Evidence for the ability of nucleophosmin/B23 to bind ATP

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By taking advantage of its ability to be retained by ATP-agarose, we have demonstrated that nucleophosmin/B23 is capable of binding ATP. The specificity of the binding was confirmed by the absence of significant binding to AMP-agarose and by its loss when nucleophosmin/B23 in nuclear extracts was preincubated with ATP. Preincubation of the nuclear extracts with other ribonucleotide triphosphates (GTP, CTP, UTP) did not compete for the binding of nucleophosmin/B23 to ATP-agarose. The purified recombinant nucleophosmin/B23 was also able to be retained by ATP-agarose. The K_d for binding of ATP to the purified recombinant nucleophosmin/B23, on the basis of retention on a nitrocellulose membrane, was $86.5 \pm 8.3 \mu\text{M}$; the

number of binding sites was 0.68 per nucleophosmin/B23 protein molecule. To determine the possible ATP-binding site of nucleophosmin/B23, various deletion clones including the two mutants in which the putative ATP-binding sequence had been deleted were constructed. Deletion of the portions of the molecule (residues 83–152 and 185–240) had little effect on the ATP binding. The C-terminal deleted mutant (residue 242 to the C-terminus deleted) lost most of its ability to be retained by ATP-agarose and to bind [α -³²P]ATP on a nitrocellulose membrane. The results indicate that the C-terminal portion (residues 242–294) contains the essential ATP-binding site of nucleophosmin/B23.

INTRODUCTION

Nucleophosmin/B23, also called protein B23, NO38 or numatrin [1–3], is a major nucleolar phosphoprotein that is significantly more abundant in tumour and proliferating cells than in normal resting cells [3,4]. Nucleophosmin/B23 is localized in granular regions of the nucleolus [5], associated with preribosomal particles [1,6], and forms hexamers [7] that might be essential for the assembly of ribosomes. A C-terminal domain of nucleophosmin/B23 has been shown to be essential for nucleolar localization, but not sufficient to target hybrid proteins to the nucleolus [8]. Subnuclear localization of nucleophosmin/B23 may therefore depend more on specific protein–protein and protein–nucleic acid interactions than on general linear targeting signals. Nucleophosmin/B23 has been ascribed various properties, including a potential role in proliferation owing to its rapid increase in synthesis during mitogenic stimulation [9]; a cytoplasmic/nuclear shuttle protein [10]; DNA-binding activity [11]; relief of transcription repression by YY1 [12]; binding of nuclear and nucleolar localization signals on the HIV Type 1 Rev protein [13] and the human T-cell leukaemia virus-1-Rex protein [14]; binding of cell cycle-regulated nucleolar protein p120 [15]; a substrate of protein kinase C [16], mitotic cdc2 kinase [17] and casein kinase II [18]; and ability to be ADP-ribosylated [19]. In addition, the N-terminal region has been shown to be a part of a fusion protein with a tyrosine kinase, a result of a chromosomal rearrangement, in some non-Hodgkin's lymphomas [20]. Studies of nucleophosmin/B23 suggest that nucleophosmin/B23 has a role in the increased nucleolar activity that is necessary for cell proliferation [3,9]. Of further interest is the ability of nucleophosmin/B23 to undergo translocation from nucleoli to nucleoplasm during the stationary phase of growth [21], which can also be induced by agents that inhibit cell growth [1,22–24]. Such drug-induced nucleophosmin/B23 translocation is blocked if ATP content is decreased by antimycin A in a glucose-free medium. Our assay *in vitro* of nucleophosmin/B23 translocation in isolated permeabilized cells instead of whole cultured cells

indicates that ATP is able to promote nucleophosmin/B23 translocation from nucleoli to nucleoplasm [25]. ATP might have an important role in regulating the nucleolar function of nucleophosmin/B23 for cell growth. This therefore raises the question of whether ATP could have a direct effect or might interact with nucleophosmin/B23. Examination of the amino acid sequence of nucleophosmin/B23 reveals a putative ATP-binding sequence [26–29]. Here we report evidence that nucleophosmin/B23 is indeed able to bind ATP. On the basis of the binding of [α -³²P]ATP to purified recombinant nucleophosmin/B23 on a nitrocellulose membrane, we determined the binding affinity and stoichiometry. The C-terminal portion of nucleophosmin/B23 is implicated as containing the ATP-binding site. The finding of ATP binding by nucleophosmin/B23 raises some possibilities for elucidating further the role of ATP in the regulation of the nucleolar activity of nucleophosmin/B23 in cell proliferation.

MATERIALS AND METHODS

Drugs and antibodies

Actinomycin D, antimycin A and ribonucleotide triphosphates (ATP, GTP, CTP and UTP) were purchased from Sigma. The monoclonal antibody against nucleophosmin/B23 (37/5.1) was kindly provided by Dr. P. K. Chan (Department of Pharmacology, Baylor College of Medicine, Houston, TX, U.S.A.). Characterization of anti-nucleophosmin/B23 MAb has been reported previously [5]. Polyclonal anti-nucleophosmin/B23 specific antibodies were purified from anti-nucleophosmin/B23 rabbit serum by an affinity column packed with *Escherichia coli* overexpressed nucleophosmin/B23–Sepharose 4B (Pharmacia).

Cell culture studies

HeLa cells were grown as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated

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fetal bovine serum, and antibiotics in a humidified incubator under air/CO₂ (19:1) at 37 °C.

Isolation of HeLa cell nuclei

Nuclei were isolated from HeLa cells with the Nonidet method as described by Yung and Chan [7]. Cells were suspended in 20 vol. of RSB buffer [0.01 M Tris/HCl/0.02 M NaCl/1.5 mM MgCl₂ (pH 7.2)] in ice for 30 min and then centrifuged at 630 *g* for 8 min. Swollen cells were resuspended in 20 vol. of RSB buffer containing 0.5 % Nonidet P40. The cells were homogenized with a Dounce homogenizer (10 strokes up and down), and the crude nuclei were collected by centrifugation at 630 *g* for 8 min. The nuclei were resuspended in 10 vol. of 0.25 M sucrose/10 mM MgCl₂, and underlayered with an equal volume of 0.88 M sucrose/0.05 mM MgCl₂. The nuclei were then collected by centrifugation at 1700 *g* for 10 min.

ATP-agarose affinity chromatography

The isolated HeLa nuclei were extracted overnight (stirred at 4 °C) with 10 vol. of imidazole buffer [20 mM imidazole/50 mM KCl/5 mM magnesium acetate/0.5 mM EDTA/1 mM PMSF (pH 7.8)] and centrifuged at 1700 *g* for 20 min. The supernatant (nuclear extract) containing the partly purified nucleophosmin/B23 was loaded at 4 °C on a 0.1 ml ATP-agarose or AMP-agarose (Sigma) column previously equilibrated with imidazole buffer. After 2 h each column was washed consecutively with 2 vol. of the following buffers: imidazole buffer, imidazole buffer containing 1 M KCl, imidazole buffer, and then imidazole buffer containing 10 mM ATP or 10 mM AMP. After these washes, the agarose in the column was resuspended in imidazole buffer up to a total volume of 0.2 ml. A 100 µl sample was withdrawn and processed for SDS/PAGE.

SDS/PAGE

SDS/PAGE was performed by the protocol of Laemmli [30].

Western Blotting

Proteins separated by SDS/PAGE were electrotransferred to Hybond-PVDF membrane (Amersham). The PVDF membrane was then soaked in a blocking solution {5 % (w/v) non-fat milk in TBST [20 mM Tris/HCl (pH 7.5)/0.5 M NaCl/0.1 % (v/v) Tween-20]} for 1 h at room temperature. The soaked PVDF membrane was then incubated with monoclonal antibody against nucleophosmin/B23 [diluted 1:5000 in 3 % (w/v) non-fat milk in TBST] or with polyclonal antibody (diluted 1:500) overnight at 4 °C, washed with TBST buffer three times for 15 min each and incubated at room temperature for 2 h with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (diluted 1:5000 in TBST buffer). The membrane was washed three times with TBST for 15 min each. Immunobands were identified by chromogenic development and/or the enhanced chemiluminescence reaction (ECL; Amersham).

Expression of nucleophosmin/B23–glutathione S-transferase fusion protein

The nucleophosmin/B23 cDNA species for full-length (T7) and the deletion mutants (RN and AA) [31] were generously provided by Dr. P. K. Chan (Baylor College of Medicine, Houston, TX, U.S.A.). The T7, RN and AA cDNA clones were amplified by PCR with specifically designed oligonucleotide primers, b23-5'p (5'-BamHI-GCCGGATCCCGATGGAAGATTCTGA-

TGGACA-3') and b23-3'p (5'-EcoRI-GCTGAATTCTTAAAGAGACTTCCTCCACTG-3'). The CD (C-terminal deletion) cDNA clone was amplified by PCR with specifically designed oligonucleotide primers, b23-5'p (5'-BamHI-GCCGGATCCCGATGGAAGATTCTGAAGACA-3') and b23-3'p (5'-EcoRI-GTCTTCTACAGAATTCTTAAGTAGGTCCTTTTGGTG-3').

The appropriate restriction enzyme sites were selected (see Figure 5A) to delete various portions of nucleophosmin/B23 without shifting the open reading frame. The schematic description of deletion clones is shown in Figure 5(B). Clone RN (a deletion between *RsaI* and *NlaIV* sites) removes 68 residues (83–152); clone AA (a deletion between the second *AhaI* and *AvaII* sites) removes 54 residues (185–240); clone CD (a deletion removes 52 residues from the C-terminal end (242 to the C-terminus). The amplified DNA fragments were digested with *BamHI* and *EcoRI*, cloned into the expression vector pGEX-3X (Pharmacia) and overexpressed in *E. coli* strain BL21(DE3). The orientation and the in-frame deletion mutation sites of all these clones were confirmed by DNA sequencing and immunodetection with nucleophosmin/B23 monoclonal antibody or polyclonal antibody.

The glutathione S-transferase–nucleophosmin/B23 recombinant proteins induced by isopropyl β-D-thiogalactoside (0.1 mM) for 2 h at 37 °C were purified by glutathione–Sephacrose 4B affinity chromatography (Pharmacia Biotech). The bound fusion proteins were removed from the carrier protein in the column by cleavage with 0.005 % biotin-labelled factor Xa in cleavage buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 mM CaCl₂]. The biotin-labelled factor Xa was separated from the recombinant nucleophosmin/B23 by washing with streptavidin gel and centrifugation.

ATP binding assay

The standard reaction mixture contained 100 µl of imidazole buffer, [α -³²P]ATP (908 c.p.m./pmol) and purified nucleophosmin/B23 (10.8 pmol) or peptides. After incubation at 0 °C for 2 h, the solution was filtered through a nitrocellulose membrane (Millipore HA, 0.45 µm pores, 24 mm diameter) soaked in imidazole buffer. The filter was washed three times with 10 ml of ice-cold imidazole buffer each time and dried under an infrared lamp; radioactivity retained on the filter was measured in a liquid-scintillation counter. The reaction without nucleophosmin/B23 provided a background value of 0.05 pmol. The *K_d* of ATP–nucleophosmin/B23 and the number of ATP-binding sites were calculated by Scatchard plot [32].

RESULTS

Nucleophosmin/B23 has ability to bind ATP

The ATP-binding activity of nucleophosmin/B23 has been tested, taking advantage of its ability to be retained by ATP-agarose. To prevent nucleophosmin/B23 from binding to the endogenous ATP, HeLa cells were treated with antimycin A in glucose-free medium for 1 h before the cellular extractions. This treatment resulted in a more than 90 % decrease in cellular ATP [25]. The results in Figure 1(A) indicate that nucleophosmin/B23 from nuclear extract was tightly retained by ATP-agarose. The specificity of the binding was confirmed by the absence of significant binding to AMP-agarose (Figure 1B) and by its loss when the nuclear extracts were preincubated with ATP (Figure 2A). Preincubation of the extracts with other ribonucleotide triphosphates (GTP, CTP, UTP) did not compete for the binding of nucleophosmin/B23 to ATP-agarose (Figure 2).

Figure 1 Ability of nucleophosmin/B23 to bind ATP

(A) Elution pattern of nucleophosmin/B23 in the HeLa nuclear extract run through an ATP-agarose column. Samples from each wash and from the columns were subjected to SDS/PAGE [10% (w/v) gel] and immunoblotted with nucleophosmin/B23 monoclonal antibody (see the Materials and methods section). Each lane contained materials corresponding to a similar number of cells. Lane T, total nuclear extract containing partly purified nucleophosmin/B23; lane F, flow through the ATP-agarose column; lane W1, first wash with imidazole buffer; lane KCl, wash with imidazole buffer containing 1 M KCl; lane W2, second wash with imidazole buffer; lane ATP, wash with imidazole buffer containing 10 mM ATP; lane W3, third wash with imidazole buffer; lane C, material retained by the ATP-agarose column after all the treatments. (B) As (A) but the sample was applied to the AMP-agarose column and the wash was with imidazole buffer containing 10 mM AMP. The line labelled 36.5 kDa indicates the position of lactate dehydrogenase (Mark12[®], Novex) on SDS/PAGE.

Quantification of ATP binding of nucleophosmin/B23 in terms of affinity and stoichiometry

The nucleophosmin/B23 cDNA was amplified by PCR with designed oligonucleotide primers. The purified recombinant nucleophosmin/B23 protein was shown to be retained by ATP-agarose (Figure 3). On the basis of the binding of [α -³²P]ATP to purified recombinant nucleophosmin/B23 on a nitrocellulose membrane, a K_d of $86.5 \pm 8.3 \mu\text{M}$ (mean $\pm$ S.D.) was determined (Figures 4A and 4B). The number of ATP-binding sites per nucleophosmin/B23 protein molecule was calculated to be 0.68. From the shape of the binding curve (Figure 4A), one can speculate that there might be another ATP-binding site on nucleophosmin/B23, probably of higher affinity. However, no significant ATP binding to nucleophosmin/B23 was observed when the binding experiment was performed with low levels (5–150 μM) of ATP (Figure 4C); neither did the result show that the radioactive counts increased linearly with ATP concentration from 0 to 150 μM . Because nucleophosmin/B23 binds to ATP, it would be useful to know whether nucleophosmin/B23 contains ATPase activity. By counting the radioactivity in the P_i spots on a TLC plate, it was found that the protein nucleophosmin/B23 contained negligible ATPase activity (less than 0.1 mol of ATP hydrolysed/min per mol of nucleophosmin/B23 protein) (results not shown). To further elucidate the specificity of ATP binding to nucleophosmin/B23, we performed a study of the nucleotide specificity for binding to nucleophosmin/B23 by a filter-binding assay. By using competition with ATP as a measure, ADP and dATP were also effective in binding nucleophosmin/B23 (Table 1). AMP was partly competitive. GTP, CTP, UTP, dGTP, dCTP and TTP all failed to compete with ATP.

Deletion of the putative ATP-binding site at the C-terminus affects the binding of ATP to nucleophosmin/B23

Attempts were made to determine the ATP-binding site(s) of nucleophosmin/B23. Examination of the amino acid sequence of nucleophosmin/B23 reveals a sequence in the middle portion (residues 110–120) of the molecule that bore a resemblance to the

Figure 2 ATP competes for binding of nucleophosmin/B23 to ATP-agarose

Details are as described in the legend to Figure 1 but the sample was preincubated with 20 mM ATP, CTP, UTP or GTP before application to the ATP-agarose column. The line labelled 36.5 kDa indicates the position of lactate dehydrogenase (Mark12[®], Novex) on SDS/PAGE.

ATP-binding consensus sequence (Table 2) [26–29]. With one amino acid mismatch (lysine instead of a hydrophobic residue), the other possible putative ATP-binding sequence is found in the C-terminal (residues 248–265) portion of nucleophosmin/B23. It can be noted that there is also one similar amino acid mismatch (aspartate instead of a hydrophobic residue) at the same position of the presumed ATP-binding sequence of Erp99 protein [29]. The mutants with the putative ATP-binding sites deleted, RN (residues 83–152 deleted) and CD (residues 242 to the C-terminus deleted) as well as a deletion mutant AA (residues 185–240 deleted) were prepared (Figure 5) and tested for their ability to be retained by ATP-agarose. The results (Figure 3) showed that, whereas there was significant binding of the mutants RN and AA to ATP-agarose, a much smaller amount of the nucleophosmin/B23 mutant CD, with the C-terminal portion deleted, was retained by ATP-agarose. Similarly to the wild-type T7 and mutant RN, the ability to be retained by ATP-agarose was largely observed in clone AA (residues 185–240 deleted). The

Figure 3 Ability of nucleophosmin/B23 and its deletion mutants to bind ATP

Elution pattern of the purified recombinant full-length (T7) or deletion mutant RN, AA or CD nucleophosmin/B23 run through an ATP-agarose column. Samples from the flow-through and from the columns were subjected to SDS/PAGE [10% (w/v) gel] and immunoblotted with nucleophosmin/B23-specific polyclonal antibody (see the Materials and methods section). Similar amounts of purified nucleophosmin/B23s were loaded on an ATP-agarose column. Lane F, flow through the ATP-agarose column; lane C, material retained by the ATP-agarose column after all the washes and treatments as in Figure 1. The line labelled 36.5 kDa indicates the position of lactate dehydrogenase (Mark12[®], Novex) on SDS/PAGE.

Figure 4 Quantification of ATP binding by nucleophosmin/B23 and its deletion mutants in terms of affinity and stoichiometry

(A) Purified recombinant nucleophosmin/B23 (10.8 pmol) was incubated with 5–1000 μ M [α - 32 P]ATP (10^3 c.p.m./pmol) at 0 °C for 2 h. The amounts of ATP–nucleophosmin/B23 protein complex were measured by filtration through Millipore membranes. Results are means $\pm$ S.D. for triplicate samples, and are representative of the results obtained in at least three independent experiments. (B) The K_d of ATP–nucleophosmin/B23 protein and the number of ATP-binding sites were calculated by a double-reciprocal plot. Points (seven of eight points) fall within the 99% confidence interval. (C) Purified recombinant nucleophosmin/B23 (10.8 pmol) was incubated with 5–150 μ M [α - 32 P]ATP (10^3 c.p.m./pmol) at 0 °C for 2 h. The amounts of ATP–nucleophosmin/B23 protein complex were measured by filtration through Millipore membranes. Results are means $\pm$ S.D. for triplicate samples, and are representative of the results obtained in at least three independent experiments. (D) The purified recombinant deletion mutant RN or CD nucleophosmin/B23 (15.5 pmol) was incubated with [α - 32 P]ATP (1700 c.p.m./pmol) at 0 °C for 2 h. The amounts of ATP–nucleophosmin/B23 protein complex were measured by filtration through Millipore membranes. Results are means $\pm$ S.D. for triplicate samples, and are representative of the results obtained in at least three independent experiments.

mutants RN and CD were further tested for the ability in binding [α - 32 P]ATP on a nitrocellulose membrane (Figure 4D). In agreement with the results of the ability to be retained by ATP-agarose, the nucleophosmin/B23 mutant RN could bind to ATP effectively, whereas mutant CD lost most of the ability to bind [α - 32 P]ATP on a cellulose membrane. Taking into consideration the different [α - 32 P]ATP specific radioactivities and the amounts

of proteins used (50% more RN molecules), the radioactive counts (for the same number of molecules) for RN nucleophosmin/B23 binding to ATP were still approx. 1.6-fold greater than those for T7 nucleophosmin/B23 (Figures 4A and 4D). The result indicates that more RN nucleophosmin/B23 molecules bind ATP. The number of ATP-binding sites per RN nucleophosmin/B23 molecule was approx. 1.0. Taken together, the

Table 1 Nucleotide specificity of binding to nucleophosmin/B23

Purified recombinant nucleophosmin/B23 (10.8 pmol) was incubated with 50 μ l of 250 μ M [α - 32 P]ATP (2050 c.p.m./pmol) and 20 mM competitor nucleotide at 0 °C for 15 min. The amounts of ATP–nucleophosmin/B23 protein complex were measured by filtration through Millipore membranes. Average values of triplicates are presented. Without a competitor, 6.9 pmol of ATP–nucleophosmin/B23 protein complex was retained on the membrane.

Competitor nucleotide	[α - 32 P]ATP–nucleophosmin/B23 (% of control)
ATP*	5.6
ADP	19.8
AMP	52.1
GTP	98.2
CTP	97.7
UTP	71.1
dATP	7.0
dGTP	85.7
dCTP	99.8
TTP	99.7

* Unlabelled ATP.

results indicate that the C-terminal portion (residues 242–294) contains the essential ATP-binding site of nucleophosmin/B23.

DISCUSSION

Intricate mechanisms that mobilize hundreds of proteins, RNA species and other elements in the nucleolus are orchestrated in a concerted manner to make ribosomes. These mechanisms are dependent on a balanced supply of rRNA and ribosomal proteins and a proper conformation of rRNA [35,36]. The biosynthesis of ribosomes involves multiple steps such as the transport of ribosomal proteins to the nucleolus and binding to nascent pre-rRNA, splicing of the 45 S RNA, packaging of ribosomal proteins into ribosome subunits, and delivery of the pre-ribosomal RNP through the nucleoplasm to the cytoplasm. Even though the function of nucleophosmin/B23 has not been defined there is ample evidence suggesting that nucleophosmin/B23 is involved in the assembly of ribosomes [1,3,7]. Electron microscopic studies have indicated that nucleophosmin/B23 is concentrated in the granular region of the nucleolus, where the processing of preribosomal particles might take place. Chromatin assembly or remodelling is a key biological process, inherently part of fertilization, DNA replication, repair, transcription and cell division (i.e. the cell cycle). The presumed chromatin assembly or remodelling role of *Xenopus* nucleoplasmin has been well documented [37]. Nucleoplasmin has recently been implicated in

the remodelling of repressive ribonucleoprotein particles containing maternal mRNA to facilitate translational activation [38]. Significant sequence similarity between the N-terminal portion of nucleophosmin/B23 and *Xenopus* nucleoplasmin [39] suggests that nucleophosmin/B23 has a function analogous to that of nucleoplasmin in chromatin assembly [40,41].

In our study of the mechanism of the translocation of nucleophosmin/B23 from nucleolus to nucleoplasm, ATP is shown to be required for drug-induced B23 translocation in whole cultured cells as well as its ability to promote B23 translocation *in vitro* [25]. Taking the advantage of its ability to be retained by ATP-agarose, the present study provides evidence that nucleophosmin/B23 is indeed able to bind ATP. On the basis of retention on a nitrocellulose membrane, ATP is bound to nucleophosmin/B23 with low affinity (K_d 86.5 ± 8.3 μ M). Our previous results show that there is a need for ATP at a level of at least 200 μ M to effect the induction of translocation of nucleophosmin/B23 from nucleoli to nucleoplasm in isolated permeabilized cells [25]. Whether the interaction of ATP with nucleophosmin/B23 is implicated in the induction of the translocation of nucleophosmin/B23 from nucleoli to nucleoplasm remains to be determined. Our present ATP binding results also appear to show that about half the nucleophosmin/B23 molecules bind ATP. It has been shown that nucleophosmin/B23 could form oligomers [7], which might be essential for its function. It is possible that oligomerization of nucleophosmin/B23 is involved in ATP binding. Furthermore the purified recombinant nucleophosmin/B23 is shown to contain negligible ATPase activity. Purified nucleophosmin/B23 purified from *E. coli* might be different from the nucleophosmin/B23 of mammalian cells in terms of post-translational modification and conformation. If no ATPase activity is involved, the conformational state of nucleophosmin/B23 bound to ATP, rather than ATP hydrolysis, might then be an important factor in the induction of the translocation of nucleophosmin/B23 from nucleoli to nucleoplasm. Nevertheless it is also likely that proteins other than nucleophosmin/B23 contain ATPase activity and provide energy for hydrolysis of nucleophosmin/B23 during its translocation from nucleoli to nucleoplasm. The putative ATP-binding sequence is found in the middle (residues 99–110) as well as in the C-terminal (residues 248–261) portions of nucleophosmin/B23 [26–29]. It is therefore likely that nucleophosmin/B23 has two ATP-binding sites. However, our present results show that deletion of residues 83–152 of the molecule has little effect on the binding of ATP. The C-terminal deleted mutant (residues 242 to C-terminus), in contrast, loses most of its ability to be retained by ATP-agarose and to bind [α - 32 P]ATP on a nitrocellulose membrane. The results suggest strongly that the C-terminal portion (residues 242–294) contains the essential ATP-binding site of nucleophosmin/B23. One can further speculate that nucleophosmin/

Table 2 Putative ATP-binding sequence of nucleophosmin/B23

Gaps are marked with full points, and consensus sequences are in bold type. Abbreviation: O, hydrophobic residue.

Protein	Reference	Residues	Sequence
Consensus sequence			K/R/H.....X(5–8).....O-X-O-O-D/E
Nucleophosmin/B23		110–120	H-I-S-G-Q-H.....L-V-A-V-E
		248–265	K-A-K-M-Q.....L-P-K-V-E
gp96	[27]	193–203	K-H-N-N-D-T.....Q-H-I-W-E
hsp108	[28]	708–719	K-T-V-M-D-L.....A-V-V-L-F-E
Erp99	[29]	324–334	K-K-V-E-K-T.....V-W-D-W-E

Figure 5 Schematic diagram (A–C) and SDS/PAGE analysis of nucleophosmin/B23 deletion clones for ATP-binding studies (D)

The full-length nucleophosmin/B23 was inserted at the *Bam*HI and *Eco*RI sites of the pGEX-3X vector. (A) The restriction enzyme sites for deletion and cloning of the nucleophosmin/B23 cDNA. (B) Various deletion clones. Numbers represent the amino acid numbers from the N-terminus; open boxes represent the open reading frame of nucleophosmin/B23; solid lines represent the non-coding sequences; broken lines represent the deleted cDNA sequences; striped boxes represent the putative ATP-binding sites. (C) The N- and C-terminal residues and the corresponding cDNA sequences of nucleophosmin/B23. The PCR primers p1, p2 and p3 are also shown. (D) The purified recombinant nucleophosmin/B23s were subjected to SDS/PAGE [10% (w/v) gel] stained with Coomassie Blue. The line labelled 36.5 kDa indicates the position of lactate dehydrogenase (Mark12[®], Novex) on SDS/PAGE.

B23 has only one ATP-binding site. Alternatively, deletion of the C-terminal portion largely affects the conformation of the molecule, resulting in its losing the middle portion's ability to bind ATP. The finding that nucleophosmin/B23 is an ATP-binding protein and the identification of its possible ATP-binding site are important steps towards the elucidation

of the role of ATP in the regulation of nucleolar function of nucleophosmin/B23 in cell proliferation.

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