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Recognition of intermolecular G-quadruplexes by full length nucleophosmin. Effect of a leukaemia-associated mutation

Sonia Bañuelos *, Benoît Lectez [1], Stefka G. Taneva [2], Georgina Ormaza [1], Marián Alonso-Mariño, Xabier Calle, María A. Urbaneja

Biophysics Unit (CSIC/UPV-EHU), Department of Biochemistry and Molecular Biology, University of Basque Country (UPV-EHU), POB 644, 48080 Bilbao, Spain

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ABSTRACT

Nucleophosmin (NPM) is a nucleolar protein involved in ribosome biogenesis. *NPM1* gene is frequently mutated in acute myeloid leukaemia (AML), correlating with aberrant cytoplasmic localization of the protein. NPM attachment to the nucleolus in physiological conditions probably depends on binding to nucleic acids, and this recognition could be altered in AML. NPM associates to guanine-rich DNA sequences, able to fold as "G-quadruplexes". We have analyzed the interaction of pentameric, full length NPM with G-rich oligonucleotides, finding that the protein binds preferentially high-order G-quadruplexes. AML-associated mutation significantly hampers DNA binding, pointing to a possible mechanism contributing to pathological mislocalization of NPM.

1. Introduction

Nucleophosmin (NPM, also termed NPM1, B23, NO38 or numatrin) is a nuclear chaperone mainly located in the nucleoli of cells. NPM performs several functions affecting cell growth: it is involved in ribosome assembly and export from the nucleolus to the cytoplasm; it controls centrosome duplication, and regulates the stability of tumor suppressors, such as p53 and Arf [1]. The multiple activities of NPM have an impact on both proliferation and apoptosis, and not surprisingly, NPM deregulation has been involved in several human cancers [2]. NPM is a 294 aminoacid protein, apparently pentameric in solution, and constituted by several domains. The core domain, corresponding to the first 120 aminoacids, folds into a β-structure, and is responsible for the oligomerization, forming a ring with pentameric symmetry [3]. The core is connected to a small C-terminal domain, built of 3 α-helices [4] through a long and flexible linker.

Although enriched in nucleoli, NPM is a "shuttling protein", its functionality requiring a continuous transit between cytoplasm, nucleoplasm and nucleolus. These transport events rely on the presence of different localization signals in NPM: nuclear localization (NLS), nuclear export (NES) and nucleolar localization signals (NoLS) [1,5]. As for nucleolar targeting, it has been described that two Trp residues in the C-terminal region of the protein are necessary for the attachment of NPM to that particular subnuclear region [6]. When the 3D structure of NPM C-terminal domain was solved [4], it was evident that those residues are essential for packing of the hydrophobic core of the domain. Therefore, a more precise view is that nucleolar localization depends on the proper folding of that domain, and probably, on its binding to nucleolar ligands (either proteins or nucleic acids), as it has been proposed for other nucleolar proteins [7].

NPM is the most frequently mutated protein in acute myeloid leukaemia (AML), constituting a hallmark of a particular subtype of the disease [8]. Mutant NPM is mainly found in the cytoplasm rather than in nucleoli of leukoblasts from these AML patients [9]. The genetic lesion implicated in AML consists in the insertion of four bases in the *NPM* gene, causing a frameshift that gives rise to a mutant NPM with a different C-terminal aminoacid sequence [9]. This alteration implies the loss of the two conserved Trp residues that had been related to nucleolar localization. In fact, it was shown that the lack of these aromatic residues prevents folding of the domain, and this, in turn, results in a failure of nucleolar retention [4]. Additionally, the novel sequence contains a NES, and both features of mutant NPM (loss of the Trp residues and acquirement of the new NES) contribute to determine the aberrant

* Corresponding author. Fax: +34 94 601 3360.
E-mail address: sonia.banuelos@ehu.es (S. Bañuelos).
[1] Present address: CIC BioGUNE, 48160 Derio, Spain.
[2] Present address: Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria.

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localization of AML-related mutant NPM to the cytoplasm instead of the nucleolus [10].

NPM binds numerous proteins [1] as well as DNA and RNA [11]. The nucleic acid binding ability of the protein has been ascribed to its C-terminal 70 residues, e.g., the C-terminal globular domain plus a previous segment of 20 residues, including several lysines [11]. Although NPM was initially described to associate with DNA in a rather unspecific manner [11], it has been more recently reported to preferentially recognize guanine rich sequences that are able to form particular DNA structures known as "G-quadruplex" (G4) [12]. These are four stranded motifs, where at least 2 planar groups of guanines ("G-tetrad") pile on top of each other, stabilized by "Hoogsteen type" hydrogen bonds and monovalent cations [13]. These structures, different from the canonical double helix, are thought to exist in vivo, and play a role in telomere maintenance and transcriptional regulation [14]. G-quadruplexes can be either intra (in a sequence with at least four tracks of three or more G) or intermolecular (bridging two or more strands), and each type can display different configurations [13]. One of the most studied G-quadruplex forming DNA is a sequence of the c-Myc oncogene promoter region, the nuclease hypersensitivity element III_1 ($NHEIII_1$). There is evidence that formation of a G-quadruplex by this sequence controls 80–90% of the transcription level of c-Myc. This DNA structure, therefore, may represent a valid target for cancer therapy [14].

Brunori et al. have described that NPM C-terminal region binds the G-rich DNA sequence (c-MYC III_1) from the c-Myc promoter and other G-rich sequences that also form G-quadruplexes [12]. Recently they have presented a structural model based on NMR of the C-terminal domain of NPM ("NPM1-C70", including the upstream extension) bound to a sequence derived from c-Myc [15]. The interaction of the protein with G-rich sequences needs to be further investigated to better understand the determinants of NPM localization and functionality. Here, we have explored the interaction of full length, oligomeric NPM with different types of G-quadruplex forming oligonucleotides. We found that NPM binds high-order (intermolecular) G4 structures with higher affinity than the monomeric intramolecular G-quadruplex previously described [15]. Furthermore, we have evaluated the consequences of pathological mutations of NPM on its DNA binding properties. Our data show that the DNA binding ability of a leukaemia-related full length mutant NPM is drastically reduced, in agreement with recently published data on the binding deficiency of the mutated C-terminal domain [16]. Therefore, alterations in nucleic acid recognition are strongly suggested to contribute to the aberrant cytoplasmic localization of NPM characteristic of AML.

2. Materials and methods

2.1. DNA oligonucleotides

Oligonucleotides G4MYC (5′-TGGGGAGGGTGGGGAGGGTGGGGAAGG) [12], G4Pu22 (5′-TGAGGGTGGGTAGGGTGGGTAA) [17], and G4Pu24I (5′-TGAGGGTGGIGAGGGTGGGGAAGG) [15] (purchased from GenScript USA Inc) are related to the c-Myc promoter sequence. To facilitate G-quadruplex formation, the oligonucleotides were resuspended in 20 mM Hepes, 100 mM KCl, pH 7.5 and annealed by heating at 95 °C for 5 min, and slowly cooling down at room temperature overnight. For some experiments, the DNA samples were resuspended without KCl and/or not subjected to annealing. Oligo G4tetra (5′-TCGCCACGTTTCGCCGTTTGCGGG GGTTTCTGCGAGGAACTTTGG) [18] (from Sigma–Aldrich) was annealed as described in Ref. [18].

2.2. Protein production

Full length NPM, both wild type and the AML-associated mutant A [9] were cloned in the plasmid pET8c-6HTEV [19], using as template a clone of human NPM kindly provided by Dr. Zhang [20]. Both constructs carry an N-terminal $(His)_6$ tag. Proteins were expressed overnight at 18 °C in *Escherichia coli*, BL21 (DE3) strain. For purification, cells were disrupted by sonication in 25 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 20 mM imidazole, 5 mM $MgCl_2$, 1 mM TCEP, 10% glycerol, supplemented with lysozyme (20 mg/L culture) and protease inhibitors (Complete, EDTA-free, Roche). The clarified extract was loaded on a Ni–NTA affinity column (Histrap FF, GE Healthcare) and the protein eluted with an imidazole gradient. NPM was then further purified by gel filtration chromatography with Superdex 200 (GE Healthcare) in 25 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT, 10% glycerol, concentrated and frozen for storage. We have observed a small proportion of degradation products in mutant A, being at most 13% as estimated by densitometry of SDS–PAGE.

2.3. Electrophoretic mobility shift assays (EMSA)

Binding assays were performed titrating either a fixed amount of NPM with increasing concentrations of oligonucleotide or vice versa. Binding reactions (20 μL) containing the appropriate amounts of NPM and DNA were incubated for 1 h at room temperature in 20 mM Hepes, 100 mM KCl, 2 mM TCEP, 10% glycerol, pH 7.5 ("Hepes/K buffer"), and loaded onto 8.5% polyacrylamide native gels. Electrophoresis was run at 120 V at room temperature, in 0.5× TBE buffer. Gels were stained with GelRed (Biotium) for DNA detection and with Coomassie afterwards. For quantification, the fluorescence intensity of the band corresponding to the complex was measured with a GelDoc EZ Imager and Image Lab software (Bio-Rad).

2.4. Size exclusion chromatography (SEC)

Mixtures of NPM and DNA (or either protein or DNA alone, as controls) were incubated for 1 h at room temperature in Hepes/K buffer. Then, 25 μL were injected in a Superdex 200 PC 3.2/30 column (GE), equilibrated in the same buffer, and chromatography was run at 4 °C.

2.5. Isothermal titration calorimetry (ITC)

ITC measurements were carried out using a VIP-ITC MicroCalorimeter (MicroCal, Inc., Northampton, MA), at 20 °C. Protein and DNA were dialyzed against Hepes/K buffer, and degassed prior to the experiment. Oligonucleotides, at 200–250 μM, were titrated onto NPM at 2–4 μM. Injections of 10 μL were performed at 240 s interval. The reaction heat of each injection is related to the calorimetric enthalpy of binding, ΔH. The binding isotherms, ΔH cursive *vs.* molar ratio, were analysed with an independent binding sites model using MicroCal Origin software. The fit of the binding curve yields the binding constant K_a ($K_d = 1/K_a$), the enthalpy ΔH and the stoichiometry of the binding reaction.

3. Results

We have characterized the interaction of full length NPM with the sequence 5′-TGGGGAGGGTGGGGAGGGTGGGGAAGG-3′ from c-Myc promoter (hereby termed G4MYC). The DNA oligo was subjected to annealing in the presence of K^+ ions to promote G-quadruplex (G4) structures formation. We followed the binding process by electrophoretic mobility shift assays (EMSA) (Fig. 1): When NPM is mixed with G4MYC, a fraction of the DNA co-migrates with the protein. Both the DNA and protein staining reflect the formation of a complex of larger size than the protein alone (Fig. 1A). Semiquantitative binding curves obtained from EMSA gels by densitometric analysis show that the amount of slowly migrating DNA