Protein NPM3 Interacts with the Multifunctional Nucleolar Protein B23/Nucleophosmin and Inhibits Ribosome Biogenesis*

Nian Huang, Sandeep Negi, Attila Szebeni, and Mark O. J. Olson‡

From the Department of Biochemistry, University of Mississippi Medical Center, Jackson, Mississippi 39216

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Protein B23/nucleophosmin is a multifunctional protein that plays roles in ribosome biogenesis, control of centrosome duplication, and regulation of p53 expression. A yeast two-hybrid screen was performed in a search for interaction partners of B23. The complementary DNA for a highly acidic protein, nucleoplasm 3 (NPM3), was found in multiple positive clones. Protein NPM3 and its interaction with B23 were further characterized. Endogenous B23 was able to be co-immunoprecipitated with NPM3, and this complex was resistant to ribonuclease treatment and high concentrations of salt. The N-terminal 35–90 amino acids of B23 were found to be required for their interaction. Separate co-immunoprecipitation studies of B23 and NPM3 suggested the existence of two different complexes, one containing B23 and 28 S ribosomal RNA (rRNA) and another composed of B23, NPM3, and other proteins, but no RNA. NPM3 was localized in the nucleolus, and its nucleolar localization depended on active rRNA transcription. In the cells overexpressing NPM3, there were decreased rates of pre-rRNA synthesis and processing. Overexpression of a mutant of NPM3 that did not interact with B23 did not alter pre-rRNA synthesis and processing, suggesting that the interaction of NPM3 with B23 plays a role in the ribosome biogenesis.

Ribosome biogenesis in eukaryotic cells is a multistep process that takes place primarily in the nucleolus where the individual stages of assembly correlate with specific subclasses of ultrastructures (1–4). The process begins with transcription of the ribosomal DNA at the border between the fibrillar center and the dense fibrillar components of the nucleolus. The product of transcription, 47 S pre-ribosomal RNA (pre-rRNA) in mammals, is processed into smaller pre-rRNA intermediates, which finally become 28, 5.8, and 18 S rRNA. The nascent pre-ribosomal particles of the dense fibrillar components eventually mature into granular components, with ribosomal proteins added at various steps in the process. Numerous non-ribosomal proteins and small nuclear RNA participate in these steps.

Protein B23 (NPM1, nucleophosmin) is an abundant nuclear non-ribosomal protein whose locations and multiple activities suggest it plays a role in ribosome biogenesis. This protein is primarily localized to the granular component region with lesser amounts in the dense fibrillar components of the nucleolus (5–8). The nucleolar localization of B23 is dependent on the presence of active rDNA transcription (9), and it is found in association with maturing pre-ribosomal RNP particles (10, 11). In vitro experiments indicate B23 has nucleic acid binding activity and ribonuclease activities (12–17). The nucleic acid binding activity has been mapped to its C-terminal end (17, 18) and is believed to be important in its nucleolar localization (15). In support of this, a splicing variant, B23.2, in which the C-terminal 35-amino acid sequence is absent, exists both in the nucleoplasm and cytoplasm (15, 17). More recent studies provide evidence for a direct role of the ribonuclease activity of B23 in processing the internal transcribed spacer 2 of pre-ribosomal RNA, especially in the production of 28 S rRNA from 32 S pre-rRNA (19). Protein B23 also has molecular chaperone activity. This chaperone activity is regulated by protein kinase CK2 phosphorylation; this activity possibly prevents protein aggregation in the nucleolus under high macromolecular concentration conditions and facilitates the ribosome assembly process (20–22).

Protein B23 has additional activities that are not directly related to ribosome biogenesis. It binds the centrosome during early prometaphase (23), and phosphorylation of B23 by CDK2/cyclin E and Polo-like kinase 1 is essential for duplication of the centrosome (24, 25). Many proteins involved in cell cycle control have also been shown to interact with B23, including p53, HDM2, ARF, and the BRCA1-BARD1 ubiquitin ligase (19, 26–28). It has been proposed that B23 also works as the protein mediating the cross-talk between ribosome biogenesis and cell cycle progression, thereby making it a potential valuable target for cancer therapy (29).

Because several other proteins have been shown to interact with protein B23 (20), we have attempted to systematically identify its interacting partners to better understand its role in the nucleolus. To this end, we performed a yeast two-hybrid screen. We found that NPM3 (nucleoplasm 3) was one of the major proteins interacting with B23. NPM3 was previously identified among the genes activated by mouse tumor virus proviral insertions and is often co-activated with proto-oncogene Fgr because of its close proximity to that gene (30). It is widely expressed in different adult mouse tissues (30–32). Experiments with deletion mutants of B23 indicate that the residue 35–90 sequence of B23 is essential for its interaction with NPM3. Evidence that NPM3 possibly regulates the function of B23 in ribosome biogenesis was provided in that ectopic expression of NPM3 slowed pre-rRNA synthesis and processing; this effect was not seen with mutants that did not interact with B23.
MATERIALS AND METHODS

Yeast Two-hybrid System Screen—The yeast two-hybrid screen was performed using the Matchmaker two-hybrid system 2 and GAL 4 Matchmaker mouse liver cDNA library (Clontech, BD Biosciences) according to the manufacturer's instructions. Briefly, full-length B23 was cloned into shuttle vector pAS2-1 and used to transform yeast strain Y190. The clone expressing BD-B23 was amplified and transformed with the DNA library. Positive clones were selected by growing the transformed yeast on S.D./-His/-Leu/-Trp (25 mM 3-amino-1, 2, 4-triazole) and detected by the β-galactosidase assay using X-gal as the substrate. The plasmids were then purified from the positive clones and sequenced.

Plasmid Construction—Mammalian transient expression vector pFLAG-CMV-5a was purchased from Sigma. Full-length NPM3 was constructed by PCR using the plasmid purified from yeast as the template, in which an overlapping primer corresponding to the missing part of the cDNA of NPM3 was designed according to the sequence reported previously (31).

Cell Culture and Transfection—HeLa or CMT3 cells (33) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units/ml of penicillin and streptomycin. Transfection was performed using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions (34). Fluorescence microscopy and cell lysis were generally performed 2 days after transfection.

To enrich NPM3-overexpressing cells, the CMT3 cells were co-transfected with FLAG-tagged NPM3 mutants or empty FLAG vector and a puromycin marker vector. After 2 days, the cells were cultured in the medium containing 2.5 μg/ml puromycin for 1 week and the puromycin-resistant cells were replated for further analysis.

Cell Lysis and Co-immunoprecipitation—HeLa or CMT3 cells cultured on 35-mm tissue culture dishes were scraped into a lysis buffer containing 50 mM Tris (pH 7.4), 100 mM NaCl, 50 mM NaF, 0.5% Nonidet P-40, 0.1% sodium deoxycholate, and 10 μg/ml proteinase inhibitor mixture (Sigma) and sonicated three times for 20 s at 20-s intervals. The supernatant was collected after centrifugation at

FIG. 1. Confirmation of interaction between protein B23 and protein NPM3 by colony-lift filter assay of β-galactosidase activities in the yeast two-hybrid system. Yeast strain Y190 was co-transformed with pACT2/NPM3 and pAS2-1/B23 (A), pACT2/NPM3 and pAS2-1 (B), or pACT2/NPM3 and pLAM5–1 (C) and selected for growth on synthetic dropout medium without leucine and histidine (upper panel). The colonies were lifted onto filter paper, treated with liquid nitrogen, and incubated with a buffer containing X-gal as the substrate for 8 h. Clones became blue when there was interaction between two proteins (lower panel).

FIG. 2. Sequence characteristics of B23 and NPM3. Functional domains of B23 were previously characterized by Hingorani et al. (18). Alignment of core regions of similarity in NPM3 and B23 was performed using the program AlignX (Infomax). Identical or similar amino acids are shaded.

Core region of similarity:

| B23.1  | SPLRPQNYLFHCCLADKDYHF--KVDRENENHOLSRLTVSLGAGAKDEL |
| Npm3  | APVTMDSFPFGCELSHTRSFFTKVEBDEDDTEPHLALMLIAGTDEC |
| Consensus | APL F FGCE A  | D DD EH LAL L L GA DE |

| B23.1  | HIVEAEAMNYEGSPIKTATLKMSQCVTSTVHLGGFEITPVPVLLRLKG |
| Npm3  | NVVEVVARHIDQIEIAVVPANLRSCQPLSVDDFQLQPVTFLRKS |
| Consensus | IVE A HD I V LA LKLQS QP LSV  | LK G |

| B23.1  | SGFITSGOHVLAVEEDAESDEEDEDKLGLSGKSREAPFAGNNK |
| Npm3  | PVRITGRHIVCINNDLSSEEDSEDEEDELKCGILPAKX |
| Consensus | ISG H I  | SEDE DDD I PA K |
FIG. 3. Co-immunoprecipitation of endogenous B23 with NPM3. CMT3 cells were transfected with either FLAG-tagged NPM3 or empty pFLAG-CMV-5 vector. Expressed NPM3 was immunoprecipitated by an anti-FLAG antibody-conjugated agarose gel. The co-immunoprecipitates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Endogenous B23 was detected by Western blotting using an anti-B23 monoclonal antibody. The same membrane was stripped and reprobed with anti-FLAG monoclonal antibody.

FIG. 4. The interaction between B23 and NPM3 is resistant to dissociation by high concentrations of salt and ribonuclease treatments. A, CMT3 cells were transfected with FLAG-tagged NPM3. Co-immunoprecipitation was performed using anti-FLAG monoclonal antibody-conjugated resin with identical aliquots of cell lysates. The resins were washed extensively with lysis buffer containing either 150, 300, or 600 mM NaCl before elution. B, cell lysates were pretreated with 100 μg/ml RNase A, 100 units/ml DNase I, or 100 units/ml micrococcal nuclease (2 mM CaCl₂) added by incubation with these enzymes for 30 min at 30 °C before co-immunoprecipitation. Co-immunoprecipitates were separated on a 12% PAGE gel and then silver-stained.
overlapping PCR method using the selected cDNA clone as the template. The cDNA was inserted into the pFLAG-CMV-5 vector, which placed a FLAG epitope at the C-terminal end of the protein. CMT3 cells were either transfected with NPM3-FLAG or the empty FLAG vector. Total cell lysates were made by sonication and clarified by centrifugation. The expressed FLAG-tagged NPM3 was immunoprecipitated by an anti-FLAG M2 affinity gel. The co-immunoprecipitates were eluted by low pH buffer and run on a SDS-PAGE gel, followed by transfer to a nitrocellulose membrane and analysis by Western blotting. Endogenous B23 detected by an anti-B23 monoclonal antibody (9) was present in the co-immunoprecipitates of NPM3 (Fig. 3). The complex containing the two proteins was very stable and resistant to dissociation by high concentrations of salt (Fig. 4A), suggesting their association was largely because of hydrophobic interactions. B23 was previously reported to be co-immunoprecipitated along with nucleolin by an anti-nucleolin antibody, and RNA was required to maintain a stable complex between the two proteins (11). To examine whether this is the case in the interaction of protein NPM3 and B23, similar nuclease/ribonuclease digestion conditions were used to treat the cell lysates before co-immunoprecipitation. No significant differences were seen before and after nuclease treatment (Fig. 4B). This suggests that B23 and NPM3 interact directly with each other in the complex.

Probing the Interacting Regions of B23 and NPM3—Several functional domains have been mapped in protein B23 (18). For example, the deletion of its N-terminal end will cause B23 to lose its ability to oligomerize and most of its chaperone activity; the 35 amino acids in its C-terminal tail are essential for the nucleic acid binding activity of B23 (17). Comparing the sequences between B23 and NPM3, about 42% of the N-terminal sequence of B23 is conserved in NPM3 (Fig. 2). This segment might contribute to the interaction between the two proteins, possibly through hetero-oligomerization. To test this possibility, a series of plasmids containing GFP-tagged deletion mutants of B23 were co-transfected into CMT3 cells with FLAG-tagged NPM3. B23 mutants were immunoprecipitated by anti-GFP monoclonal antibody-conjugated beads, and the co-immunoprecipitated NPM3 was detected by an anti-FLAG monoclonal antibody. All of the mutants tested, except B23ΔH9004N90, interacted with NPM3 (Fig. 5). In the mutant B23ΔH9004N90, in which the oligomerization domain of B23 was almost completely deleted, the interaction with NPM3 was abolished. In contrast, the deletion mutants B23ΔH9004C35 and B23ΔH9004C132 that had lost the nucleic acid binding domain were still able to bind NPM3. Thus, the interaction between B23 and NPM3 is largely dependent on the oligomerization domain of B23 and not on its nucleic acid binding region.

Similarly, the deletion mutants of NPM3, NPM3ΔH9004N30 and NPM3ΔH9004N90, were also made and tagged with FLAG. Endogenous B23 was able to be co-immunoprecipitated with NPM3ΔN30 but not by NPM3ΔN90, suggesting amino acids 30–90 in the N-terminal homologous region of NPM3 were required for interaction with B23.

B23, but not NPM3, Is Associated with 28 S rRNA—One of the most prominent differences between the sequences of protein NPM3 and protein B23 is that only protein B23 has a nucleic acid binding domain located in its C-terminal tail (Fig. 2). In vitro experiments using Escherichia coli-expressed pro-
tein B23 showed that it binds DNA and RNA nonspecifically (12). To compare the possible association of B23 and NPM3 with nucleic acids in the cell, we performed co-immunoprecipitation experiments separately for B23 and NPM3. As shown in Fig. 6, the complexes containing B23 showed strong association with 28 S rRNA, but no RNAs were found in the complexes formed by NPM3. Because a portion of B23 is also co-precipitated with NPM3, it seems likely that this portion of B23 is not bound to RNA (also see “Discussion”).

**Localization of NPM3 in Cells**—B23 is primarily located in the nucleoli of interphase cells (34, 37). Similarly, most of the NPM3 was located in the nucleoli of the cells, although the signal of NPM3 was more diffuse than that of B23 (Fig. 7, before Actinomycin D treatment). The nucleolar localization of B23 requires active rRNA gene transcription; treatment of cells with inhibitors of pre-rRNA synthesis, including actinomycin D, results in translocation of the protein from the nucleolus to the nucleoplasm (9, 37). NPM3 also has translocation characteristics similar to those of B23. As shown in Fig. 7, HeLa cells transfected with either B23-FLAG or NPM3-FLAG were incubated with 0.5 μg/ml actinomycin D for 1 h. Cells were then stained with anti-FLAG antibody and counterstained with Hoechst.

**Ectopic Expression of NPM3 Inhibits Pre-rRNA Transcription and Processing**—Recent studies have implicated protein B23 in pre-rRNA processing (19). Considering the interaction of NPM3 with B23 and correlation between rRNA synthesis and NPM3 localization (Fig. 7), we evaluated the effect of overexpression of NPM3 on ribosome biogenesis. Cells transfected with NPM3 and its deletion mutants were enriched by drug selection. Using an antibody to the N-terminal sequence of NPM3 (kindly provided by Dr. Marion Schmidt-Zachmann), the amount of NPM3 in the cells transfected with NPM3 was about twice that of the control cells as determined by Western blot analysis (data not shown). The cells were pulse-labeled using [3H]uridine and chased for 0, 2, and 4 h; their RNA was extracted, and the RNA from equal numbers of cells was loaded on the gel. As shown in Fig. 8A, 4-h chase (Fig. 8A, 4-h chase) in the cells overexpressing NPM3 and NPM3ΔN30. The reduction of rRNA production could result from the inhibition of transcription and/or processing. The 0-min chase times of incorporation approximately represent the relative rates of 47 S pre-rRNA transcription; in cells expressing NPM3 and NPM3ΔN30, this rate was reduced to

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2. K. Hingorani, unpublished data.
an average of 33 and 35% of the control cells, respectively (Fig. 8B). No significant change in rRNA production was seen for the cells expressing NPM3H9004N30.

To better examine differences in the processing rates in these cells, we adjusted the amount of RNA loaded so that each lane contained the same amount of radioactivity. L-[methyl-3H]methionine was used for labeling because of the rapid turnover of the cellular methionine pool (35). In cells expressing NPM3 and NPM3H9004N30, the half-life of 47 S pre-rRNA was increased to at least twice that of the control cells (Fig. 8C and D), which suggests that the processing of pre-rRNA in NPM3-expressing cells is also suppressed.

DISCUSSION

In this study we have provided evidence that protein NPM3 is an interacting partner of nucleolar phosphoprotein B23 and that it may regulate B23 function in ribosome biogenesis. First, the yeast two-hybrid screen and the co-immunoprecipitation of B23 with NPM3 indicated that these two proteins interacted with each other in vitro and in vivo. Second, we detected an RNP complex that contained 28 S rRNA and protein B23, but there was no RNA found in the complex containing NPM3. Third, both of these proteins normally had nucleolar localization, but they translocated into the nucleoplasm after inhibition of rRNA transcription. Finally, expression of NPM3, but not its mutants that do not interact with B23, down-regulated pre-rRNA synthesis and processing, suggesting that interaction between NPM3 and B23 might be involved in regulating ribosome biogenesis.

Unlike the association between B23 and nucleolin in which the presence of ribonucleic acid is required for the stable formation of the complex (11), the binding between B23 and NPM3 most likely results from direct interaction between the proteins (Fig. 4A). Considering that both proteins have very low pIs (B23, 4.62; NPM3, 4.71) and the complex is resistant to dissociation by high salt concentrations (Fig. 4B), hydrophobic forces could be the major contributor to this interaction. Coincidentally, the co-immunoprecipitation of protein NPM3 with deletion mutants of B23 suggests that the Asn-35-Asn-90 region of protein B23 plays an important role between their interactions; this appears to be the most hydrophobic region of B23. The functional domains of B23 have been well characterized (18). The residue 35–90 segment also covers most of the sequence mapped as essential for the chaperone activity and the oligomerization of B23. Therefore, it seems likely that
residue 35–90 region of B23 is responsible for its interaction with proteins, including its own oligomerization. Although B23 and NPM3 have many features in common, one striking difference is that the former seems to be associated with RNA whereas the latter is not. The interaction between B23 and nucleic acids has been extensively investigated with proteins, including its own oligomerization.

residue 35–90 region of B23 is responsible for its interaction with B23. Therefore, the association of B23 with 28 S rRNA is consistent with its location.

Comparison of the B23 and NPM3 sequences reveals that the relatively short sequence of NPM3 does not have a segment similar to the nucleic acid binding tail of B23. This is consistent with no detectable RNAs being found in the complexes co-immunoprecipitated by NPM3 (Fig. 6). Because a portion of protein B23 is also co-precipitated with NPM3, this part of the protein B23 pool that forms a complex with NPM3 does not contain RNA. We concluded that there are at least two different complexes associated with B23, one of which contains 28 S rRNA whereas the other does not. NPM3 may form a complex with those "free" portions of B23 that do not associate with 28 S rRNA. Because B23 normally is not found in the mature ribosome, it has to be released from this rRNA complex before mature ribosomes are transported to the cytoplasm. The question then is, what are the factors that dissociate B23 from the maturing RNP particle? One candidate could be NPM3. Another candidate might be the variant form of B23, B23.2, which also does not have nucleic acid binding activity and has been reported to inhibit the rRNA binding activity of B23 (15).

Many characteristics of protein B23 suggest that it participates in ribosome biogenesis. B23 is localized to the nucleolus during interphase when ribosomes are actively produced but translocates from the nucleolus to the nucleoplasm after actinomyein D treatment, suggesting that it is associated with the pre-rRNA transcriptional apparatus (9). This distribution of B23 also affects those proteins associated with it, such as the human immunodeficiency virus-1 Rev protein (37). Although NPM3 also showed similar translocation after actinomyein D treatment (Fig. 7), it is unknown whether this is due to the direct effect of inhibition of pre-rRNA transcription or because of its association with B23. In vitro studies also suggest B23 is a pre-rRNA processing factor because it has ribonuclease activity and preferentially cleaves at specific sites in the second internal transcribed spacer (16). However, the most direct evidence for this is that down-regulation of B23 by short interfering RNA caused a defective 32 S processing pathway and preferentially inhibited 28 S rRNA product formation (19). How these findings are related to the association of B23 with 28 S rRNA is unknown. In this study we found that expressing NPM3 inhibits both transcription and processing of pre-rRNA. This effect is likely related to the interaction between NPM3 and B23 in that no significant inhibition was observed in the cells expressing the NPM3 mutants not interacting with B23. It is possible that overexpressed NPM3 interferes with the function of B23 by altering the balance between the complexes formed by B23, with the effect of drawing B23 to a complex that does not contain 28 S rRNA. However, considering the interactions between B23 and cell signaling molecules (19, 26, 27), it is possible that other mechanisms are also involved in the inhibition of ribosome biogenesis by NPM3.

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B23 and nucleic acids has been extensively investigated with proteins, including its own oligomerization. Although B23 and NPM3 have many features in common, one striking difference is that the former seems to be associated with RNA whereas the latter is not. The interaction between B23 and nucleic acids has been extensively investigated in vitro (12, 13). This binding activity resides in its C-terminal end (17, 18). The RNA binding activity of B23 is modulated by cyclin B/cdc2-mediated phosphorylation and has been implicated in its translocation from nucleoli to cytoplasm during mitosis (15). Until now there has been no preferred sequence found that B23 binds with in vitro. However, our co-immunoprecipitation data showed that in vivo B23 is preferentially associated with 28 S rRNA (Fig. 6). This preference is possibly rendered by other proteins residing in the same RNP complex with B23. Furthermore, this association with 28 S rRNA, which is one of the last species released during the processing of pre-rRNA, matches the ultrastructural location of B23 in the nucleolus. Protein B23 is primarily found in the granular component that contains the more mature pre-ribosomal RNP particles (5–8). Therefore, the association of B23 with 28 S rRNA is consistent with its location.

Comparison of the B23 and NPM3 sequences reveals that the relatively short sequence of NPM3 does not have a segment similar to the nucleic acid binding tail of B23. This is consistent with no detectable RNAs being found in the complexes co-immunoprecipitated by NPM3 (Fig. 6). Because a portion of protein B23 is also co-precipitated with NPM3, this part of the protein B23 pool that forms a complex with NPM3 does not contain RNA. We concluded that there are at least two different complexes associated with B23, one of which contains 28 S rRNA whereas the other does not. NPM3 may form a complex with those "free" portions of B23 that do not associate with 28 S rRNA. Because B23 normally is not found in the mature ribosome, it has to be released from this rRNA complex before mature ribosomes are transported to the cytoplasm. The question then is, what are the factors that dissociate B23 from the maturing RNP particle? One candidate could be NPM3. Another candidate might be the variant form of B23, B23.2, which also does not have nucleic acid binding activity and has been reported to inhibit the rRNA binding activity of B23 (15).

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