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An intrabody specific for the nucleophosmin carboxy-terminal mutant and fused to a nuclear localization sequence binds its antigen but fails to relocate it in the nucleus

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ABSTRACT

The cytoplasmic accumulation of NPM1 (NPMc+) is found in acute myeloid leukemia (AML) with NPM1 mutation. NPM1 must shuttle between nucleus and cytoplasm to assure physiological protein synthesis and, therefore, the elimination of NPMc+ is not a suitable therapeutic option. We isolated, characterized, and produced a functional scFv intrabody fused to nuclear localization signal(s) (NLS) that does not recognize NPM1 but binds to the mutant-specific C-terminal NES (nuclear export signal) of NPMc+, responsible for its cytoplasmic accumulation. The scFv-NLS fusion accumulated in the nuclei of wild type cells and strongly bound to its antigen in the cytoplasm of NPMc+ expressing cells. However, it failed to relocate the majority of NPMc+ in the nucleus, even when fused to four NLS. Our results show the technical feasibility of producing recombinant intrabodies with defined sub-cellular targeting and nuclear accumulation but the lack of information concerning the features that confer variable strength to the signal peptides impairs the development of biomolecules able to counteract pathological sub-cellular distribution of shuttling proteins.

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1. Introduction

Nucleophosmin (NPM1) is a nucleolar multifunctional phosphoprotein involved in RNA metabolism [1–3], regulation of the p19/ARF-p53 tumor-suppressor pathway [4,5] and c-Myc turnover through Fbw7γ [6]. Under physiological conditions, the protein shuttles between nucleus and cytoplasm. In about one-third of adult patients with AML with normal karyotype, it has been demonstrated that AML cells bear mutations in the last coding exon of the NPM1 gene (exon 12) [7–9]. More than 40 heterozygous different mutations have been described. The mutations result in frame shift and the loss of the two tryptophan residues located in the C-terminal portion of the protein that are necessary for nucleolar localization. The insertion of short nucleotide stretches of eleven amino acids generates the *de novo* formation of a Chromosomal Region Maintenance 1 (CRM1)/Exportin 1-dependent NES responsible for mutant NPM1 cytoplasmic delocalization (NPMc+) [10–12]. Although a correlation between NPM1 cytoplasmic accumulation and leukemia initiation and progression has been recently demonstrated *in vivo* in murine models [13,14], so far there is no direct molecular evidence of the mechanism by which NPMc+ can induce pathological conditions. It has been suggested that NPMc+ could form hetero-octamers with NPM1 inducing its delocalization and that of proteins normally associated to NPM1, such as p19/ARF and Fbw7γ [4–6,15]. A monoclonal antibody (T26) specific for the cytoplasmic mutation has been demonstrated helpful to confirm the connection between NPMc+ expression and AML in patients [16]. However, when we performed a double staining to identify both NPM1 and NPMc+ localization, it turned out that a significant portion of the wild type protein was still located in the nucleoli [17], questioning the hypothesis of a massive NPM1 migration to the cytoplasm. Nevertheless, both the shuttling and the residential activities of NPM1 are necessary for the normal metabolism since NPM1 seems to be the rate-limiting nuclear export shuttle

Abbreviations: AML, acute myeloid leukemia; CRM1, Chromosomal Region Maintenance 1; GST, glutathione S-transferase; MBP, maltose binding protein; NES, nuclear export signal; NLS, nuclear localization signal; NPM1, nucleophosmin; NPMc+, cytoplasmic nucleophosmin; scFv, single-chain variable fragment.

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for ribosome components in mammalian cells and an indispensable regulator of protein synthesis [18]. The diminished NPM1 shuttling capacity impairs the regular ribosome assembly, places genetic pressure upon p19/ARF/p53 pathway, and leads to mutations resulting in cellular transformation [18]. This means that NPM1 shuttling must be preserved as well as its predominant nucleolar accumulation. Consequently, any therapeutic perspective should re-establish this equilibrium rather than inactivate NPMc+ by means of neutralizing drugs.

Intrabodies have been successfully used in the past to knockout their targets or sequester their antigen in specific sub-cellular compartments [19–21]. Similarly, we isolated a scFv antibody specific for the *de novo* exclusive NES motif present in the mutated NPMc+, confirmed its correct folding when it was expressed as an intrabody, and fused it to a sequence corresponding to a repeat of nuclear localization signals (NLS). Despite the effective binding to NPMc+ and the transient relocation into the nucleus, our data showed that the antigen–antibody complex remained statistically localized in the cytoplasm, a result that seems to confirm some previous reports underlining the large efficiency variability existing among nuclear localization signal peptides [22,23].

2. Materials and methods

2.1. Expression and purification of recombinant NPMc+

Full-length NPMc+ was expressed as a GST (glutathione S-transferase) fusion from pGEX4T vector and purified by affinity chromatography [24] using GSTrapFF column and ÄKTA Explorer (GE Healthcare). The C-terminal NPMc+ fragment corresponding to the 45 amino acids from 255 to 298 was synthesized by PCR, cloned in pETM44 vector [25] as MBP (maltose binding protein)-6× His tag fusion and transformed in BL21 cells. Cultures were grown in ZYP-5052 auto-inducing medium [26]. Purification was performed combining HisTrapHP column and ÄKTA Explorer (GE Healthcare).

2.2. Selection and subcloning of phage displayed scFvs

Human monoclonal scFv antibodies specific to NPMc+ were isolated from the synthetic ETH-2 Gold phage display library [27]. A pre-panning incubation step of the library against MBP at a concentration of 100 μg mL^{-1} was performed before each panning round to deplete anti-MBP binders. Three rounds of panning were performed on Nunc-Immuno™ Maxisorp™ tubes (Nunc) coated with the fusion construct NPMc+–MBP at a concentration of 25 μg mL^{-1} in 50 mM sodium carbonate buffer, pH 9.6 [28] and scFvs were screened by ELISA [27]. Six clones with an absorbance value higher than 0.49 and negative for the fusion tag were considered positive (Supplementary Fig. 1A) and sequenced using the following primers: Fdseq1 5′-GAATTTTCTGTATGAGG-3′ and PelbBack 5′-AGCCGCTGGATTGTTATTAC-3′. The results indicated that all the six clones shared the same sequence, suggesting a high selective pressure toward one specific binder (Supplementary Fig. 1B). It was produced in large scale in TG1 cells and purified on HiTrapMabSelectSuRE ProteinA column followed by size exclusion chromatography on HiLoad 16/60 Superdex 200 using ÄKTA Explorer (GE Healthcare). The mouse anti-Myc monoclonal antibody 9E10 (8 μg mL^{-1}) was used as a primary antibody in ELISA test. The NLS corresponding to the SV40 large T-antigen was fused to scFv by PCR using the following primers: FW: 5′-CCAAGCTTCCATGGAGGTGCAGCTGTTGGAGTCTGGG-3′; REV: 5′CTAGGCGC GGCCGCATACCCCT ACGACGTGCCCGACTACCC-CAAAAAGAAACGAAAAGTA TAGTCTAGACTAG-3′ and the product was cloned HindIII-XbaI in the pcDNA3.1 vector (Invitrogen) to obtain NLS-HA fusions. The same approach was used to insert till to four SV40 NLS sequences. Flag tag and GFP-scFv fusion were obtained by cloning HindIII-XbaI the antibody cDNA in the pcDNA3.1 and in the pEGFP-C1 (Clontech) vectors, respectively.

Supplementary Fig. S1 related to this article can be found, in the online version, at doi:10.1016/j.btre.2014.05.008.

2.3. Cell culture and transfection

HeLa cells were grown in Dulbecco's modified Eagle Medium (Lonza) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 U mL^{-1}), streptomycin (100 mg mL^{-1}). OCI-AML2 and OCI-AML3 [29] cell lines were grown in MEM Alpha + GlutaMAX™-I medium (Gibco) supplemented with 20% FBS, glutamine and antibiotics. Transient transfections were performed using Lipofectamine™ 2000 (Invitrogen). Sf9 (*Spodoptera frugiperda*) insect cells were cultured at 27 °C in Sf 900 II SMF medium (Gibco) and transfected with pFastBacDual plasmids (Invitrogen) expressing either wild type NPM1 or NPMc+ using Insectogene T030-1.0 (Biontex). Baculoviral supernatant was collected after 96 h and used for two cycles of infection.

2.4. Immunoblotting and immunoprecipitation

For immunoprecipitation, cells were lysed in 50 mM Tris–HCl, pH 8, 150 mM NaCl, 0.5% NP40, and protease inhibitors. Ten micrograms of scFv were added overnight at 4 °C to HeLa and OCI-AML3 cell lysates followed by protein A/G-sepharose (GE Healthcare). For co-immunoprecipitation experiments, total cell lysate was incubated with mouse M2 anti-Flag agarose beads (Sigma) and with anti-mouse IgG agarose beads (Sigma) for 4 h at 4 °C. Precipitated recombinant purified proteins and cell lysates were separated by SDS-PAGE gel and immunoblotted over a nitrocellulose membrane (Whatman). After incubation with primary antibodies in 5% skimmed milk, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies (Bio-Rad). Primary antibodies used in WB were mouse monoclonal anti-Myc 9E10, mouse monoclonal anti-Flag M2, mouse monoclonal anti-NPMc+ T26, mouse 338 [30] anti-NPM1 C-terminal end, mouse 376 [30] anti-NPM N-terminal region, and supernatant from recombinant scFv. ECL Plus was used as a substrate for chemiluminescent-based protein immune detection (Pierce). Primary antibodies used in IP were mouse monoclonal anti-NPMc+ T26 and recombinant scFv.

2.5. Immunofluorescence assay

Cells grown on cover slips were fixed in paraformaldehyde, washed twice in PBS, permeabilized 5 min in 0.2% Triton X-100, washed again in PBS and blocked in 2% BSA for 30 min at room temperature. Slides were incubated 1 h in blocking buffer containing primary antibodies, washed extensively in PBS, and incubated with CY3-conjugated donkey anti-mouse immunoglobulin (Jackson ImmunoResearch) for 30 min. After washing, slides were counterstained with DAPI, rinsed in distilled water, mounted with mowiol, and assessed at the DAPI, GFP and CY3 channels. Images were acquired using an Olympus AX70 microscope equipped with a CoolSNAP *EZ* Turbo 1394 camera (Photometrics) and processed using ImageJ 1.43 software (Wayne Rasband, NIH). Leptomycin B experiments were performed as previously described [10]. Confocal microscopy was performed on a Leica TCS SP5 equipped with violet (405 nm) and blue (488 nm) excitation laser lines. Primary antibodies used for IF were mouse monoclonal anti-Myc 9E10, mouse monoclonal anti-HA, mouse monoclonal anti-NPMc+ T26, and recombinant scFv.

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