Distinct domains of a nucleolar protein mediate protein kinase binding, interaction with nucleic acids and nucleolar localization

Arpita Das\textsuperscript{1,2}, Jeong-Hyun Park\textsuperscript{1,2}, Christopher B. Hagen\textsuperscript{1} and Marilyn Parsons\textsuperscript{1,2,*}

\textsuperscript{1}Seattle Biomedical Research Institute, 4 Nickerson St, Seattle, WA 98109, USA and \textsuperscript{2}Department of Pathobiology, School of Community Medicine and Public Health, University of Washington, Seattle, WA 98195, USA

*Author for correspondence at address 1 (e-mail: mparsons@u.washington.edu)

Accepted 22 June; published on WWW 13 August 1998

SUMMARY

Nopp44/46 is a phosphoprotein of the protozoan parasite \textit{Trypanosoma brucei} that is localized to the nucleolus. Based on the primary sequence, Nopp44/46 appears to be a protein composed of distinct domains. This communication describes the relationship of these domains to the known functional interactions of the molecule and suggests that the amino-terminal region defines a novel homology region that functions in nucleolar targeting. We have previously shown that Nopp44/46 is capable of interacting with nucleic acids and associating with a protein kinase. Using in vitro transcription and translation, we now demonstrate that the nucleic acid binding function maps to the carboxy-terminal domain of the molecule, a region rich in arginine-glycine-glycine motifs. Our experiments reveal that a central region containing a high proportion of acidic residues is required for association with the protein kinase. Analysis of transfectants expressing epitope-tagged Nopp44/46 deletion constructs showed that the amino-terminal 96 amino acids allowed nuclear and nucleolar accumulation of the protein. This region of the molecule shows homology to several recently described nucleolar proteins. Deletion of a 27-amino-acid region within this domain abrogated nucleolar, but not nuclear, localization. These studies show that Nopp44/46 is composed of distinct modules, each of which plays a different role in molecular interactions. We suggest that this protein could facilitate interactions between sets of nucleolar molecules.

Key words: Nucleolus, RNA binding protein, Protein kinase, \textit{Trypanosoma brucei}

INTRODUCTION

The nucleolus is the site of eukaryotic ribosome biogenesis, with transcription of rDNA, processing of pre-rRNA and formation of pre-ribosomal particles occurring within this compartment. Well-defined structural regions of the nucleolus (fibrillar center, dense fibrillar component, and granular region) that correspond to these functions have long been recognized in higher eukaryotes (Melese and Xue, 1995), but morphologically distinct regions have only been recently defined in yeast (Léger-Silvestre et al., 1997). Nucleoli contain many proteins with functions intimately tied to rRNA transcription, processing and ribosome assembly (e.g. RNA polymerase I, fibrillarin and ribosomal proteins), as well as others whose relationships to ribosome biogenesis are less direct (e.g. Nopp140 and casein kinase II) (Meier and Blobel, 1992; Allende and Allende, 1995; Olson, 1990). Although many nucleolar proteins contain nuclear localization signals (see for example Schmidt et al., 1995; Peculis and Gall, 1992; Henriquez et al., 1990), a consensus sequence for nucleolar accumulation has not emerged. Since the nucleolus is not separated from the rest of the nucleus by a membrane, it is likely that mechanisms for localization to this subnuclear compartment follow a different paradigm than those operating to sequester proteins to membrane-bounded organelles. Thus, one current hypothesis is that, once within the nucleus, a protein may not be specifically targeted to the nucleolus, but rather is retained there following interaction with other nucleolar components (Melese and Xue, 1995; Peculis and Gall, 1992).

Nucleolar proteins often have an acid-rich domain (Jantzen et al., 1990; Bergès et al., 1994; Lapeyre et al., 1987) or a region rich in arginine-glycine-glycine (RGG) motifs (Lapeyre et al., 1990, 1987; McGrath et al., 1997). While the function of the acidic domains in nucleolar proteins has not been elucidated, some RGG domains in these and other proteins have been shown to participate in binding to nucleic acids (Kiledjian and Dreyfuss, 1997; Jantzen et al., 1990; Ghisolfi et al., 1992; Kumar et al., 1990). We have previously shown that a major tyrosine-phosphorylated protein, Nopp44/46, of the protozoan parasite \textit{Trypanosoma brucei} is a nucleolar protein (Das et al., 1996). Antibodies to this protein co-precipitate a protein kinase activity (Parsons et al., 1994). In addition, Nopp44/46 purified from parasites or generated by in vitro translation can bind to nucleic acids in vitro (Das et al., 1996). Nopp44/46 is capable of binding single- and double-stranded DNA, as well as RNA, and it shows a preference for poly(U). Analyses of Nopp44/46 cDNAs predict a set of almost identical
35-37 kDa proteins (Das et al., 1996) (see GenBank accession number U53863). These proteins migrate anomalously on SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with apparent molecular masses of 44-46 kDa.

The Nopp44/46 isoforms share a common structure, comprising four regions that have very distinct characteristics. The amino-terminal unique region (residues 1-96) has no distinct amino acid composition. The junction region (residues 97-168) has a high proportion of acidic residues interspersed with other amino acids. This region is followed by the 61-residue acidic region and finally the repeat region that contains over 20 RGG motifs. It is the last region which varies in length among the different isoforms.

We hypothesized that the regions of discrete sequence characteristics played different functional roles. We therefore examined various deletion constructs for their role in the three known properties of Nopp44/46: binding to nucleic acids, interaction with a protein kinase and nucleolar localization. Each property maps to a different region of the molecule, supporting the contention that Nopp44/46 is built of distinct functional modules. Sequences related to the amino-terminal module, which functions in nuclear and nucleolar targeting, were found in several nucleolar proteins.

MATERIALS AND METHODS

Trypanosomes

Procyclic form trypanosomes, strain TREU667, were grown in Cunningham’s medium (Cunningham, 1977). For the production of Procyclic form trypanosomes, strain TREU667, were grown in

In vitro transcription and translation of Nopp proteins

The full-length coding region of Nopp44/46, the unique region through to the acidic region, and the repeat region were amplified from Nopp44/46 cDNA by PCR. These were cloned into the pCR2.1 vector (Invitrogen). The amplified fragments were first cloned into the pCR2.1 vector, the amplified fragments were first cloned into the pCR2.1 vector (Invitrogen). The cloned inserts were released by cleavage with BamHI and cloned into the XbaI site of the vector pTbmyc2 (see Fig. 1C for constructs generated). Construct UJA-A5-71-myc was generated by digestion of the UJA-myc plasmid with EcoRV and HpaI followed by religation. The new construct encoded a protein spanning the unique through to the acidic regions, but lacking residues 45-71 of the unique region. All of the constructs encode proteins with the myc-tag at the carboxy terminus. All constructs were checked by a combination of restriction enzyme digestion and DNA sequence analysis prior to transfection.

Western blotting, immunoprecipitations and in vitro kinase assays of myc-tagged proteins

For total cell protein analyses, cells were lysed in SDS-PAGE sample buffer. For immunoprecipitations, cells were extracted with immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 2 mM EGTA, 1 mM phenyl methyl sulfonyl fluoride, 10 µg/ml aprotinin, 50 µg/ml leupeptin), and insoluble proteins were removed by centrifugation.

Table 1. PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
<th>Product/Constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 Unique N</td>
<td>5’ GACCTAAATAGCAGCTCATTAGGGCGCCAGAATGGAGGT 3’</td>
<td>T7 UJA, T7 UJAR</td>
</tr>
<tr>
<td>Acidic C stop</td>
<td>5’ TCTAGAACTTCTATGAGGGCGTTGACAT 3’</td>
<td>T7 UJA</td>
</tr>
<tr>
<td>T7 Repeat N</td>
<td>5’ GACCTAAATAGCAGCTCATTAGGGCGCCAGAATGGAGGT 3’</td>
<td>T7 R</td>
</tr>
<tr>
<td>Repeat C stop</td>
<td>5’ GCTTAGAACTTCTATGAGGGCGTTGACAT 3’</td>
<td>T7 R, T7 UJAR</td>
</tr>
<tr>
<td>Unique N</td>
<td>5’ GCTTAGAACTTCTATGAGGGCGTTGACAT 3’</td>
<td>U, UJ, UJA</td>
</tr>
<tr>
<td>Unique C</td>
<td>5’ GCTTAGAACTTCTATGAGGGCGTTGACAT 3’</td>
<td>U</td>
</tr>
<tr>
<td>Junction N</td>
<td>5’ GCTTAGAACTTCTATGAGGGCGTTGACAT 3’</td>
<td>J</td>
</tr>
<tr>
<td>Junction C</td>
<td>5’ GCTTAGAACTTCTATGAGGGCGTTGACAT 3’</td>
<td>UJ, J</td>
</tr>
<tr>
<td>Acidic C</td>
<td>5’ GCTTAGAACTTCTATGAGGGCGTTGACAT 3’</td>
<td>UJA</td>
</tr>
<tr>
<td>Repeat N</td>
<td>5’ GCTTAGAACTTCTATGAGGGCGTTGACAT 3’</td>
<td>R</td>
</tr>
<tr>
<td>Repeat C</td>
<td>5’ GCTTAGAACTTCTATGAGGGCGTTGACAT 3’</td>
<td>R</td>
</tr>
</tbody>
</table>

* XbaI restrictions sites are underlined; start codons are indicated in bold.
Solubilized proteins were analyzed by western blot using a monoclonal antibody against Nopp44/46 (ID2; Parsons et al., 1994) or anti-myc (9E10; Evan et al., 1985). Immunoprecipitations were performed using cell lysates containing 50 µg of soluble cell protein and 1 µg of monoclonal ID2 anti-Nopp or 1 µl anti-myc ascites fluids. The complexes were collected with Protein A-Sepharose, washed as previously described, and subjected to immunoblot and immune kinase analysis. In some cases, immunoprecipitations were performed on lysates from cells incubated overnight in phosphate-free medium supplemented with [32P] (Das et al., 1996). Immune kinase assays were carried out following the previously described protocol (Parsons et al., 1994) using the anti-myc antibody 9E10 to immunoprecipitate the tagged protein. Myelin basic protein was used as an exogenous substrate in the assay.

Immunofluorescence analysis

Cells were washed and resuspended in phosphate-buffered saline (PBS). Following a 20-minute incubation on glass slides in a humid chamber, cells were fixed in 1.5% formaldehyde in PBS at room temperature for 5 minutes. Cells were washed three times in PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, and washed three times. After blocking with 5% non-fat milk plus 0.05% Tween-20 for 30 minutes at room temperature, the incubation was continued for 90 minutes with antibody 9E10. Following three washes with PBS, cells were incubated in fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 1 hour. Finally cells were washed three times in 0.05% Nonidet P-40 in PBS, counterstained in 4,6-diamino-2-phenylindole (DAPI) (Das et al., 1996) and mounted in three times in 0.05% Nonidet P-40 in PBS, counterstained in 4,6-conjugated goat anti-mouse IgG for 1 hour. Finally cells were washed with PBS, cells were incubated in fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 1 hour. Finally cells were washed three times in 0.05% Nonidet P-40 in PBS, counterstained in 4,6-diamino-2-phenylindole (DAPI) (Das et al., 1996) and mounted in Prolong antifade media (Molecular Probes). Slides were viewed on a Nikon Microphot-FX microscope and photographed.

RESULTS

We have previously shown that Nopp44/46 is composed of an amino-terminal unique region, a central acidic region (containing 75% acidic amino acids) and a carboxy-terminal repeat region rich in RGG motifs (Fig. 1A). Further analysis led us to designate the last 73 residues of the unique region as the junction region, since this region contains a high proportion of acidic amino acids (approximately 43% aspartic and glutamic acids). In this communication, these regions will be denoted as U (unique), J (junction), A (acidic) and R (repeat). The identification of three distinct functional attributes of Nopp44/46 (nucleolar localization, interaction with nucleic acids and protein kinase binding) led us to investigate which portions of the molecule conferred these properties.

The RGG-rich repeat region is capable of binding to nucleic acids in vitro

To determine which domain of the molecule is responsible for binding to nucleic acids, we amplified, transcribed and translated R region also bound to ssDNA, while the in vitro product containing the UJA regions did not. No radiolabeled proteins bound to Protein A-agarose, demonstrating that the proteins do not bind the matrix itself. Like the full-length molecule, the in vitro-translation product of the UJA regions

![Fig. 1. Nopp44/46 proteins and fragments used in this study.](image)

(A) Primary structure of full-length Nopp44/46, drawn to scale. The regions are defined by their differences in amino acid composition. U, the unique region (residues 1-96); J, the junction region (residues 97-168); A, the acidic region (residues 169-229); R, the repeat region containing multiple RGG motifs (residues 230-325). (B) Schematics of the proteins produced by in vitro transcription and translation. The shading of the domains corresponds to A, and the names of the products reflect the domains they contain. (C) Proteins specified by constructs used in transfection experiments. Domains are indicated as above. Construct UJAΔ45-71 is missing residues 45-71 of the unique region as well as the R region. All of the proteins contain a double myc tag at the carboxy terminus (asterisk). In the text, the names of these proteins include the myc designation (e.g. UJ-myc). (D) The T. brucei expression plasmid pTbmyc2. The PARP sequence contains the promoter (flag) and splice acceptor for trans-splicing. This is followed by an XbaI site (X), two myc epitope coding sequences in tandem, and a BamHI site (B). The HYG gene allows for selection in trypanosomes and the AMP gene allows for selection in E. coli. The tubulin intergenic sequence contains an MluI site (M), allowing for linearization of the plasmid and integration into the βt intergenic region of the tubulin array.
migrated significantly slower than expected from its predicted molecular mass.

**Expression of myc-tagged Nopp proteins**

To determine which domains of the molecule are responsible for nucleolar localization and protein kinase binding, several truncated versions of Nopp44/46 were expressed as myc-fusion proteins in *T. brucei* using the vector pTbmyc2 (Fig. 1D). Stable transfectants were selected by virtue of the hygromycin resistance gene carried on the transfected DNA. The expression of the tagged proteins was tested by immunoblot analysis of total cell lysates (Fig. 3). As anticipated, untransfected cells (WT) and all of the transfectants reacted with the monoclonal anti-Nopp 44/46 ID2, showing the characteristic doublet at 44-46 kDa. The abundance of Nopp44/46 showed no reproducible differences in the various cell lines. Anti-Nopp also detects some lower molecular mass bands in all samples. The origin of these smaller molecules is unclear, but unlike Nopp44/46 they do not immunoprecipitate with the monoclonal antibodies (unpublished data).

Wild-type cells did not show any significant reactivity with anti-myc 9E10. Transfectants carrying UJA-myc, UJ-myc and UJAΔ45-71-myc (the UJA construct with codons 45-71 deleted) expressed proteins that were recognized by both ID2 (Fig. 3A) and anti-myc (Fig. 3B). The R-myc and U-myc transfectants expressed proteins that were recognized by anti-myc but not by anti-Nopp ID2. (Parenthetically, this suggests that the J region is required for expression of the epitope recognized by antibody ID2.) Thus, all of these cell lines expressed the myc-tagged proteins. All of the myc-tagged proteins that contained repeats or highly acidic segments migrated aberrantly on SDS-PAGE (e.g. UJA-myc migrated at 34 kDa, as opposed to the predicted molecular mass of 29 kDa, and R-myc migrated at 18 kDa as opposed to 14 kDa), as was previously observed with the full-length molecule (Das et al., 1996). No obvious detrimental effects of expression of these Nopp44/46 fragments in *T. brucei* was observed.

An additional construct was made in which the myc tag was appended to the J region alone. Although hygromycin-resistant transfectants were readily obtained, no proteins were observed on immunoblot analysis using anti-myc. Finally, several different approaches were used in attempts to clone the full-length Nopp44/46 in plasmid vectors. None of the approaches, which included in vivo excision from λ Zap, cloning of PCR products in various vectors and piecing together the gene from component fragments, were successful.

**Myc-tagged Nopp proteins do not interact with native Nopp44/46**

Certain nucleolar proteins, such as NO38 and B23 (nucleoplasm), form homo-oligomeric complexes (Yung and Chan, 1987; Schmidt-Zachmann et al., 1987; Zirwes et al., 1997). The glycine-rich domain of hnRNP A1, which contains multiple RGG motifs, promotes oligomerization of this molecule (Cobianchi et al., 1994). The potential for Nopp44/46 multimers has not been explored. If such multimers were to form, then truncated versions of the protein might be capable of localization to the nucleolus or interaction with the protein kinase activity by piggybacking onto the native molecule. Therefore we tested...
whether the myc-tagged proteins interact with native Nopp44/46 by immunoprecipitation with anti-myc, followed by immunoblotting with either anti-myc (Fig. 4A) or anti-Nopp ID2 (Fig. 4B). As shown in Fig. 4A, the myc-tagged proteins were efficiently precipitated. The sole exception was R-myc, which was found to be insoluble in the immunoprecipitation buffer (Fig. 3B, inset) and therefore was excluded from the analysis. The absence of the 44-46 kDa bands in the anti-myc immunoprecipitates demonstrates that Nopp44/46 did not co-precipitate with any of the myc-tagged proteins (Fig. 4A). Similarly, no evidence of interaction between Nopp44/46 and UJA-myc was observed in immunoprecipitation experiments performed under very mild conditions (no detergents and at physiological salt concentrations, data not shown). These results show that the properties attributed to these truncated molecules (see below) are not mediated by virtue of an interaction with the full-length Nopp44/46.

**Protein kinase association requires the junction region**

We have previously demonstrated that protein kinase activity is associated with Nopp44/46 and that this kinase can use both Nopp44/46 and myelin basic protein as substrates (Parsons et

---

**Fig. 4.** Analysis of myc-tagged Nopp protein associations. Lysates of wild-type cells (WT) or of transfectants expressing the indicated myc-tagged proteins were incubated with the listed antibodies for immunoprecipitation (IP). The antibodies used included anti-myc 9E2, anti-Nopp44/46 ID2 and the isotype-matched control for the latter, IgG2A UPC10. The antigen-antibody complexes were collected with Protein A Sepharose. The migration of molecular mass markers is indicated by hash marks and identified in A. (A) Anti-myc immunoblot analysis of Nopp proteins. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with anti-myc. Reactive proteins were revealed with \(^{125}\)I-labelled Protein A. The migration of molecular mass markers (kDa) is indicated. (B) Anti-Nopp44/46 immunoblot analysis of immunoprecipitates. Immunoprecipitates collected in parallel with those shown in A were analyzed by immunoblotting with anti-Nopp44/46 ID2. (C) Immune kinase reactions. Immunoprecipitates collected in parallel with those shown above were analyzed by immune kinase assays as described in Materials and methods. Myelin basic protein was used as an exogenous substrate. Asterisks mark the migration of the myc-tagged Nopp fragment present in each transfectant.

**Fig. 5.** Phosphorylation of Nopp44/46 fragments. (A) T. brucei cells expressing the indicated Nopp44/46 fragments were incubated overnight in phosphate-free medium containing \(^{32}\)P\(_{i}\) and lysates prepared. The Nopp proteins were precipitated with the indicated antibodies and analyzed by SDS-PAGE, autoradiography and phosphorimaging analysis. (B) Parallel immunoprecipitates were performed on cultures incubated in the same medium, but without \(^{32}\)P\(_{i}\). These samples were analyzed by immunoblotting using anti-myc followed by autoradiography and phosphorimaging analysis. This allowed normalization for the differing expression levels of the various Nopp fragments. (C) Relative levels of \(^{32}\)P\(_{i}\) associated with the Nopp fragments. The phosphorimaging data obtained from A and B were used to calculate the relative labeling of each myc-tagged protein. For each sample, the level of \(^{32}\)P\(_{i}\) labeling was divided by the relative abundance of the myc-tagged protein to yield a normalized value. This value for the most highly phosphorylated protein, UJA-myc, was designated as 100%.
In parallel with the studies shown in Fig. 4A,B, the myc-tagged proteins were immunoprecipitated and subjected to in vitro kinase assays using myelin basic protein as an exogenous substrate (Fig. 4C). Anti-myc immunoprecipitates of the wild type (untransfected) cells showed no associated kinase activity. However, substantial protein kinase activity was co-precipitated with both the UJA-myc protein and the UJ-myc protein. This result demonstrates that our previous observation of kinase activity co-precipitating with Nopp44/46 is not attributable to cross-reactivity of the anti-Nopp44/46 antibodies with a protein kinase, since anti-myc also co-precipitates this activity. The protein kinase activity that co-precipitated with each of the proteins (as measured by myelin basic protein phosphorylation) was quantified by phosphorimaging, as was the amount of proteins binding anti-myc and anti-Nopp. This allowed us to normalize our data to determine the protein kinase activity associated with each of the proteins. UJA-myc, UJ-myc and U-myc were associated with 55%, 25% and less than 3%, respectively, of the protein kinase activity of the full-length molecule. These data show that the J region is required for co-precipitation of protein kinase activity with Nopp44/46, and suggest that the interaction or activity may be enhanced by the presence of the adjacent A and R regions. Alternatively it is possible that multiple protein kinase activities are associated with Nopp44/46, each of which interacts with a different region of the molecule.

**Myc-tagged UJ and UJA proteins are phosphorylated**

The myc-tagged proteins described above were examined for their level of phosphorylation. Parallel cultures were incubated overnight in phosphate-free medium, with one tube containing trace amounts of $^{32}$P$_i$. The myc-tagged proteins were immunoprecipitated with anti-myc and the $^{32}$P-labeled samples analyzed by SDS-PAGE and phosphorimaging (Fig. 5A). Western blot analysis of the immunoprecipitates from the unlabeled cultures were used to quantify the relative amount of Nopp fragments that precipitated with anti-myc (Fig. 5B, last three lanes). Thus we could normalize the levels of $^{32}$P associated with myc-tagged Nopp fragments immunoprecipitated from the radiolabeled cultures to the total amount of immunoprecipitated myc-tagged molecules (Fig. 5C). Although U-myc was abundantly expressed, little phosphorylation was observed. In contrast, both UJ-myc and UJA-myc were phosphorylated to a much higher degree. Normalized to the abundance of the proteins in the immunoprecipitates, it appeared that UJA-myc contained about twice as much $^{32}$P as UJ-myc, which in turn contained tenfold more than U-myc.

**The unique region of Nopp44/46 confers nuclear and nucleolar localization**

We have previously utilized the myc-tag to study the subcellular localization of various trypanosome proteins, including those residing in the cytoplasm (Peterson et al., 1997) glycosomal microbodies (Peterson et al., 1997) and mitochondria (Anderson et al., 1998). In these studies, the myc-tag was neutral with respect to localization of the tagged proteins. To determine which region of Nopp44/46 confers nucleolar localization, we investigated the localization of the myc-tagged proteins in transfectants by immunofluorescence microscopy (see Fig. 6). Staining of wild-type cells with anti-Nopp44/46 ($\alpha$-Nopp) shows a bright spot that is smaller than the nucleus, which coincides with a weaker area of staining with the DNA-specific dye DAPI. Our previous immunoelectron microscopic studies identified this compartment as the nucleolus (Das et al., 1996). When the same, untransfected cells were stained with anti-myc ($\alpha$-myc), little fluorescence was observed. However, anti-myc staining of the transfectants expressing tagged proteins containing the intact unique region (U-myc, UJ-myc and UJA-myc) showed a fluorescence pattern identical to that seen with wild-type cells.
and anti-Nop44/46. Since there are no nucleolar protein markers for T. brucei apart from Nop44/46, we were able to perform direct colocalization studies only with the U-myc transfectant (it does not react with anti-Nop44/46). As shown in Fig. 6, the same region of the nucleus is identified by both anti-myc and anti-Nop44/46 in these transfectants. Thus, the U region of Nop44/46 is sufficient for nucleolar accumulation. The cells expressing UJA445-71-myc showed a different pattern, with staining of both the nucleolus and the nucleus. Finally, R-myc transfectants were diffusely stained throughout the cell. Since R-myc was not soluble in immunoprecipitation buffer, we speculate that it may be associated with the cytoskeleton.

Iterative database analysis using the Psi-Blast program (Altschul et al., 1997) revealed a moderate level of homology between the U region of Nop44/46 and the amino-terminal region of a recently described class of nucleolar histone deacetylases (Lusser et al., 1997) (Fig. 7). Additional proteins also showed similarity to this region of Nop44/46, albeit at a reduced level. These included B23, a nucleolar protein (Schmidt-Zachmann and Franke, 1984) and certain nuclear FK506 binding proteins, one of which is known to be nucleolar (Benton and Zhang, 1994; Shan and Melese, 1994).

**DISCUSSION**

The studies presented here indicate that each domain plays a distinct function. Phosphorylation of the R region has not been tested.

**Fig. 8.** Model of Nop44/46 domain functions. Studies presented here indicate that each domain plays a distinct function. Phosphorylation of the R region has not been tested.
well conserved between the different proteins identified in the homology search (Fig. 7). However, it is quite possible that additional residues are required for targeting as well. Clearly further experimental analysis of the U homology regions will be required before the nucleolar retention motifs can be defined.

The mechanism by which Nopp44/46 enters the nucleus is a question that has not been resolved. No consensus nuclear localization sequences are obvious on the molecule (either of the basic type (LaCasse and Lefebvre, 1995) or of the M9 type (Pollard et al., 1996). As a rather small molecule (the predicted size of the native molecule is 35-37 kDa), Nopp44/46 may be able to enter the nucleus freely and then be sequestered in the nucleolus by its interactions with nucleolar molecules. However, the localization of UJA45-71-myc to the nucleus (without accumulation in the nucleolus or cytoplasm) suggests that this may not be the case and that a specific mechanism is involved in the nuclear import of Nopp44/46. For example, it may have a novel type of nuclear localization sequence or it may interact with another protein in the cytoplasm and piggyback into the nucleus by virtue of that protein’s nuclear localization sequence.

The last function explored in this communication is the interaction of Nopp44/46 with a protein kinase. Our previous work has shown that monoclonal anti-Nopp44/46 co-precipitates a protein kinase activity, indicating that Nopp44/46 is associated with a protein kinase. This hypothesis was verified in the current work, which shows that a completely unrelated antibody, antimmuc, also co-precipitates a protein kinase from cells expressing specific myc-tagged Nopp proteins. The deletion constructs tested show that the J region is required for co-precipitation of protein kinase activity, which in turn is enhanced by the presence of the adjacent A region. These regions may function in either interaction of the protein kinase with Nopp44/46 and/or in the modulation of the kinase activity. The nature of the Nopp-associated protein kinase has not yet been determined, apart from the fact that both tyrosine and serine phosphorylating activity is found in the immunoprecipitates (Parsons et al., 1994). Since Nopp44/46 is a highly phosphorylated protein, with both serine and tyrosine phosphorylation (but no apparent threonine phosphorylation), it is possible that it is a substrate for the associated kinase. This conjecture would fit with the observation that only those Nopp fragments that associated with the protein kinase were efficiently phosphorylated. The U-myc protein, which resides in the same compartment within the cell, did not become phosphorylated to a significant degree, although it possesses six serines and two tyrosines (out of a total of 21 serines and tyrosines in the full-length molecule). Another, not mutually exclusive, possibility is that the specific sites for phosphorylation are located only in the J and A regions. It is interesting that three consensus sites for phosphorylation by casein kinase II (S-X-X-D/E embedded in a region of acidic residues; Allende and Allende, 1995) are present in the J and A regions. Casein kinase II has been shown to phosphorylate several nucleolar proteins in other organisms (Olson, 1990). Although normally considered a serine/threonine kinase, a recent report indicates that in yeast cells casein kinase II can phosphorylate immunophilin Fpr3 on tyrosine, provided that an adjacent serine is phosphorylated or is converted to an acidic residue (Wilson et al., 1997). One tyrosine in the A region of Nopp44/46 is surrounded by acidic residues and thus might also be a substrate for such phosphorylation. It should be noted however that the T. brucei casein kinase II has not yet been characterized.

The studies presented here demonstrate that the different functional attributes of Nopp44/46 can be mapped to distinct domains of the molecule. Because Nopp44/46 is a relatively abundant protein, we speculate that it may play a structural role. Its ability to interact with both proteins and nucleic acids may allow it to facilitate the interaction of other molecules within the trypanosome nucleolus. The definition of the segments of Nopp44/46 that mediate these interactions should facilitate the identification of its molecular partners. This may prove to be the most efficient route to clarifying the function of Nopp44/46, since null lines lacking this multigene family may be difficult to construct.

The authors thank Dr Steven Anderson for providing the pTbmyc2 plasmid. This work was supported in part by NIH AI31077 and NIH S10 RR11865-01.

REFERENCES

Functional domains of a nucleolar protein

overlap DNA- or RNA-binding domains in nucleic acid-binding proteins. 


