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Original Article

Mutation of cysteine 21 inhibits nucleophosmin/B23 oligomerization and chaperone activity

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Abstract: Nucleophosmin (NPM/B23) is a multifunctional nucleolar protein to which both tumor-suppressor and oncogenic functions have been attributed. NPM/B23 has a variety of binding partners including ribosomes, nucleic acids, the centrosome and tumor suppressors such as p53 and p19ARF. These disparate functions are likely due to its ability to oligomerize and display molecular chaperone activity. In this report we identify a single amino acid residue, Cys^{21}, of nucleophosmin as important for the oligomerization and chaperone activity. Mutation of Cys^{21} to aromatic hydrophobic residues (e.g., Phe or Try), but not to a conserved polar residue (e.g., Ser) inhibited the pentameric oligomerization of NPM/B23. However, only Phe substitution of Cys^{21} drastically inhibited NPM/B23 chaperone activity. Interestingly, expression of Cys21Phe mutant in MCF7 cells demonstrated that this mutant protein does not co-polymerize with endogenous wild-type NPM/B23 and acts as negative dominant by destabilizing the endogenous dimer, trimer oligomerization. Taken together, the results in this study identify Cys^{21} as critical residue for NPM/B23 oligomerization and chaperone functions. In addition, Cys^{21} mutant provide a strong link between the oligomerization and chaperone functions of NPM/B23.

Keywords: Nucleophosmin (NPM/B23), nucleolar protein, tumor-suppressor, oncogene, nucleophosmin/B23 oligomerization, chaperone

Introduction

Nucleophosmin (NPM, B23, numatrin) is an abundant nucleolar phosphoprotein involved in multiple essential cellular functions [1]. NPM/B23 is shown to be involved in aspects of ribosomal biogenesis by binding to pre-ribosomal ribonucleoparticles [2] possibly through its ability to bind rRNA [3,4]. Down-regulation of NPM/B23 levels by RNA interference inhibits the processing of pre-ribosomal RNA [5]. In addition, NPM/B23 shuttles between the nucleus and cytoplasm suggesting a role in the nucleocytoplasmic transport of proteins [6]. This is further supported by its ability to bind nuclear localization signal peptides and stimulate nuclear import of proteins [7]. NPM/B23 interacts with several nucleolar proteins, including nucleolin, p120, and the tumor suppressors Rb, IRF-1, $p19^{ARF}$ and p53, modulating their subcellular localization and thus affecting their activities [5, 8-12].

NPM/B23 has been shown to act as a molecular chaperone by preventing protein aggregation and enzyme denaturation [13]. It is thought to function as a nucleosome chaperone by binding to histones [14,15]. The N-terminus of NPM/B23 shows extensive homology to the nucleoplasmin family of acidic histone chaperones [16]. Nucleoplasmin and NPM/B23 have both been shown to form oligomers, which are required for histone binding and assembly [14-16]. Thus it was suggested that the oligomerization status of NPM/B23 protein might be necessary for its chaperone activity [17].

The levels of NPM/B23 are markedly higher in tumor cells than in normal cells [18] and its expression is increased in association with mitogenesis [19]. Moreover, overexpression of NPM/B23 results in malignant transformation of NIH-3T3 cells [11]. NPM/B23 translocations are found in various myeloid and lymphoid cancers [20-22]. Interestingly, in all these translo-

cations, the N-terminal part of NPM/B23 is fused to different genes. Further support for the importance of NPM/B23 in tumorigenesis was provided recently by the generation of NPM/B23 knockout mice, which display haematological defects, centrosome abnormalities and genomic instability [23]. Together these data strongly implicate NPM/B23 in cancer and cell cycle control.

Given that many chaperones act as oligomers we hypothesized that the chaperone activity of NPM/B23 might be linked to its ability to form oligomers. Here we report that the N-terminal cysteine residue (Cys^{21}) of NPM/B23 is required for its chaperone activity. Mutation of Cys^{21} to Phe or Trp severely impairs the ability of the protein to form higher order oligomers, while only Phe substitution inhibits its chaperone activity. Moreover, Phe^{21} mutant expressed *in vivo*, in MCF7 cells, does not oligomerize with wild-type NPM/B23.

Materials and methods

Cloning of NPM/B23 constructs and site-directed mutagenesis

The full-length NPM/B23 cDNA was amplified by RT-PCR from total RNA isolated from CEM T lymphocytes tumor cells (ATCC, Manassas, VA). The primers used were: NPM/B23-F 5'ATACGCGGATCCACCATGGAAGATTCGATGGAC 3' and NPM/B23-R 5'TTTAATTAAGCGGCCGCTTAGCTAGCGTAATCTGGTACGTCGTATGGGTAAAGAGACTTCCTCACTGC 3'. The forward primer has a BamHI linker whereas the reverse primer includes a Not I linker and the sequence encoding an HA-epitope tag (YPYDVPDYA; Influenza hemagglutinin-HA). RT-PCR was performed with the Ready-to-Go RT-PCR beads from GE Healthcare. The PCR product was verified by sequencing, digested with BamHI and NotI enzymes and subcloned into the pTRE2-hygro vector (BD Biosciences, Bedford, MA). The cDNA encoding the full length NPM/B23 sequence was digested with BamHI and NotI enzymes and ligated into the BamHI and NotI sites of the pGEX-6P1 vector (GE Healthcare, Baie d'Urfe Quebec) for recombinant protein expression. Mutagenesis of the nucleophosmin Cysteine residues was performed by PCR using the QuickChange Site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The mutagenic primers were: for C21F/C21W/C21S, 5' CTTTTCGGTTTC/GG/CCGAACTAAAGGCC and 5' GGCCTTTAGTTCGA/CC/GGAACCGAAAAG (underlined residues are mutated). Screening for the mutations was facilitated by the introduction of novel restriction sites in the mutagenic primers (where possible).

Recombinant NPM/B23 protein expression and purification

The NPM/B23 ORF was directionally subcloned into the BamHI and NotI sites of pGEX-6P1 vector. This construct was transformed into *E. Coli* strain BL21 Star DE3 (Invitrogen, La Jolla, CA) for GST-fusion protein expression. Fusion protein was induced with 0.1 mM IPTG for 3 hours at 37°C. GST-NPM/B23 fusions were batch purified using glutathione sepharose 4B beads (GE Healthcare) according to the manufacturer's protocol. The GST-NPM/B23 fusion proteins were cleaved on the beads with 2 units Prescission Protease. The purity and yield of the recombinant NPM/B23 were evaluated by 10% SDS-PAGE followed by Gel Code stain (Pierce, Nepean, Ontario). The protein concentration of samples was estimated by the Coomassie Plus Protein assay (Pierce).

Cell lines and transfection of NPM/B23 plasmids

MCF-7 Tet off cells were obtained from Clontech and were maintained in DMEM supplemented with 10% FBS, 4 mM L-glutamine, 100 μg/ml G418. PTRE2-hygro NPM/B23 plasmid transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were selected with 100 μg/ml hygromycin for 1-2 weeks and the expression of various NPM/B23 transfectants was verified by Western blotting using murine anti-NPM/B23 (Zymed Laboratories, San Francisco, CA) or anti-HA tag monoclonal antibodies.

SDS PAGE and Western Blotting

Cells were lysed using a lysis buffer containing 100 mM Tris-HCL pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and protease inhibitors. Crude cell lysates (50 μg), or 1 μg of purified recombinant NPM/B23, was suspended in 1X Laemmli sample buffer (containing β-mercaptoethanol) and loaded onto 10% SDS PAGE without boiling to preserve the oligomeric status of NPM/B23 complex as de-

scribed previously by Yung and Chan [24]. Resolved proteins were either stained using Gel Code (Pierce) or transferred onto Hybond nitrocellulose membranes for Western blot analysis. Membranes were blocked in 5% non-fat dried milk in PBS and hybridized with a mouse anti-NPM/B23 or anti-HA tag mAbs (Zymed) diluted in blocking buffer at 1000 dilution (v/v) in PBS. Nitrocellulose membrane was washed and incubated with HRP-linked goat anti-mouse antibody diluted 1/3000 v/v with PBS. Immuno-reactive proteins were visualized by chemiluminescence using Femto-super signal kit from Pierce.

Chaperone assay

The chaperone assay was performed as described by Buchner et al. [25] using porcine heart citrate synthase from Roche (Mississauga, ON). The thermal denaturation reaction consisted of 1 μM citrate synthase solution in 40 mM HEPES-KOH, pH 7.5 incubated at 43 °C in the presence or absence of 1 μM recombinant NPM/B23. The chaperone activity of NPM/B23 is determined by the ability of normal and mutated protein to prevent the thermal aggregation of citrate synthase. Aggregation of citrate synthase is measured by changes in light scattering of the protein solution monitored at 360 nm with readings recorded every 5 min (up to 70 minutes).

Results and discussion

Previous studies have mapped the oligomerization and chaperone domains to the first 119 N-terminal residues of NPM/B23 using deletion mutants [17,26,27]. In this study it was of interest to examine the role of a highly conserved cysteine "Cys21" residue on the oligomerization and chaperone activities of NPM/B23. Sequence comparisons revealed that the Cys21 residue is well conserved from Xenopus to human nucleophosmin and between different proteins of this family [16]. Using site directed mutagenesis; Cys21 was mutated to Phe, Trp and Ser residues. **Figure 1** shows the expression of wild type (Cys21), and mutant NPM/B23 proteins purified from *E.Coli* as detected by Western blotting using anti-NPM/B23 mAb. The results in **Figure 1** (lane 1) show the expression of purified wild type NPM/B23 migrating as a monomer, trimer and pentamer (or oligomer) as previously observed with either recombinant [17,27] or native NPM/B23 [24,26]. Interest-

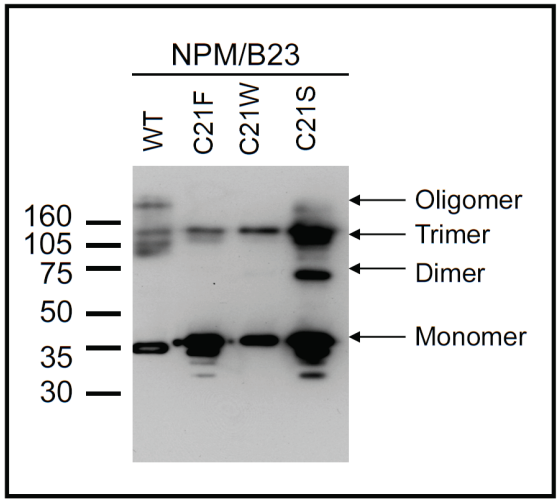

Figure 1. Effects of Cys21 mutations on oligomerization of recombinant NPM/B23. Wild type and three Cys21 mutants of NPM/B23 were expressed and purified from BL21 E. Coli. Purified WT and mutant NPM/B23 proteins were resolved on 10% SDS-PAGE and subjected to Western blotting and probed with anti-NPM/B23 mAb. Lanes 1- 4 show WT, C21F, C21W and C21S mutant NPM/B23. Note the absence of oligomer band in lanes 2 and 3 (C21F and C21W) mutants of NPM/B23.

ingly, mutating Cys21 to Phe or Trp (e.g. C21F or C21W) impaired the formation of pentamer NPM/B23, as evidenced the absence of ~175 kDa signal band on SDS PAGE (lanes 2 and 3 of fig. 1). By contrast, Cys21 mutation to Ser (e.g. C21S) did not affect the pentamer oligomerization of NPM/B23. Moreover, the C21S mutation appears to have stabilized the dimer state of NPM/B23. Taken together, our results are in agreement with earlier structure function analysis of NPM/B23, which revealed that the oligomerization domain maps to the amino-terminal half of NPM/B23 [17]. In addition, the crystal structure of the Xenopus homologue, NO38, has revealed that the N-terminal 123 amino acid residues are sufficient for oligomerization and histone chaperone functions [15]. Crystal structure analysis of NPM/B23, a structurally related family member, has revealed that the Cys21 residue is part of an important a-helix that resides in the subunit-subunit interface within NPM/B23 pentamer [16]. The formation of NPM/B23 dimers with C21S mutant suggests that the serine replacement increases the stability of NPM/B23 oligomerization, possibly through specific interactions or steric fit. In con-

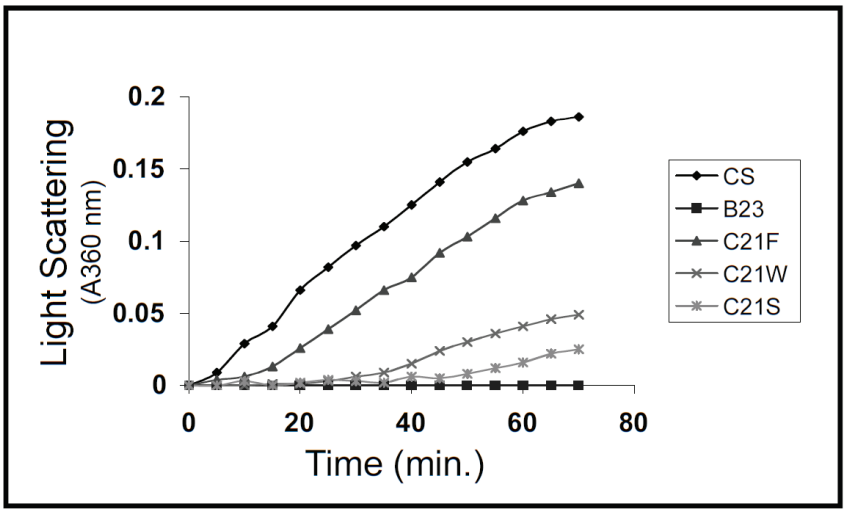

Figure 2. Effects of Cys21 mutations on NPM/B23 chaperone activity. Wild-type NPM/B23 inhibits the thermal denaturation and aggregation of citrate synthase. The thermal denaturation of citrate synthase is determined in the absence (u) or presence of WT (■), C21F (p), C21W (☚ and C21S (☚ NPM/B23 mutants. The aggregation of citrate synthase at 43°C is measured spectrophotometerically at 360 nm. An increase in light scattering due to citrate synthase aggregation indicates a reduced NPM/B23 chaperon activity.

trast, mutation of Cys21 to Phe or Trp, two bulky hydrophobic residues, inhibits higher order oligomerization possibility to steric effect or destabilization of a-helix that resides in the subunit-subunit interface.

Since the oligomerization ability of NPM/B23 protein was previously shown to correlate with its chaperone activity [17], we investigated the ability of the three Cys21 mutations on the molecular chaperone function of NPM/B23. In this assay, the ability of wild type and mutant NPM/B23 to inhibit the thermal denaturation of citrate synthase was measured over a period of 70 minutes as previously described by Buchner *et al.* [25]. The increase in light scattering at 360 nm is a measure of protein aggregation due to the thermal denaturation of citrate synthase as observed in the absence of NPM/B23 (**Figure 2**). The presence of wild-type NPM/B23 lead to a compete inhibition of citrate synthase denaturation (**Figure 2**). By contrast the presence of mutant NPM/B23 provided some protection against the thermal denaturation of citrate synthase, with C21S mutant having the highest protection followed by C21W and C21F (**Figure 2**). The C21F mutant was nearly inactive as a chaperone, while C21S mutant was similar to wild type. Interestingly, the chaperone activity of C21F and C21S NPM/B23 mutants is consistent with the oligomerization results obtained for both mutants (**Figure 1**). The chaperone activity of C21W mutant are interesting as it was expected to be less active as chaperone based on the oligomerization results (**Figure**s **1** versus **2**). However, close examination of the oligomerization results in **Figure 1** shows higher ratio of trimer to monomer NPM/B23 for C21W than C21F (**Figure 1**). Alternatively, unlike the Phe21 substitution which affects both the oligomerization and chaperone activity, Trp21 substitution appears to have greater impact on the oligmerization of NPM/B23.

Having demonstrated the effects of C21F on pentamer formation and chaperone activities *in vitro*, it was of interest to determine if this mutation also inhibits the oligomerization of NPM/B23 *ex vivo.* The results in **Figure 3** show wild-type and NPM/B23 mutant over-expressed in MCF-7 cells using the tetracycline inducible system. To distinguish between exogenous and endogenously expressed NPM/B23, the recombinant NPM/B23 wild-type and C21F mutant were tagged with an HA epitope at their C-terminus. The C21F mutant behaved essentially the same *ex vivo* as *in vitro*: it prevented NPM/B23 oligomer formation (**Figure 3**). Since MCF-7 cells express quite high levels of endogenous NPM/B23, this result implies that the C21F mutated protein cannot oligomerize with wild-type endogenous NPM/B23 possibly acting in a

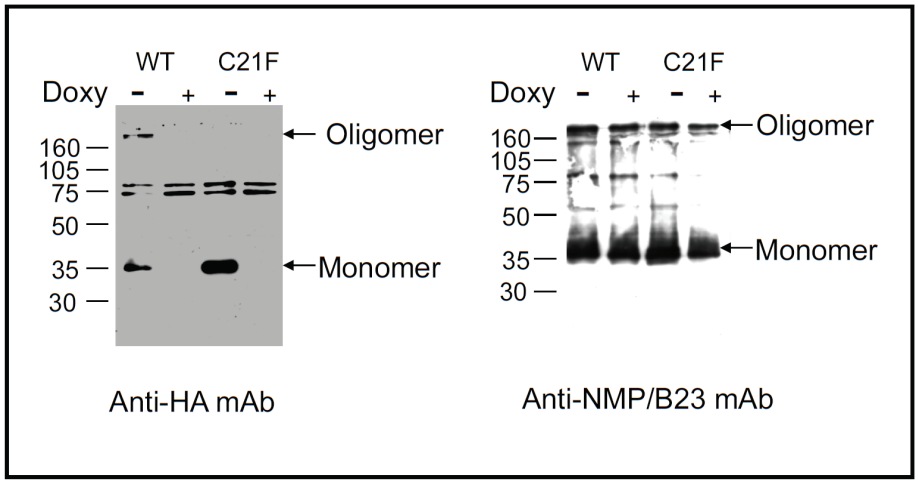

Figure 3. Expression and characterization of NPM/B23 Cys21 mutants in a breast tumor cell line. Cell lysates from MCF7 cells expressing wild-type (WT) and Cys21 (C21F) mutant full length NPM/B23 were resolved on SDS-PAGE and transferred to nitrocellulose membrane. Western blot of cell lysates from MCF-7 tet off stably transfected cells probed with an anti-HA tag (Panel A) or anti-NPM/B23 monoclonal antibody (Panel B) showing the doxycycline-regulated expression of WT and C21F mutant NPM/B23 (addition of doxycycline turns off the vector). Notice the presence of the monomer and pentamer in the WT while C21F mutant NPM/B23 is found exclusively as a monomer. The doublet band around 75 kDa is non-specific and serves as a loading control.

dominant negative fashion over the endogenous protein, which is able to form multimers (**Figure 3B**). This possibility is further supported by a decrease in the amount of total endogenous oligomerized NPM/B23 in the presence of the C21F mutation in MCF-7 cells (**Figure 3B**).

The findings in this report are of interest with respect to earlier studies describing the genomic translocations of NPM/B23 gene in lymphomas and the importance of the N-terminal part of NPM/B23 in mediating oligomerization. Bischof *et al.* [28] have elegantly demonstrated that the 117 amino-terminal amino acid residues of NPM/B23 that are fused to the ALK oncogene in non-Hodgkin's lymphoma, are required for oligomerization and activation of the ALK tyrosine kinase activity; and deletion of this domain leads to inability of the fusion protein to oligomerize and cause transformation [29]. Furthermore, this translocation alters the cellular distribution of the NPM-ALK fusion protein [30], raising the possibility that NPM/B23 oligomerization can affect the cellular distribution of the endogenous protein and its binding partners. For example, the binding of NPM/B23 to p19ARF tumor suppressor protein targets ARF to the nucleoli and inhibits its function [31,32]. In summary, the results in this report demonstrate the effects amino acid Cys21 substitutions on the oligomerization and chaperone activities of NPM/B23. Work is underway to examine the effects of Cys21 substitution on NPM/B23 chaperone functions and protein interactions *in vitro* and *ex vivo*, in addition to the effects of this mutant on other normal functions of NPM/B23.

Abbreviations: Nucleophosmin, NPM/B23; Cysteine, Cys; Glutathione S-transferase, GST; Cysteine 21 mutation to Phenylalanine, C21F; Cysteine 21 mutation to Tryptophan, C21W; Cysteine 21 mutation to Serine, C21S.

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