Mapping the Functional Domains of Nucleolar Protein B23

Kamini Hingorani, Attila Szebeni and Mark O. J. Olson‡

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

2500 North State street, Jackson, MS 39216

Running title: Structure-Function Relationships in Protein B23

* This work was supported in part by grants from the National Institutes of Health and by the Medical Guardian Society of the University of Mississippi.

‡ To whom correspondence should be addressed. Tel: 601-984-1500;

Email: molson@biochem.umsmed.edu
Summary

Protein B23 is a multifunctional nucleolar protein whose cellular location and characteristics strongly suggest it is a ribosome assembly factor. The protein has nucleic acid binding, ribonuclease and molecular chaperone activities. To determine the contributions of unique polypeptide segments enriched in certain classes of amino acid residues to the respective activities, several constructs that produced N- and C-terminal deletion mutant proteins were prepared. The C-terminal quarter of the protein was shown to be necessary and sufficient for nucleic acid binding. Basic and aromatic segments at the N- and C-terminal ends, respectively, of the nucleic acid binding region were required for activity. The molecular chaperone activity was contained in the N-terminal half of the molecule, with important contributions from both nonpolar and acidic regions. The chaperone activity also correlated with the ability of the protein to form oligomers. The central portion of the molecule was required for ribonuclease activity and possibly contains the catalytic site; this region overlapped with the chaperone-containing segment of the molecule. The C-terminal, nucleic acid binding region enhanced the ribonuclease activity but was not essential for it. These data suggest that the three activities reside in mainly separate, but partially overlapping segments of the polypeptide chain.
INTRODUCTION

Ribosome assembly is a multistep process that utilizes numerous proteins and small nucleolar RNAs (1, 2). One candidate for a ribosome assembly factor is an abundant protein called B23 [also known as nucleophosmin/NPM (3), NO38 (4) or numatrin (5)] whose location, abundance and multiple activities suggest that it plays a major role in ribosome biogenesis. This is supported by the ability of protein B23 to bind nucleic acids (6, 7), and by its association with maturing preribosomal RNP\(^1\) particles (4, 8, 9). Treatment of cells with drugs that inhibit preribosomal RNA processing or synthesis (10, 11), causes translocation of B23 to the nucleoplasm, which further suggests its presence in nascent preribosomal particles. Finally, protein B23 possesses intrinsic ribonuclease activity that has been implicated in the processing of preribosomal RNA in the ITS2 region (12, 13).

Protein B23 interacts with other nucleolar proteins, including nucleolin (14), protein p120 (15) and the HIV-1 Rev protein (16). Its ability to shuttle between the nucleus and cytoplasm (17), bind NLS containing peptides (18) and stimulate import of proteins into the nucleus (18) suggested a role in nuclear import. The latter activity might be explained by its ability to act as a molecular chaperone (19). In normal cells, this activity may aid in the transport of ribosomal or

\(^1\) The abbreviations used are: RNP, ribonucleoprotein; ITS2, internal transcribed spacer region 2; NLS, nuclear localization signal; PCR, polymerase chain reaction; GST, glutathione S-transferase; LB, luria bertani; TB, terrific broth; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PMSF, phenyl methyl sulfonyl fluoride; DTT, dithiothreitol; ETS, external transcribed spacer region; HPLC, high performance liquid chromatography; LADH, liver alcohol dehydrogenase; CS, citrate synthase; kDa, kilo dalton; MDa, mega dalton; His, histidine.
other nucleolar proteins from their site of synthesis into the nucleus or nucleolus. Alternatively, protein B23 could serve as a chaperone in preventing aggregation of proteins in the very crowded environment of the nucleolus during ribosome assembly. Under native conditions protein B23 probably exists as a hexamer and or larger oligomer (4, 20). Since many chaperones are oligomers (21, 22), the chaperone activity of protein B23 could be related to its oligomerization properties.

Although the activities of protein B23 are commonly found in many proteins, it lacks similarities to any sequence motifs normally correlated with these activities. Specific functions are often contained in discrete structural regions or domains of multifunctional proteins. In such cases, functional studies can be facilitated by characterization of the activities residing in individual domains. In protein B23 there are distinctive sequence motifs along the polypeptide chain, which suggest the presence of functional domains; i.e., the N-terminal region has relatively high density of hydrophobic residues, the central region contains two highly acidic segments and the C-terminal third of the molecule carries a net positive charge. Thus, it might be possible to characterize segments of the molecule responsible for particular activities and thereby identify novel structural-functional motifs. In this study we generated a series of N- and C-terminal deletion mutants of protein B23 to facilitate the dissection of the molecule into possible functional domains. It was found that different activities reside in mainly independent but slightly overlapping segments of the polypeptide chain.

**EXPERIMENTAL PROCEDURES**

*Preparation and cloning of B23 constructs* - To create fusion constructs containing the entire B23 cDNA or fragments encoding different domains of protein B23, appropriate primers
were synthesized (Cybersyn) for use in polymerase chain reaction amplification using AmpliTaq DNA polymerase (Perkin Elmer) and rat B23 cDNA as template. The following oligonucleotides were used for the synthesis of each construct: B1N (5'-ATGGAAGACTCGATGGACATG-3') and B1C (5'-TTAAAGAGACTTCCTCCACTG-3') for full length B23, B2N (5'-GATGAAAATGAGCACCAG-3') and B1C for ∆N35, B3N (5'-GGCTTCGAAATTACACCA-3') and B1C for ∆N90, B4N (5'-GAGGAAGATGCAGAGTCAG-3') and B1C for ∆N119, B5N (5'-GGAAAGAGATCTTGCTCCC-3') and B1C for ∆N139, B6N (5'-GAAGAAAGGTTCCAGTGGAAG-3') and B1C for ∆N185, B7N (5'-ACACCAAGGTCAAAGGGT-3') and B1C for ∆N216, B1N and B2C (5'-ACCACCTTTTCTATATCTTG-3') for ∆C35, B1N and B3C (5'-TTTCAATCAAGTTTTTACTTTTCTTG-3') for ∆C132, B1N and B4C (5'-ATCTTCTCATCTTTACTCTCTC-3') for ∆C161, B1N and B5C (5'-CAAGACCACAGGTGGTGAAT-3') for ∆C192. ∆N and ∆C indicate N- and C-terminal mutants and the numbers specify the number of amino acids deleted from the respective end. PCR products were excised from the gel and ligated into the pCR2.1 vector (Invitrogen). All constructs were sequenced and subsequently subcloned into the pQE-30 vector (Qiagen), with the N-terminal His tag. The fragments were excised by XhoI and HindIII sites and subcloned into the SalI and HindIII sites of the pQE-30 vector. For the N-terminal GST- tagged deletion constructs (GST-∆N255, GST-∆N240), the fragments were excised by EcoRI restriction endonuclease and ligated into the EcoRI site of pGEX-3X (Amersham Pharmacia Biotech.) vector. Nucleotide sequences were verified so that the correct reading frame was preserved for each clone.
Expression and purification of His- tagged recombinant proteins - The recombinant plasmids were transformed into M15 bacterial cells and grown in 1 liter volumes of LB media. The cells were grown to an A_{600} of 0.6 and induced with 1 mM IPTG for 3 h. A few of the mutants including full length B23, ΔN35, ΔN90, ΔN139 and ΔC35 grew poorly in LB media and were grown in terrific broth (TB) instead. Cells were collected by centrifugation and kept at -20°C overnight. Harvested cells were resuspended in buffer B (8 M urea, 0.01 M Tris, 0.1 M NaH_{2}PO_{4}, pH 8.0), and mixed gently on a rocker for 45 min. The homogenate was then spun at 15,000 rpm for 20 min. To the supernatant, 1 ml of pre-equilibrated Ni^{2+}-Nitrilotriacetic acid superflo flow resin was added and mixed gently for 2 hrs. After three washes with buffer C (8 M urea, 0.01 M Tris, 0.1 M NaH_{2}PO_{4}, pH 6.3) tagged peptide was eluted with elution buffer (buffer C + 250 mM imidazole). All of the eluates were tested for protein purity using SDS-PAGE and were dialyzed against a modified H1 buffer (50 mM Tris, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol, 0.1 mM PMSF, pH 7.9) and concentrated to 5-10 mg/ml of protein using Amicon centriprep concentrators with the desired cut-off range. Protein concentrations were calculated using the BioRad protein assay (23).

Expression and purification of the GST- tagged recombinant proteins - The recombinant plasmids were transformed into the bacterial strain BL21. The protocol used for protein expression and purification is as described in Smith and Johnson (24). Purified proteins (GST alone, GST-ΔN255, GST-ΔN240) were dialyzed against H1 buffer (described above) and were tested for purity using SDS-PAGE.

Nucleic acid filter binding assay - Plasmid pGEM-4Z was labeled using random priming (Megaprime DNA labeling kit) and 50 μCi [α-^{32}P]dCTP (New England Nuclear) as per the manufacturers instructions (Amersham Pharmacia Biotech). Binding to DNA by protein B23 and
its mutants was measured using a nitrocellulose filter binding assay as described previously (25). The reaction mixture, which included labeled DNA and mutant proteins (0-60 µM) was incubated in a TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA) at 37°C for 15 min. The reactions were stopped by filtration through a prewetted nitrocellulose filter (Schleicher & Scheull 0.45 µM), followed by washes with 1 ml TBE. The amount of radioactivity retained on the filter was quantified using a Molecular Dynamics phosphorimager.

*In vitro transcription of rat rDNA plasmid* - The substrate used for ribonuclease digestions was obtained by *in vitro* transcription of the rDNA plasmid (pXKDF15) as described previously (13). The plasmid contained 1.3 kb of the 5' ETS sequence with positions +638 to +1880 from the transcription start site. Plasmid pXKDF15 was linearized with *Xho*I and *in vitro* transcription was performed using a bacteriophage T7 RNA polymerase (26). Transcripts were uniformly labeled with $^{32}$P by the addition of 50 µCi [α-$^{32}$P]UTP (New England Nuclear). Synthesized transcripts were treated with DNase I and proteinase K, followed by a phenol extraction. To the supernatant, sodium acetate (pH 5.5) was added to a final concentration of 0.3 M and the mixture was ethanol precipitated at -70°C. Transcripts were washed with 70% EtOH, dried under vacuum and resuspended in 10 mM Tris-HCl, pH 7.5.

*Perchloric acid precipitation ribonuclease assay* - The perchloric acid precipitation assay used was a modification of the method used by Eichler and Eales (27). Reaction mixtures (20 µl) containing radiolabeled RNA (40 µg/ml) and protein (50 µM concentration) in a buffer containing 50 mM Tris HCl (pH 7.5), 50 mM NaCl, 0.5 mM MgCl$_2$ and RNasin at a final concentration of 0.5 U/µl were digested at 37°C for 15 min. The assays were initiated by addition of proteins and terminated by the addition of 15 µl 2.5 mg/ml yeast RNA, 7 µl uranyl acetate and 80 µl of 10% perchloric acid. Reactions were placed on ice for 20 min, followed by
centrifugation at maximum speed for 10 min. 100 µl aliquots of the supernatant were taken and the amount of non-precipitable nucleotides was determined by liquid scintillation counting. The control in this assay was a catfish T cell receptor β protein cloned into pQE30 vector that was processed in a manner identical to protein B23 and its mutants to account for endogenous ribonuclease activity carried through the purification process.

Molecular chaperone assay - The chaperone activity of protein B23 and its mutants was measured using bovine liver rhodanese (Sigma) as a substrate. The anti-aggregation effect was measured using a turbidity assay (28) essentially as previously described (19). Briefly, reaction mixtures contained ice-cold rhodanese solution at a concentration of 300 µM in 20 mM Tris-HCl (pH 7.4) with or without added protein B23 or mutants thereof. Aggregation was monitored by spectrophotometrically recording the absorbance at 360 nm at 1 min intervals after addition of the sample to the cuvette held at 65°C. The relative activity was calculated from the ratio of the absorbance of the reaction mixture containing the mutant protein with the absorbance of the sample containing protein B23.1 (100%) at the 15 min time point with a substrate to protein molar ratio of 1:0.5. Under the latter conditions there was a linear relationship between reduction in turbidity and protein concentration.

Size exclusion chromatography - Purified proteins (30 µl of a 100 µM concentration protein solution in 20 mM Sodium Phosphate buffer (pH 7) were loaded onto a Superose 200 column (Amersham Pharmacia Biotech) equilibrated with sodium phosphate buffer (pH 7). The column was run using a Varian HPLC system at a flow rate of 0.6 ml/min at room temperature and the elution was monitored at an absorbance of 280nm. Molecular weight standards were purchased from Sigma and the column was calibrated using the following protein markers; blue dextran (2,000 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa),
alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (9.6 kDa).

RESULTS

Protein B23.1 has several distinctive segments in its primary structure. These include a nonpolar N-terminal domain, two highly acidic regions in the central portion and a C-terminal region that is basic (Fig. 1A). To determine whether these segments correlate with functional domains of the protein, we generated a series of N- and C-terminal His- and GST-tagged deletion mutants (Fig. 1A). The purified mutant proteins were analyzed by SDS-PAGE. All of these fusion proteins were efficiently expressed at their predicted sizes, without any significant degradation (Figs. 1B and C).

Aromatic and basic segments within the C-terminal 76 residues are required for nucleic-acid binding - Previous studies showed that protein B23 binds both DNA and RNA and that the C-terminal end is essential for this activity (6). To determine which parts of the C-terminal sequence are required for nucleic acid binding, we initially assayed the series of His-tagged N-terminal deletion mutants. Nitrocellulose filter binding assays were performed with double-stranded DNA (plasmid pGEM) uniformly labeled with $^{32}$P. The N-terminal deletion mutants, ∆N35, ∆N185 and ∆N216 had DNA binding curves very similar to that of the full length B23 (Fig. 2A). Likewise, mutant proteins ∆N90, ∆N119 and ∆N139 also had normal DNA binding curves (data not shown). These results clearly indicated that the C-terminal 76 amino acids (∆N216) are sufficient for nucleic acid binding activity. The C-terminal deletion mutants ∆C35, ∆C132, ∆C161 and ∆C192 were also inactive in DNA binding (Fig. 2A), further supporting the importance of the C-terminal end for this activity.
To further dissect the nucleic-acid binding domain, more deletion constructs were tested. The GST (glutathione-S-transferase) fusion tag was used to facilitate expression of the small mutant proteins (GST-ΔN255, GST-ΔN240). The ΔN255 mutant contains the 37 amino acids that are unique to isoform B23.1 and essential for DNA binding activity as shown with ΔC35 (see above). However, ΔN255 did not bind DNA, indicating that this segment alone is not sufficient for activity (Fig. 2B). The mutant ΔN240, which lacks the basic amino acid cluster at the N-terminal end of the 76 residue segment (Fig. 1A) is also devoid of DNA binding activity. Thus, both ends of the 76 residue C-terminal domain are critical for nucleic acid binding.

The ribonuclease activity requires segments from the central and the C-terminal regions - To determine the location of the B23 endoribonuclease activity in the polypeptide chain, the 5' ETS region of pre-ribosomal RNA was used as a non-specific RNA substrate (13). The ribonuclease activity of the individual deletion mutants was assessed using the perchloric acid precipitation assay with 32P-labeled RNA. Preliminary studies were performed with varying protein and constant substrate concentrations; this provided information on the linear range of protein and substrate concentrations that could be used for initial rate assays. A control protein not known to have any ribonuclease activity, expressed and purified under conditions identical to those used for the B23 mutants, was used to assess the background ribonuclease level of bacterially produced protein.

Figure 3 shows that a high level of ribonuclease activity is maintained even after deletion of the first 139 amino acids from the amino terminal end of the protein. Interestingly, deletion of the amino terminal 139 amino acids (ΔN139) including the first acidic domain enhances the ribonuclease activity significantly. Conversely, deletion of the C-terminal 35 amino acids (ΔC35) decreases the ribonuclease activity of the protein almost 2 fold, whereas deletion of 132 amino
acids from the C-terminal end of the protein (ΔC132) reduces the activity by about 3-4 fold. Mutant proteins with substantial deletions in either the N- or C-terminal ends (ΔN185, ΔN216, ΔC161, ΔC192) show little or no activity. These results suggest that the central portion of protein B23 and the C-terminal end play crucial roles in maintaining the ribonuclease activity.

The molecular chaperone activity requires nonpolar and acidic segments in the N-terminal half of protein B23 - Protein B23 has been shown to have molecular chaperone activity toward substrates typically used in anti-aggregation assays (19). Light scattering studies performed with rhodanese at a concentration of 300 µM showed that when the temperature was raised from 4°C to 65°C, the protein aggregated and achieved maximum turbidity in 30 minutes (Fig. 4A). However, the aggregation was almost completely suppressed by adding B23 in a 1:1 molar ratio. The aggregation as measured by turbidity decreased in a linear manner as concentrations of added protein B23 were increased (data not shown). To determine the segments of the polypeptide chain that contribute to the molecular chaperone activity, aggregation assays were performed with the mutants using a substrate to protein molar ratio of 1:0.5 to account for both positive and negative effects.

As portions of the N-terminal region of B23 were deleted, there were moderate reductions in chaperone activity; i.e., the anti aggregation effect of ΔN35 and ΔN90 relative to the full-length protein was reduced to 84% and 66% respectively (Fig. 5B). However, deletion of an additional 30 amino acids (ΔN119) reduced the activity to approximately 10% of the control. The remaining N-terminal deletion mutant proteins (ΔN139, ΔN185, ΔN216) had no anti-aggregation activity (Fig. 5B). Upon analysis of the C-terminal mutant proteins, ΔC35 showed 100% activity indicating that the C-terminal 35 amino acids did not contribute to the chaperone activity. However, deletion of larger portions of the C-terminal end decreased the anti-
aggregation effect, with mutant proteins ΔC132, ΔC161 and ΔC192 having 80%, 57% and 30% of the control activity, respectively. Similar studies were performed using liver alcohol dehydrogenase and citrate synthase as substrates with results generally similar to those obtained with rhodanese (data not shown).

It has been suggested that molecular chaperones suppress aggregation by making appropriately-placed hydrophobic surfaces available to the denaturing protein substrates (29-31). The N-terminal region of protein B23 is relatively rich in nonpolar amino acids. The substantially decreased anti-aggregation effect seen after removal of the N-terminal region (ΔN119) suggests the chaperone activity is dependent on this nonpolar region. However, deletion of the acidic regions also results in loss of activity, indicating that the N-terminal region is not sufficient for maintaining full chaperone activity. In other words, both of these segments of the protein seem to be important for the chaperone activity.

*The molecular chaperone activity correlates with the oligomerization state of protein B23* - Several laboratories have previously observed that protein B23 is capable of oligomerization and probably exists as a hexamer or larger oligomer in the cell (20, 32, 33). Gel filtration chromatography was used to assess the oligomeric states of the mutant proteins to determine the possible relationship of this property with chaperone activity. Examples of gel filtration elution profiles using full length B23, ΔN139 and ΔN90 are shown in Fig. 5A. All of these proteins elute primarily as single peaks; protein B23 has an apparent molecular weight of 350 kDa, which approximates a decameric complex. Similarly, ΔN139 has an apparent molecular mass of 24 kDa compared to a theoretical monomer molecular weight of 20,130 Da, suggesting that this mutant protein mainly exists as a monomer. Conversely, protein ΔN90 forms a very large complex that elutes near the void volume. Although it was not possible to estimate the molecular weight of
this complex, it is clearly larger than 700 kDa. Other mutant proteins that aggregate into larger complexes are ΔN35, ΔC132 and ΔC161.

Table I provides estimates of molecular weights and oligomerization states of the constructs based on the data presented in Fig. 5B. The data indicate that the N-terminal deletion mutants ΔN35 and ΔN90 form very large oligomers, whereas ΔN119 elutes as a trimer and the remaining N-terminal mutants ΔN139, ΔN185 and ΔN216 elute primarily as monomers. The C-terminal mutants ΔC35, ΔC132, ΔC161 elute as oligomers, whereas ΔC192 is a mixture of large oligomeric complexes and monomers. These studies indicate that the N-terminal third of the molecule is essential for oligomerization.

Analysis of these data suggests a link between oligomerization state and chaperone activity. The effect is pronounced with the N-terminal mutants ΔN119, ΔN139, ΔN185 and ΔN216 that clearly exist as monomers and do not possess any chaperone activity. However, ΔN35 and ΔN90 are oligomers and show about 60-80% retention of chaperone activity. The C-terminal mutants exist mainly as oligomers and show varying degrees of activity. These studies suggest that the chaperone activity requires the ability to oligomerize for maximal activity. The N-terminal region seems to play a dual role in the chaperone activity and oligomerization of protein B23.

DISCUSSION

The current studies show that the molecular chaperone, ribonuclease and nucleic acid binding activities of protein B23 reside in nearly independent, but partially overlapping segments of the polypeptide chain (Fig. 6). Two adjacent segments in the polypeptide chain are important for the molecular chaperone activity. The first of these is the nonpolar region in the N-terminal
end; deletion of this region results in nearly complete abolition of chaperone activity, suggesting that the nonpolar residues play a crucial role in the chaperone activity. The second important segment is the acidic region in the center of the molecule. Removal of this part of the molecule also results in proteins with greatly reduced chaperone activities. Thus, both charge-charge and hydrophobic interactions seem to be essential for the chaperone activity of protein B23. The same combination of interactions is important for the activity of members of the small heat shock class of chaperones; e.g. αB-crystallin (29).

Molecular chaperones are divided into several groups on the basis of similarity of structural characteristics and/or similarity of functions. Although there is little or no sequence homology between B23 and the small heat shock proteins (sHsps), there are other interesting similarities (22). The monomeric sizes of these proteins are 15-40 kDa, but they exist as large oligomeric complexes of up to 50 subunits within cells, with molecular weights ranging from 280 kDa to 2 MDa. Their secondary structures are predominantly β-sheet (40-50%) with some α-helix (10-20%). These proteins have sequence homology with each other in the C-terminal halves (including nonpolar residues), and also contain conserved, flexible and solvent-exposed C-terminal extensions. Protein B23 shares several features in common with the sHsps/αB-crystallins including a) the dependence on both nonpolar and charged regions for activity, b) secondary structures composed mostly of β-sheets and β-turns (34) and c) a tendency to oligomerize (20).

There is mounting evidence for a relationship between oligomerization state and chaperone activity (35, 36). Although protein B23 is known to oligomerize (20, 32, 33), this is the first study to correlate its oligomerization with molecular chaperone activity. Clearly, no chaperone activity was retained in mutant proteins that were monomeric; however it cannot be
ruled out that these mutants simply lacked a substrate binding site since this seems to reside in
the N-terminal third of the molecule.

The mutagenesis studies provided clues about the general sequence requirements within
the 76 residue nucleic acid binding segment. Deletion of either end of this segment results in
complete loss of nucleic acid binding activity. The C-terminal 37 residue segment, which is
required but is not sufficient for activity, is relatively rich in aromatic amino acids. In the C-
terminal end of this sequence there are five aromatic residues: FINYVKNCFRMTDQEAIQDLWQWRKSL.
The placement of the aromatic residues, especially
the two tryptophans is highly conserved in analogous proteins including starfish nucleolar
protein ANO39 (37), sea urchin mitosis apparatus protein p62 (38) Xenopus NO38 (4) and B23
from chickens (39) and humans (3). The requirement for the two tryptophans is reinforced by
experiments in which they were replaced by leucines; the resulting mutant protein did not bind
DNA. The spacing of the basic residues in the N-terminal end of the 76 residue segment is also
highly conserved; this portion is also necessary but not sufficient for activity. Thus, the aromatic
and basic side chains at the two ends of the nucleic acid binding domain seem to act in
combination to serve this function. The N-terminal end of this segment also contains the two
putative cdc2 phosphorylation sites (40), which could regulate nucleic acid binding during
various stages of the cell cycle.

Finally, analysis of the two isoforms of protein B23 for ribonuclease activity reveals that
although these mutants differ only in their C-terminal end, the shorter form shows a significant
decrease in activity, suggesting that the C-terminal 35 amino acids are important for substrate
binding. This is not surprising since this region is essential for nucleic acid binding activity.

2 A. Baumann and M. O. J. Olson, unpublished observations.
Since the shorter isoform possesses a relatively high level of activity, it follows that the catalytic site is in another part of the molecule. Interestingly, deletion of the first acidic domain causes a substantial increase in ribonuclease activity; this effect may be due to exposure of the region between the two acidic segments. It is possible that this increase in activity upon deletion of the acidic domain is due to a decrease in electrostatic repulsion, making RNA-protein interactions more favorable. Alternatively, the shift to the monomeric state resulting in decreased steric hindrance and more rapid diffusion could cause this enhancement. Since deletion of the short region between the acidic segments results in the complete loss of ribonuclease activity, it seems likely that it contains the catalytic site. Curiously, deletion of the N-terminal end of the molecule, which contains all of the histidine residues, does not abolish activity. Although histidine residues are part of the catalytic sites in many ribonucleases (41) this is clearly not the case in the B23 ribonuclease. Determining the catalytic mechanism of the B23 ribonuclease may be facilitated by a crystallographic structure of the protein along with its bound substrate.

What is the advantage to the organism of having a protein with multiple activities that are seemingly unrelated in the same polypeptide chain? The C-terminal nucleic acid binding domain of B23 seems to be involved with recognition at various levels. First, this region has been shown to be essential for nucleolar localization in the Xenopus version of the protein (42) and in a similar protein from sea urchin (43). Because of its association with pre-ribosomal particles in the nucleolus, the most likely mode of recognition is through RNA binding. Since other parts of the B23 molecule are also important for nucleolar targeting (33), this may be a cooperative process involving interactions with other proteins. The recognition process could target proteins bound to protein B23 to specific sites during the ribosome assembly process. The C-terminal domain could also be important in substrate recognition for the protein's ribonuclease activity.
Preferential cleavage of a narrow region of the pre-rRNA transcript by the B23 ribonuclease (13) could possibly utilize the C-terminal end of B23 for recognition.

The catalytic region of the ribonuclease seems to reside in the center of the molecule and overlaps with the chaperone-containing segment. It is conceivable that the binding of other proteins; e.g., ribosomal proteins, to the chaperone region of B23 could alter the catalytic site and regulate the ribonuclease activity to provide temporal regulation of steps in ribosome biogenesis. Thus, seemingly independent activities could be related to each other through targeting and regulatory processes. Confirmation of this hypothesis will require development of more satisfactory systems for studying ribosome biogenesis.

Acknowledgements

We gratefully acknowledge Siddhartha De and Drs. Aurita Antao and Donald Sittman for their helpful discussions regarding the project. We would also like to thank Mike Wallace for technical assistance.

REFERENCES

1. Hadjiolov, A. A. (1985) The nucleolus and ribosome biogenesis. Springer-Verlag, New York, NY

2. Maxwell, E. S., and Fournier, M. J. (1995) Annu. Rev. Biochem. 64, 897-934

3. Chan, W. Y., Liu, Q. R., Borjigin, J., Busch, H., Rennert, O, M., Tease L. A., and Chan, P. K. (1989) Biochemistry 28, 1033-1039
4. Schmidt-Zachmann, M. S., Hügle-Dörr, B., and Franke, W. W. (1987) *EMBO J.* 6, 1881-1890

5. Feuerstein, N., and Mond, J. J. (1987) *J. Immunol.* 139, 1818-1822

6. Wang, D., Baumann, A., Szebeni, A., and Olson, M. O. J. (1994) *J. Biol. Chem.* 269, 30994-30998

7. Dumbar, T. S., Gentry, G. A., and Olson, M. O. J. (1989) *Biochemistry* 28, 9495-9501

8. Prestayko, A. W., Klomp, G. R., Schmoll, D. J., and Busch, H. (1974) *Biochemistry* 13, 1945-1951

9. Olson, M. O. J., Wallace, M. O., Herrera, A., Carlson-Marshall, L., and Hunt, R. C. (1986). *Biochemistry* 25, 484-495

10. Yung, B. Y. M., Busch, H., and Chan, P. K. (1985) *Biochim. Biophys. Acta* 826, 167-173

11. Yung, B. Y. M., Busch, R. K., Busch, H., Mauger, A. B., and Chan, P. K. (1985) *Biochim. Pharmacol.* 34, 4059-4063

12. Herrera, J. E., Savkur, R., and Olson, M. O. J. (1995) *Nucleic. Acids Res.* 23, 3974-3979

13. Savkur, R. S., and Olson, M. O. J. (1998) *Nucleic Acids Res.* 26, 4508-4515

14. Li, Y. P., Busch, R. K., Valdez, B. C., and Busch, H. (1996) *Eur. J. Biochem.* 237, 153-158

15. Valdez, B. C., Perlaky, L., Henning, D., Saijo, Y., Chan, P. K., and Busch, H. (1994) *J. Biol. Chem.* 269, 23776-23783

16. Fankhauser, C., Izaurralde, E., Adachi, Y., Wingfield, P., and Laemmli, U. K. (1991) *Mol. Cell. Biol.* 11, 2567-2575

17. Borer, R. A., Lehner, C. F., Eppenberger, H. M., and Nigg, E. A. (1989) *Cell* 56, 379-390

18. Szebeni, A., Herrera, J. E., and Olson, M. O. J. (1995) *Biochemistry* 34, 8037-8042

19. Szebeni, A., and Olson, M. O. J. (1999) *Protein Sci.* 8, 905-912
20. Herrera, J. E., Correia, J. J., Jones, A. E., and Olson, M. O. J. (1996) *Biochemistry* **35**, 2668-2673

21. Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., and Sigler, P. B. (1994) *Nature* **371**, 578-586

22. Ehrnsperger, M., and Buchner, J. (1998) Molecular chaperones in the life cycle of proteins, Marcel Dekker, Inc., New York, NY

23. Bradford, M. M. (1976) *Anal Biochem.* **72**, 248-254

24. Smith, D. B., and Johnson, K. S. (1988) *Gene* **67**, 31-40

25. Riggs, A. D., Suzuki, H., and Bourgeois, S. (1970) *J. Mol. Biol.* **48**, 67-83

26. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984) *Nucleic Acids Res.* **12**, 7035-7056

27. Eichler, D. C., and Eales, S. J. (1982) *J. Biol. Chem.* **257**, 14384-14389

28. Buchner, J., Grallert, H., and Jakob, U. (1998) *Meth. Enzymol.* **290**, 323-338

29. Plater, M. L., Goode, D., and Crabbe, M. J. C. (1996) *J. Biol. Chem.* **271**, 28558-28566

30. Guha, S., Manna, T. K., Das, K. P., and Bhattacharya, B. (1998) *J. Biol. Chem.* **273**, 30077-30080

31. Gibbons, D. L., and Horowitz, P. M. (1995) *J. Biol. Chem.* **270**, 7335-7340

32. Yung, B. Y. M., and Chan, P. K. (1987) *Biochim. Biophys. Acta* **925**, 74-82

33. Zirwes, R. F., Kouzmenko, A. P., Peters, J. M., Franke, W., and Schmidt-Zachmann M. S. (1997) *Mol. Biol. Cell.* **8**, 231-248

34. Umekawa, H., Chang, J. H., Correia, J. J., Wang, D., Wingfield, P. T., and Olson, M. O. J. (1993) *Cell. Mol. Biol. Res.* **39**, 635-645
35. Yonehara, M., Minami, Y., Kawata, Y., Nagai, J., and Yahara, I. (1996) *J. Biol. Chem.* **271**, 2641-2645

36. Leroux, M. R., Melki, R., Gordon, B., Batelier, G., and Candido, P. M. (1997) *J. Biol. Chem.* **272**, 24646-24656

37. Nakajima, H., Matoba, K., Matsumoto, Y., Hongo, T., Kiritaka, K., Sugino, H., Nagamatsu, Y., Hamaguchi, Y., and Ikegami, S. (2000) *Eur. J. Biochem.* **267**, 295-304

38. Ye, X., and Sloboda, R. D. (1997) *J. Biol. Chem.* **272**, 3606-3614

39. Maridor, G., and Nigg, E. A. (1990) *Nucleic Acids Res.* **18**, 1286

40. Peter, M., Nakagawa, J., Doree, M., Labbe, J. C., and Nigg, E. A. (1990) *Cell* **60**, 791-801

41. Gerlt, J. A. (1993) Nucleases, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

42. Peculis, B. A., and Gall, J. G. (1992) *J. Cell. Biol.* **116**, 1-14

43. Warner, A. K., and Sloboda, R. D. (1999) *Cell Mot. and Cytoskeleton* **44**, 68-80

**FIGURE LEGENDS**

**FIG. 1.** **Protein B23 deletion mutants.** A, Schematic representation of protein B23.1 and N- and C-terminal deletion mutants. The characteristic segments are indicated as follows in the diagram: nonpolar region, dark gray; acidic regions, black; bipartite NLS, diagonal dark stripes; moderately basic regions, light gray; basic cluster, hatched box; aromatic rich region and segment unique to the B23.1 isoform, thin striped box. B, Electrophoretic analyses of His-tagged mutant proteins. Purified His-tagged proteins were run on 15% SDS-PAGE. Molecular weight markers and the different mutant forms of protein B23 were loaded on the lanes indicated. C,
Electrophoretic analyses of GST-tagged mutant proteins. Purified GST-tagged proteins of B23 and molecular weight markers were loaded on the lanes indicated on a 15% SDS-PAGE.

FIG. 2. Nitrocellulose filter binding assay for binding of double stranded DNA to protein B23 and its deletion mutants. 32P-labeled double stranded DNA (15 µM nucleotide concentration) was incubated with increasing protein concentrations (0-60 µM) in TBE binding buffer at 37°C for 20 min. Reactions were terminated by filtration through pre-wetted nitrocellulose filters. A, filter binding curves of proteins ■, B23.1; ○, ∆N35; ▲, ∆N185; ▼, ∆N216; □, ∆C35; ●, ∆C132; △, ∆C161; ▼, ∆C192. (B) filter binding curves of proteins ■, B23.1; ●, GST; ▲, GST-∆N255; ▼, GST-∆N240.

FIG. 3. Detection of ribonuclease activity of protein B23 and its deletion mutants using perchloric precipitation assay. Reaction mixtures containing radiolabeled RNA (40 mg/ml) and proteins (50 µM) were incubated at 37°C. Reactions were terminated after 15 min. by addition of yeast tRNA, uranyl acetate and perchloric acid (PCA). The reactions were placed on ice for 20 min. and centrifuged at 13,000 r.p.m for 10 min. Aliquots of the supernatants were withdrawn and the amount of non precipitable nucleotides was determined by liquid scintillation counting. Standard deviations are represented as error bars.

FIG. 4. Effect of B23 N- and C-terminal deletion mutant proteins on the thermal denaturation of rhodanese. Rhodanese (300 µM) was subjected to thermal denaturation at 65°C for 30 min in the presence and absence of the wild type or mutant B23.1 proteins. The aggregation of rhodanese was monitored by the absorbance of the sample at 360 nm in a spectrophotometer. A, Absorbance in the presence (○) and absence (●) of equimolar amounts of protein B23.1. B, Effect of N- and C-terminal deletion mutant proteins on the thermal denaturation of rhodanese. The substrate to protein ratio was maintained at 1:0.5 and the
absorbance profile of each mutant was obtained. The absorbance of the sample in the presence of the individual mutant proteins after 15 min. of thermal denaturation was plotted relative to the absorbance in the presence of protein B23.1 (100%). Standard deviations are shown as error bars.

FIG. 5. Size exclusion chromatography of protein B23 and its deletion. A, Elution profiles for proteins ΔN90, B23.1 and ΔN139 on a Superose 200 column. Elution volumes of selected standards, blue dextran (Vo), thyroglobulin (669 kDa) and carbonic anhydrase (29 kDa) are indicated by arrows. B, Elution profiles of deletion mutants (o) relative to a series of molecular weight protein standards (■), thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (9.6 kDa) are shown.

FIG. 6. Summary of activities of protein B23 N- and C-terminal deletion mutants. The characteristic segments of the proteins are indicated as described in Figure 1A. The chaperone and ribonuclease activities are relative to those of B23.1 (100%). For DNA binding, + represents binding activity comparable to B23.1, and – represents minimal to no binding affinity.
Table I

Molecular mass estimation of N- and C-terminal deletion mutants by analytical gel filtration chromatography. The molecular size of the mutants was estimated from a plot of Ve/Vo versus molecular weight constructed from standard proteins of known molecular weight (Fig. 5).

| Protein | Ve/Vo | Estimated molecular weight | Monomer molecular weight | Approximate number of subunits |
|---------|-------|-----------------------------|--------------------------|-----------------------------|
| B23.1   | 1.9285| 354,656                     | 35,420                   | 10                          |
| ΔN35    | 1.6429| > 700,000<sup>a</sup>       | 32,670                   | >21                         |
| ΔN90    | 1.1071| > 700,000<sup>a</sup>       | 25,520                   | >27                         |
| ΔN119   | 2.25  | 63,716                      | 22,330                   | 2-3                         |
| ΔN139   | 2.4286| 24,551                      | 20,130                   | 1                           |
| ΔN185   | 2.5   | 16,768                      | 15,070                   | 1                           |
| ΔN216   | 2.5714| 11,751                      | 11,660                   | 1                           |
| ΔC35    | 1.9285| 354,656                     | 32,670                   | 11                          |
| ΔC132   | 1.7857| > 700,000<sup>a</sup>       | 20,900                   | >34                         |
| ΔC161   | 1.8214| 628,309                     | 17,710                   | 35                          |
| ΔC192   | 1.0357| > 700,000<sup>a</sup>       | 14,300                   | >49                         |

<sup>a</sup> Because these samples eluted out of the linear range of the standards, no effort was made to determine their molecular weights.
FIG. 1.

A

B23.1

ΔN35

ΔN90

ΔN119

ΔN139

ΔN185

ΔN216

ΔC35

ΔC132

ΔC161

ΔC192

ΔN240

ΔN255

B

kDa

212

122

83

52

35

28

20

M

B23.1

ΔN35

ΔN90

ΔN119

ΔN139

ΔN185

ΔN216

ΔC35

ΔC132

ΔC161

ΔC192

ΔN240

ΔN255

C

kDa

212

122

83

52

35

28

20

7.2

M

GST

GST

GST

GST

ΔN240

ΔN255

M
FIG. 3.
FIG. 4.

A

B

% Inhibition Relative to B23.1

Protein

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
FIG. 5.

A

![Graph showing time (min) vs. absorbance (A at 280 nm). Peaks labeled Vo, 669, and 29, with annotations ∆N90 and B23.1.](http://www.jbc.org"

B

![Graph showing log (MW) vs. Ve/Vo. Points labeled 669, 443, 200, 150, 66, 29, with annotations ∆N90, ∆N139, ∆N119, ∆N185, ∆C35, ∆N185, and ∆C192.](http://www.jbc.org)
FIG. 6.

| Mutant   | Chaperone Activity | Oligomerization | Nucleic Acid Binding Activity | RNase Activity |
|----------|--------------------|-----------------|------------------------------|----------------|
| B23.1    | 100%               | oligomer        | +                            | 100%           |
| ΔN35     | 84%                | oligomer        | +                            | 102%           |
| ΔN90     | 66%                | oligomer        | +                            | 94%            |
| ΔN119    | 10%                | trimer          | +                            | 75%            |
| ΔN139    | 0%                 | monomer         | +                            | 150%           |
| ΔN185    | 0%                 | monomer         | +                            | 0%             |
| ΔN216    | 0%                 | monomer         | +                            | 0%             |
| ΔC35     | 97%                | oligomer        | -                            | 63%            |
| ΔC132    | 80%                | oligomer        | -                            | 29%            |
| ΔC161    | 57%                | oligomer        | -                            | trace          |
| ΔC192    | 30%                | oligomer/monomer| -                            | 0%             |
| ΔN240    |                    | GST             |                              |                |
| ΔN255    |                    | GST             |                              |                |
Mapping the functional domains of nucleolar protein B23
Kamini Hingorani, Attila Szébeni and Mark O J Olson

J. Biol. Chem. published online May 26, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M003278200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts