serve to regulate expression of the NOPP44/46 proteins.

p34 and p37 do not regulate NOPP44/46 mRNA levels in vivo. We wished to determine at which level p34 and/or p37 acts to regulate NOPP44/46 expression. In order to determine whether p34 and p37 affect NOPP44/46 transcript abundance, steady-state levels of transcript were analyzed in both wild-type and p34/p37 RNAi cells. Total RNA was isolated from an equivalent number of wild-type and p34/p37 RNAi cells, followed by Northern blot analysis. No significant differences were found in the levels of NOPP44/46 transcripts in wild-type versus p34/p37 RNAi cells (Fig. 3, top panel, compare wild type to RNAi) compared to the small and large subunit rRNA controls (middle and bottom panels, respectively). An average of at least three separate experiments demonstrated only a 1.17-fold difference in NOPP44/46 transcript abundance between wild-type and p34/p37 RNAi cells. These results indicate that p34 and p37 do not regulate the steady-state level of NOPP44/46 transcript.

Another possibility was that p34 and p37 serve to regulate NOPP44/46 transcript stability, leading to an increase in protein levels in the p34/p37 RNAi cells. In order to determine this, RNA stability assays were performed. Actinomycin D was added to both wild-type and p34/p37 RNAi cells to inhibit RNA polymerase II activity, and total RNA was isolated at increasing time points (Fig. 4). NOPP44/46 mRNA (Fig. 4, top panel) was quite stable in both wild-type and p34/p37 RNAi cells (Fig. 4, top panel, compare wild type to RNAi) compared to the SSU rRNA loading control (Fig. 4, lower panel), showing very little degradation over 4 hours. Upon quantification of three separate experiments, we found an average of only a 1.26-fold difference in stability of the NOPP44/46 transcript between wild-type and p34/p37 RNAi cells over a 4-hour period, which does not account for the differences in protein levels seen in Fig. 1 and 2. Additionally, all forms of NOPP44/46 (10) are equally stable in these experiments. Overall, our results suggest that NOPP44/46 transcript steady-state levels and stability are not changed in the p34/p37 RNAi cells.
compared to wild-type cells. We thus conclude that p34 and p37 do not regulate NOPP44/46 at the level of transcript abundance or stability (Fig. 3 and 4), and so we next wished to determine whether they regulate the total cellular levels of NOPP44/46 protein. An equivalent number of wild-type and p34/p37 RNAi cells were lysed, and protein levels were examined via Western blot analysis. The results shown are representative of four separate experiments. Surprisingly, no change in the overall protein levels of NOPP44/46 occurred within the p34/p37 RNAi cells compared to wild-type cells (Fig. 5, middle panel). This is in contrast to what was seen within the nuclear fraction of p34/p37 RNAi cells (Fig. 2, middle panel, compare wild type to RNAi), suggesting that p34 and p37 act to regulate NOPP44/46 cellular localization.

Loss of p34 and p37 leads to a change in localization of NOPP44/46. Using the protocol of Rout and Field (33), we isolated nuclei to analyze NOPP44/46 protein localization within T. brucei cells. This technique yields more highly purified nuclei than the technique utilized in conjunction with sucrose gradient sedimentation analyses, thus allowing for better evaluation of the nucleocytoplasmic distribution of NOPP44/46. The results in Fig. 6 are representative of three separate experiments. Analysis of phosphoglycerate kinase (a cytoplasmic marker) and RNA polymerase II large subunit (a nuclear marker) indicates that nuclear preparations obtained from both wild-type and p34/p37 RNAi cells yielded clearly distinct subcellular fractions (Fig. 6A and B, lower two panels). Although some nuclear contamination of cytoplasmic extracts may be present (Fig. 6B, third panel), the nuclear fraction is substantially free of cytoplasmic contamination (Fig. 6A, bottom panel). We first confirmed that p34 and p37 are no longer detectable in cell extracts upon induction of p34/p37 dsRNA expression (top panel). Using the nuclear and cytoplasmic fractions, we demonstrated a clear enrichment in NOPP44/46 abundance within the nuclear extracts, concomitant with loss of p34 and p37 expression (Fig. 6A, second panel, compare wild type to RNAi). This increase occurs even though overall protein levels of NOPP44/46 remain unaltered within these cells (Fig. 5), indicating that p34 and p37 do indeed affect NOPP44/46 protein cellular localization.

The p34 and p37 proteins associate with exportin 1. p34 and p37 each contain several domains, including an N-terminal alanine, proline, lysine-rich domain (APK), two RBD, and a C-terminal lysine, lysine, glutamic acid, “X” repeat motif (Fig. 7A). The last 16 amino acids of each protein comprise a bipartite nuclear localization signal (NLS) (Fig. 7A), and these proteins were shown to be predominantly nuclear in our previous work (39). We next wanted to determine if p34 and p37 contain any additional motifs that may be involved in regulating the nuclear export of the NOPP44/46 proteins. Using the NetNES program website (24), we found that p34 and p37 each contain a leucine-rich nuclear export signal (NES) (Fig. 6B).
7A and B). This type of NES is known to mediate the interaction of a subset of nucleocytoplasmic shuttling proteins with the nuclear export factor exportin 1 (14). Figure 7B shows the p34 and p37 NES compared to the canonical sequence, which is based on the human immunodeficiency virus type 1 Rev protein (7). We also show the NES from TcUBP-1, a T. cruzi RNA binding protein (12), to demonstrate the similarities between these two kinetoplastid NES sequences.

In order to determine whether p34 and p37 interact with exportin 1, IC experiments were performed. Wild-type nuclear extracts were incubated with anti-Xpo1-cross-linked Dynabeads, and Western blot analysis was performed using antip34/p37 antiserum. The data shown are representative of five separate experiments. The results of these experiments show that p34 and p37 are present in the IC pellet fraction to the same extent that they are present in the nuclear extract (Fig. 8, left top panel, compare nuclear extract and pellet fractions), demonstrating an interaction of both proteins with exportin 1. Neither p34 nor p37 interacted with the beads alone (Fig. 8, left top panel). When these experiments were repeated using p34/p37 RNAi cells, no p34 or p37 was found in the IC pellet or supernatant fractions (Fig. 8, left top panel), indicating that NOPP44/46 are associated with exportin 1 in a p34- and/or p37-dependent manner, we hypothesized that they are subsequently transported out of the nucleus. To determine whether this is the case, we treated wild-type cells with LMB, a specific inhibitor of the exportin 1 pathway. LMB acts to inhibit exportin 1-dependent nuclear export by irreversibly modifying a conserved cysteine residue required for the interaction with NES-containing cargoes (23). Cells were treated with LMB at a final concentration of 0.001 ng/cell, as previously described for T. brucei (38). Cells were left untreated (control) or treated for 12, 24, or 48 h, after which cells were fractionated into cytoplasmic and nuclear extracts. A 500-μg aliquot of nuclear extract was used for subsequent immune capture of exportin 1. Samples were subjected to SDS-PAGE and Western blot analysis to detect p34 and p37 (upper panel). Membranes were stripped and reprobed for detection of NOPP44/46 (middle panel). As a control, membranes were stripped again and reprobed for PABP (lower panel). B, IC beads-alone control; NE, 50 μg nuclear extract; P, IC pellet fraction; S, IC supernatant fraction.

![Figure 8](image) FIG. 8. NOPP44/46 associate with exportin 1 through an interaction with p34/p37. Wild-type and RNAi cells were fractionated into cytoplasmic and nuclear extracts. A 500-μg aliquot of nuclear extract was used for subsequent immune capture of exportin 1. Samples were subjected to SDS-PAGE and Western blot analysis to detect p34 and p37 (upper panel). Membranes were stripped and reprobed for detection of NOPP44/46 (middle panel). As a control, membranes were stripped again and reprobed for PABP (lower panel). B, IC beads-alone control; NE, 50 μg nuclear extract; P, IC pellet fraction; S, IC supernatant fraction.

7A and B). This type of NES is known to mediate the interaction of a subset of nucleocytoplasmic shuttling proteins with the nuclear export factor exportin 1 (14). Figure 7B shows the p34 and p37 NES compared to the canonical sequence, which is based on the human immunodeficiency virus type 1 Rev protein (7). We also show the NES from TcUBP-1, a T. cruzi RNA binding protein (12), to demonstrate the similarities between these two kinetoplastid NES sequences.

In order to determine whether p34 and p37 interact with exportin 1, IC experiments were performed. Wild-type nuclear extracts were incubated with anti-Xpo1-cross-linked Dynabeads, and Western blot analysis was performed using anti-p34/p37 antiserum. The data shown are representative of five separate experiments. The results of these experiments show that p34 and p37 are present in the IC pellet fraction to the same extent that they are present in the nuclear extract (Fig. 8, left top panel, compare nuclear extract and pellet fractions), demonstrating an interaction of both proteins with exportin 1. Neither p34 nor p37 interacted with the beads alone (Fig. 8, left top panel). When these experiments were repeated using p34/p37 RNAi cells, no p34 or p37 was found in the IC pellet or supernatant fractions (Fig. 8, left top panel), indicating that NOPP44/46 are associated with exportin 1 in a p34- and/or p37-dependent manner, we hypothesized that they are subsequently transported out of the nucleus. To determine whether this is the case, we treated wild-type cells with LMB, a specific inhibitor of the exportin 1 pathway. LMB acts to inhibit exportin 1-dependent nuclear export by irreversibly modifying a conserved cysteine residue required for the interaction with NES-containing cargoes (23). Cells were treated with LMB at a final concentration of 0.001 ng/cell, as previously described for T. brucei (38). Cells were left untreated (control) or treated for 12, 24, or 48 h, after which cells were fractionated into cytoplasmic and nuclear extracts. A 500-μg aliquot of nuclear extract was used for subsequent immune capture of exportin 1. Samples were subjected to SDS-PAGE and Western blot analysis to detect p34 and p37 (upper panel). Membranes were stripped and reprobed for detection of NOPP44/46 (middle panel). As a control, membranes were stripped again and reprobed for PABP (lower panel). B, IC beads-alone control; NE, 50 μg nuclear extract; P, IC pellet fraction; S, IC supernatant fraction.

![Figure 9](image) FIG. 9. p34 and/or p37, exportin 1, and NOPP44/46 are involved in a common complex. Single immune capture experiments demonstrated that p34 and/or p37 is required for the association of NOPP44/46 with exportin 1. Thus, we wished to determine whether p34 and/or p37, NOPP44/46, and exportin 1 form a molecular complex. The results shown in Fig. 9 are representative of five separate experiments. We performed single IC experiments to capture p34 and p37 (Fig. 9, left panel, p34/p37 IC) and, in a separate experiment, exportin 1 (Fig. 9, left panel, Xpo1 IC). These experiments confirmed the interaction between NOPP44/46 and p34 and p37, and also between NOPP44/46 and exportin 1 (Fig. 9, compare IC-P lanes between p34/p34 IC and Xpo1 IC). Sequential immune capture experiments, capturing exportin 1 and then p34 and p37, demonstrated that a subset of nuclear NOPP44/46 are in complex with p34 and/or p37 and with exportin 1 simultaneously (Fig. 9, right panel). This may not represent the entire population of NOPP44/46 involved in this complex, as more protein was released from the beads upon a second elution (Fig. 9, right panel).

A large proportion of NOPP44/46 is present in the IC supernatant sample (Fig. 9, right panel), representing either free protein or protein involved in another complex(es). Thus, p34 and/or p37, NOPP44/46, and exportin 1 are part of a common complex which may also contain additional proteins and/or RNA.

NOPP44/46 proteins accumulate in the nucleus upon treatment of wild-type procyclic cells with leptomycin B. Since NOPP44/46 are associated with exportin 1 in a p34- and/or p37-dependent manner, we hypothesized that they are subsequently transported out of the nucleus. To determine whether this is the case, we treated wild-type cells with LMB, a specific inhibitor of the exportin 1 pathway. LMB acts to inhibit exportin 1-dependent nuclear export by irreversibly modifying a conserved cysteine residue required for the interaction with NES-containing cargoes (23). Cells were treated with LMB at a final concentration of 0.001 ng/cell, as previously described for T. brucei (38). Cells were left untreated (control) or treated for 12, 24, or 48 h, after which cells were fractionated into cytoplasmic and nuclear extracts. A 500-μg aliquot of nuclear extract was used for subsequent immune capture of exportin 1. Samples were subjected to SDS-PAGE and Western blot analysis to detect p34 and p37 (upper panel). Membranes were stripped and reprobed for detection of NOPP44/46 (middle panel). As a control, membranes were stripped again and reprobed for PABP (lower panel). B, IC beads-alone control; NE, 50 μg nuclear extract; P, IC pellet fraction; S, IC supernatant fraction.
nuclear and cytoplasmic extracts. Extracts were subjected to SDS-PAGE for subsequent Western blot analysis of NOPP44/46. As shown in Fig. 10 (representative of four separate experiments), we detected a steady increase in nuclear NOPP44/46 protein abundance throughout the time course of the experiment (right panel), whereas cytoplasmic NOPP44/46 levels remained relatively constant (left panel). Using exposures from each experiment in the linear range, there was an average 5.5-fold ± 0.3-fold increase in nuclear NOPP44/46 protein levels after 48 h of treatment. These results correlate with those shown in the middle panels of Fig. 2, where there was an increase in nuclear NOPP44/46 proteins in the absence of p34 and p37. As a control, we used an antibody directed against the large subunit of RNA polymerase II (Fig. 10, bottom panel), since it does not contain a nuclear export signal. The levels of this protein remained steady throughout the time course of the experiment, demonstrating that the increase in nuclear NOPP44/46 abundance is specific to their interaction with exportin 1. Together, our results demonstrate that NOPP44/46 exit the nucleus through an exportin 1-dependent mechanism, which is mediated by their interaction with p34 and/or p37.

**DISCUSSION**

We have previously identified two nearly identical RNA binding proteins, p34 and p37, which are essential for Trypanosoma brucei survival (19, 40). These trypanosome-specific proteins (18) interact with NOPP44/46 (30), a family of trypanosome-specific nuclear phosphoproteins (29), suggesting that this protein-protein interaction serves a unique function within *T. brucei*. In the experiments presented here, we knocked down the expression of both p34 and p37 proteins using RNA interference to further characterize their association with the NOPP44/46 proteins.

Results presented here indicate that loss of p34 and p37 proteins leads to the disruption of a cellular complex(es) that normally contains these proteins. In sucrose gradient analysis of RNAi cell nuclear extracts, an upward shift in the sedimentation profile of the NOPP44/46 proteins occurred (Fig. 1B, bottom panel), indicating that loss of p34 and p37 resulted in alterations within a complex(es) that contains both proteins.

Upon induction of p34/p37 dsRNA, an increase in nuclear NOPP44/46 protein levels occurred compared to wild type (Fig. 2, middle panels). A 12-fold increase in NOPP44/46 protein levels was observed upon loss of p34 and p37 (Fig. 6A, second panel), suggesting that p34 and p37 regulate NOPP44/46 expression. We detected no change in the abundance or stability of NOPP44/46 transcript in the RNAi cells (Fig. 3 and 4), indicating that p34 and p37 do not regulate NOPP44/46 transcript expression. Unexpectedly, when we examined the overall cellular protein levels of NOPP44/46, we found that they remained unchanged in p34/p37 RNAi versus wild-type cells (Fig. 5). Taken together with our cellular fractionation results (Fig. 2 and 6), this strongly suggests that p34 and p37 act to regulate NOPP44/46 cellular localization.

Previous studies using immunoelectron microscopy and immunofluorescence analyses have indicated that NOPP44/46 are localized to the nucleolus (10). However, this does not preclude the possibility that a small subset of these proteins rapidly shuttle out of the nucleolus/nucleus, since their cytoplasmic abundance may be below the level of detection with these methods. The NOPP44/46 proteins do not contain a nuclear localization or export signal sequence, although their amino-terminal unique (U) sequence has been shown to be required for nucleolar localization. This suggests that the cellular localization of NOPP44/46 may be dependent on other nuclear and/or nucleolar proteins.

In p34 and p37, the last nine residues of the second RBD comprise a putative NES sequence (Fig. 7A), which fits the consensus sequence found in the human immunodeficiency virus type 1 Rev protein (13). It is also very similar to the NES found in the *T. cruzi* RNA binding protein TecUBP-1 (Fig. 7B) (12), indicating a sequence conservation within trypanosomes. Figure 8 demonstrates that p34 and p37 interact with the nuclear export factor exportin 1. A variety of cellular components undergo exportin 1-dependent nuclear export. These include the large (60S) and small (40S) ribosomal subunits, many cellular proteins, several U snRNAs, all rRNAs, and a subset of mRNAs (7, 15, 20, 26, 36, 37). Exportin 1 has been identified in trypanosomes and is indicated as having a role in the nuclear export of spliced leader RNA in *T. brucei* as well as a subset of mRNA in *T. cruzi* (6, 38).

The p34 and p37 proteins appear to act as adapter proteins allowing for the export of the NOPP44/46 proteins out of the nucleus in an exportin 1-dependent manner. Examples of proteins and protein complexes that depend on adapter proteins for their association with exportin 1 and subsequent nuclear export include the mammalian 14-3-3 proteins and the yeast 60S ribosomal subunit (5, 20). The 60S ribosomal subunit is exported to the cytoplasm via the exportin 1 nuclear export pathway, which requires the nonribosomal adapter protein Nmd3p (20). Nmd3p acts to provide the nuclear export signal to mediate the association between the 60S ribosomal subunit and exportin 1. It is important to note that, in addition to Nmd3p, several other nonribosomal factors are required for nuclear export of the 60S ribosomal subunit, including Nop53p, Nug1p, and Nug2p (1, 35). This suggests that nuclear export may involve large complexes and that it is regulated by several different types of proteins. Thus, p34 and p37, possibly in concert with unidentified factors, may act in a similar manner to regulate NOPP44/46 cellular localization. This is further supported by the p34/p37 RNAi immune capture results, which demonstrated the loss of the NOPP44/46-exportin 1 association in the absence of p34 and p37 (Fig. 8, right middle panel). The accumulation of NOPP44/46 in the nucleus following
LMB treatment (Fig. 10) further suggests that NOPP44/46 are exported from the nucleus via exportin 1. Together, these data suggest that p34 and p37 may function as a control mechanism to shuttle excess NOPP44/46 proteins out of the nucleus or, alternatively, they may serve to regulate the nuclear export of a larger complex containing the NOPP44/46 proteins.

We have previously established that p34 and p37 associate with 5S rRNA (31), a component of the 60S ribosomal subunit. Recent work from this laboratory using the same p34/p37 RNAi cells has demonstrated a dramatic decrease in the levels of 5S rRNA, in addition to disruption of a cellular complex(es) containing 5S rRNA (19). These results suggest an important role for p34 and p37 in stabilizing 5S rRNA. The loss of 5S rRNA in these cells leads to a significant decrease in protein synthesis efficiency, which appears to be the result of a defect in the assembly of the 60S ribosomal subunits into the 80S complex (19). At this time, we do not know whether the disrupted complex(es) in the p34/p37 RNAi cells contains both NOPP44/46 and 5S rRNA or whether they are separate and distinct complexes. The Parsons laboratory has recently shown that NOPP44/46 are involved in the later stages of 60S ribosomal subunit biogenesis (21). Experiments utilizing NOPP44/46 RNAi cells have demonstrated aberrant processing of pre-60S rRNA, disruption of 60S assembly, and cell death (21). Incorporation of 5S rRNA is one of the final steps in the assembly pathway of the 60S ribosomal subunit (11). Thus, p34 and p37 may function to coordinate 5S rRNA incorporation into assembling pre-60S ribosomal subunits through an interaction with the NOPP44/46 proteins.

We do not currently know whether the interaction of p34 and p37 with 5S rRNA and the NOPP44/46 proteins reflects one common or two distinct roles for these proteins. Thus, these interactions could occur independently of one another. Overall, the results presented here indicate that the protein-protein interaction of p34 and p37 with NOPP44/46 plays an important role in NOPP44/46 protein cellular distribution, possibly in the context of a larger complex. Experiments are currently under way to more precisely define the components and function of this complex.

ACKNOWLEDGMENTS

We thank Marilyn Parsons (Seattle Biomedical Research Institute) for the NOPP44/46 monoclonal antibody as well as antisera to T. brucei phosphoglycerate kinase. We also thank Vivian Bellofatto (University of Medicine and Dentistry of New Jersey) for polyclonal antiserum to phosphoglycerate kinase. We also thank Vivian Bellofatto (University of Medicine and Dentistry of New Jersey) for polyclonal antiserum to phosphoglycerate kinase. We also thank Marilyn Parsons (Seattle Biomedical Research Institute) for the NOPP44/46 monoclonal antibody as well as antisera to T. brucei phosphoglycerate kinase. We also thank Vivian Bellofatto (University of Medicine and Dentistry of New Jersey) for polyclonal antiserum to phosphoglycerate kinase. We also thank Marilyn Parsons (Seattle Biomedical Research Institute) for the NOPP44/46 monoclonal antibody as well as antisera to T. brucei phosphoglycerate kinase. We also thank Marilyn Parsons (Seattle Biomedical Research Institute) for the NOPP44/46 monoclonal antibody as well as antisera to T. brucei phosphoglycerate kinase. We also thank Marilyn Parsons (Seattle Biomedical Research Institute) for the NOPP44/46 monoclonal antibody as well as antisera to T. brucei phosphoglycerate kinase.
families of RNA binding proteins from *Trypanosoma brucei* associate in a direct protein-protein interaction. Mol. Biochem. Parasitol. 122:81–89.

31. Pitula, J., W. T. Ruyechan, and N. Williams. 2002. Two novel RNA binding proteins from *Trypanosoma brucei* are associated with 5S rRNA. Biochem. Biophys. Res. Commun. 290:569–576.

32. Roberts, T. G., N. R. Sturm, B. K. Yee, M. C. Yu, T. Hartshorn, N. Agabian, and D. A. Campbell. 1998. Three small nucleolar RNAs identified from the spliced leader-associated RNA locus in kinetoplastid protozoans. Mol. Cell. Biol. 18:4409–4417.

33. Rout, M. P., and M. C. Field. 2001. Isolation and characterization of subnuclear compartments from *Trypanosoma brucei*: identification of a major repetitive nuclear lamina component. J. Biol. Chem. 276:38261–38271.

34. Seed, J. R. 2001. African trypanosomiasis research: 100 years of progress, but questions and problems still remain. Int. J. Parasitol. 31:434–442.

35. Thomson, E., and D. Tollervey. 2005. Nop53p is required for late 60S ribosomal subunit maturation and nuclear export in yeast. RNA 11:1215–1224.

36. Trotta, C. R., E. Lund, L. Kahan, A. W. Johnson, and J. E. Dahlberg. 2003. Coordinated nuclear export of 60S ribosomal subunits and NMD3 in vertebrates. EMBO J. 22:2841–2851.

37. Tschochner, H., and E. Hurt. 2003. Pre-ribosomes on the road from the nucleolus to the cytoplasm. Trends Cell Biol. 13:255–263.

38. Zeiner, G. M., N. R. Sturm, and D. A. Campbell. 2003. Exportin 1 mediates nuclear export of the kinetoplastid spliced leader RNA. Eukaryot. Cell 2:222–230.

39. Zhang, J., W. Ruyechan, and N. Williams. 1998. Developmental regulation of two nuclear RNA binding proteins, p34 and p37, from *Trypanosoma brucei*. Mol. Biochem. Parasitol. 92:79–88.

40. Zhang, J., and N. Williams. 1997. Purification, cloning, and expression of two closely related *Trypanosoma brucei* nucleic acid binding proteins. Mol. Biochem. Parasitol. 87:145–158.