B23 interacts with PES1 and is involved in nucleolar localization of PES1

Jianhua Zhang¹, Ying Yang¹, and Jiarui Wu¹,²*

¹Key Laboratory of Systems Biology, State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
²Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei 230027, China
*Correspondence address. Tel: +86-21-54921128; Fax: +86-21-54920787; E-mail: wujr@sibs.ac.cn

PES1, the human homolog of zebrafish pescadillo, is a nucleolar protein that is essential for cell proliferation. We report herein that a nucleolar marker protein B23 physically interacts with PES1 and is involved in the nucleolar localization of PES1. In vivo interaction between B23 and PES1 was verified by co-immunoprecipitation of endogenous B23 and PES1 proteins, and they showed cellular co-localizations under both normal and actinomycin D-induced stress conditions. Furthermore, we mapped their interaction domains via in vitro pull-down assays. When B23 was knocked down by RNA interference, there appeared an increased nucleoplasmic distribution of PES1. Our results support a previous hypothesis that B23 might be a nucleolar hub protein for protein targeting to the nucleolus, and shed light on the nucleolar localization mechanism of PES1. The physical interaction between B23 and PES1 implies that they may participate in ribosome biogenesis in a protein complex.

Keywords B23; PES1; cell nucleolus

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Introduction

B23 (also known as nucleophosmin, NPM1, or NO38) is an abundant nucleolar protein taking part in various distinct cellular activities including ribosome biogenesis, centrosome duplication, molecular chaperoning and genomic stability control [1–3]. B23 is located primarily in the granular regions of the nucleolus, while many cellular proteins physically interact with B23, such as tumor suppressor ARF [4,5] and P53 [6], ribosomal proteins L5 and S9 [7,8], and interestingly some viral nucleolar targeting proteins [9–11]. B23 was proposed to be a nucleolar hub protein [12–14]. Notably, B23 is overexpressed in various tumors, and it has been proposed as a marker for gastric, colon, ovarian, and prostate carcinomas. In addition, disruption of the B23 gene by translocation is frequently found in human hematopoietic malignancies. About one-third of adult acute myeloid leukemia (AML) contains B23 mutants with aberrant cytoplasmic distribution [1–3].

Pescadillo, which was originally identified in a mutagenesis screen in zebrafish [15], is highly conserved from yeast to human. Yeast, mouse and human homologs of pescadillo were named as Yph1p, Pes1 and PES1, respectively. Pes1 knockout mice showed embryonic lethality due to a disruption in ribosome biogenesis [16]. Subsequent investigations revealed that PES1 interacted with Bop1 and WDR12, and the three proteins formed a stable complex in the nucleolus involving pre-rRNA processing and maturation of the large ribosomal subunit [17–19]. In addition, pescadillo was reported to regulate gene transcription [20] and induce large-scale chromatin unfolding [21]. Mis-regulation of pescadillo has been associated with cancer and chromosomal instability [22–24].

PES1 is predominantly localized in the nucleolus, with slight distribution in the nucleoplasm [22]. Nucleolar localization is fundamental for PES1 functioning in ribosome biogenesis, since PES1 mutants of domain deletions or highly conserved residue point mutations, which showed diffused nucleoplasmic distribution, failed to replace the function of endogenous PES1 [25]. Despite the importance of nucleolar localization for PES1, what factors maintain PES1 in the nucleolus remain largely unknown.

We report herein that the nucleolar marker protein B23 physically interacts with PES1 and is involved in its nucleolar localization.
Materials and Methods

Plasmid construction

Full-length PES1 amplified from human cDNA by PCR was cloned into pEGFP-C1 (Clontech, Mountain View, USA) and pGEX-4T-1 (GE Healthcare, Piscataway, USA) generating GFP-PES1 and GST-PES1, respectively. Full-length B23 cloned from human cDNA was inserted into pET-28a (Novagen, Madison, USA) to encode 6× His-tagged B23. Deletion mutants of GST-PES1 were generated by PCR with the following primers: P-001-F (5′-CGGGATCCATGGGAGGCTT-GAGAAG-3′) and P-415-R (5′-CGGAATTCCTCAAGAGAAAGTT-3′) for PES1 (1–415); P-101-F (5′-CGGGATCCATGTGGAACACTGGAG-CGT-3′) and P-588-R (5′-CGGAATTCTCACCGCCCTTGCCCTT-3′) for PES1 (101–588); P-220-F (5′-CGGGATCCATGGCACAAGAAGCCTTTTGAG-3′) and P-588-R for PES1 (220–588); P-322-F (5′-CGGGATACCTCGCCACCGG-3′) and P-588-R for PES1 (322–588). PCR fragments were digested with BamHI and EcoRI, purified, and cloned into pGEX-4T-1. PES1 (Δ322–415) was generated using Takara MutanBEST kit with the primes BRCT upper (5′-GGGGTGCAGCTGCCACAC-3′) and BRCT lower (5′-CTCCTGCGCCTCCAGCTCC-3′) and P-588-R for PES1 (220–588); P-322-F (5′-CGGGATACCTCGCCACCGG-3′) and P-588-R for PES1 (322–588). PCR fragments were digested with BamHI and EcoRI cloning sites of the pET-28a vector. DNA fragments encoding full-length PES1 (1–588) and mutant PES1 (322–588) were sub-cloned into a modified vector pcDNA3 expressing HA-tagged PES1 (1–588) and PES1 (322–588), respectively. Full-length B23 was subcloned into pmRFP-C1 (Clontech) expressing RFP-tagged B23.

Cell culture, transfection and stable cell line generation

HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (Gibco) at 37°C in 5% CO₂-containing atmosphere. Cells were transfected with plasmids by jetPEI (Polyplus, Illkirch, France) according to the manufacturer’s instructions. To generate stable HeLa cell line expressing GFP-PES1, HeLa cells were transfected with pEGFP-C1-PES1 and then selected in the presence of 400 μg/ml G418 (Promega, Madison, USA).

Immunoprecipitation and immunoblotting

For immunoprecipitation, cells were scraped into a lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 5% glycerol, and 10 μl/ml protease inhibitor cocktail (Sigma, St. Louis, USA) and lysed at 4°C for 1 h with gentle rotation. Cell lysates were cleared by centrifugation at 15,300 g for 10 min. The supernatants were incubated with antibodies or control IgGs for 4 h at 4°C. Then, protein G agarose beads (Upstate, Lake Placid, USA) were added for 1 h. The beads were washed three times with the lysis buffer and boiled in 2× SDS-loading buffer (100 mM Tris–HCl, pH 6.8, 200 mM DTT, 4% SDS, 20% glycerol and 0.2% bromophenol blue) for 5 min before loading for immunoblotting analysis. For immunoblotting, protein samples were subjected to SDS-PAGE and then transferred onto PVDF membrane (Millipore, Billerica, USA). The blots were incubated with primary antibodies, followed by incubation with horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, USA). Signals were detected by enhanced chemiluminescence system (ECL, GE Healthcare).

Protein purification and pull-down assays

All GST-PES1 and His-B23 proteins were expressed in E. coli BL21(DE3) cells. Expressions of all GST-PES1 proteins were induced with 0.1 mM IPTG at 28°C for 5 h. GST fusion proteins were purified by binding to glutathione-Sepharose 4B (GE Healthcare). Expression of His-B23 full-length and deletion mutants were all induced by 0.1 mM IPTG at 37°C for 3 h. Ni-NTA agarose (Qiagen, Hilden, Germany) was used to purify 6× His-tagged B23 proteins. For pull-down assays, HeLa cells were lysed in phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3) plus 1% Triton X-100 and 10 μl/ml protease inhibitor cocktail (Sigma), and then centrifuged at 15,300 g for 10 min. The supernatants were collected and incubated with bacterially purified proteins which had been coupled to beads, at 4°C for 2 h. Pull-down beads were washed four times with the lysis buffer, and boiled in 2× SDS-loading buffer for 5 min prior to immunoblotting assays.

RNA interference and isolation of nucleoli

The siRNA targeting B23 was prepared as described previously [26]. The siRNA oligos were transfected into cells by Lipofectamine 2000 (Invitrogen) and maintained for 3 days prior to biological assays. Nucleoli were isolated from HeLa cells as previously described [27], with detailed protocol at http://www.lamondlab.com/f7nucleolarprotocol.htm.
**Immunofluorescence**

Cells were grown on pre-coated glass coverslips (Fisher Scientific, Itasca, USA), fixed with 4% paraformaldehyde for 20 min, permeabilized and blocked with blocking buffer (PBS plus 0.2% Triton X-100 and 3% BSA) for 30 min, and then incubated with anti-B23 antibody (Zymed, South San Francisco, USA) for 1 h, followed by incubation with Cy3-conjugated goat anti-mouse secondary antibody (Jackson, Bar Harbor, USA) for 1 h. For HA tag immunostaining, cells were incubated with anti-HA antibody (Sigma) for 1 h and then FITC-conjugated goat anti-mouse secondary antibody (Jackson) for 1 h. Antibodies were diluted in the blocking buffer. All the procedures were performed at room temperature since cell fixation. Image observations were performed with a TCS-SP2 Leica confocal microscopy (Leica, Mannheim, Germany).

**Results**

**B23 associates with PES1 in vivo and they are co-localized under normal and stress conditions**

Previous reports indicated that PES1 was predominantly localized in the nucleolus [22], while it is not clear what factors maintain nucleolar localization of PES1. Since B23 was proposed as one of the hub proteins in the nucleolus that were able to interact with multiple nucleolar proteins [14], we tested whether there was physical interaction between B23 and PES1 with a reciprocal immunoprecipitation assay. Proliferating HeLa cells were lysed and whole-cell lysates were immunoprecipitated with anti-B23 or anti-PES1 antibody (8E9) (Ascenion, Munich, Germany). Results indicated that endogenous B23 and PES1 could be co-precipitated, suggesting a physical association between B23 and PES1 in vivo [Fig. 1(A)].

Next we examined the cellular localizations of B23 and PES1. To facilitate localization studies, a HeLa cell line stably expressing GFP-PES1 was generated. Consistent with previous studies [22], GFP-PES1 exhibited the same nucleolar localization pattern as endogenous PES1. Under normal physiological conditions, B23 and GFP-PES1 showed co-localization in the nucleolus [Fig. 1(B), Con]. Since B23 had been reported to translocate out of the nucleolus after exposure of cells to a variety of stresses [28,29], we treated the cells with actinomycin D (0.5 μg/ml) for 12 h before immunostaining.

Figure 1 In vivo interaction and co-localization of B23 and PES1 (A) Co-immunoprecipitation of endogenous B23 and PES1. HeLa cells were lysed with the lysis buffer and 5% of the lystate was kept as input. On the left panel, equal amounts of lysate were immunoprecipitated (IP) with anti-B23 antibody or normal mouse IgG, and then the precipitates were immunoblotted (IB) against PES1 and B23. Reverse immunoprecipitation using the anti-PES1 antibody was shown on the right panel. (B) Co-localization of B23 and PES1 under normal and stress conditions. HeLa cells stably expressing GFP-PES1 were treated with or without actinomycin D (ActD) (0.5 μg/ml) for 12 h, fixed and then immunostained against B23 (red). The overlay images of the two proteins were labeled ‘Merge’. Cell nuclei were visualized by DAPI staining. Bar = 10 μm.
Results showed that GFP-PES1 was also highly co-localized with B23 under the stress caused by actinomycin D [Fig. 1(B), ActD], indicating that PES1 and B23 might have co-localization under stress situations.

Mapping interaction domains between B23 and PES1

To determine the domains involved in B23–PES1 interaction, we constructed a series of deletion mutants of each protein and tested their binding activities utilizing GST and His pull-down assays. The human PES1 protein has 588 amino acids and it contains an evolutionarily highly conserved N-terminal pescadillo-like protein domain (NPLP-domain, Pfam-database PF06732), a BRCT domain (aa 322–415), and two stretches of acidic domains near the C-terminus [Fig. 2(A), upper panel] [30]. In search of domains in PES1 required for B23 binding, bacterially purified GST and GST-PES1 proteins were used to pull-down endogenous B23 from HeLa cell lysate. Results showed that N-terminus (aa 1–322) of PES1 was essential for B23 binding as GST-PES1 (322-588) lost binding activity to B23 [Fig. 2(A), lane 5]. BRCT domain of PES1 is dispensable for B23 binding since the BRCT domain deletion mutant, GST-PES1 (Δ322–415), was able to bind to B23 [Fig. 2(A), lane 6]. In addition, binding activity of GST-PES1 (101–588) and GST-PES1 (220–588) gradually decreased when more truncation was made to the protein [Fig. 2(A), lanes 3, 4].

Similar His pull-down approach was employed to map domains of B23 critical for PES1 interaction. Various functional domains have been identified within B23 protein, including N-terminal oligomerization domain bearing most of the in vitro chaperone activity, the C-terminal domain mediating nucleic acid binding, and two central acidic domains for histone binding [Fig. 2(B), upper panel] [5]. Pull-down data revealed that neither His-B23 (1–119) nor His-B23 (191–294) could significantly pull-down endogenous PES1 [Fig. 2(B), lanes 4 and 6], indicating that the two central acidic domains (aa 120–190) were required for PES1 binding. Interestingly, His-B23 (1–257) and His-B23 (1–195) exhibited enhanced PES1 binding activity compared with B23 full-length protein [Fig. 2(B), lanes 1–
Thus the C-terminal domain of B23 probably plays an inhibitory role in the interaction between B23 and PES1.

**B23 is involved in nucleolar localization of PES1**

The physical interaction between B23 and PES1 and the proposed role of B23 as a hub protein in the nucleolus strongly suggest that B23 might regulate the nucleolar localization of PES1. Using RNA interference, we specifically down-regulated B23 level in GFP-PES1 stable cell line cells [Fig. 3(B)], and then analyzed the localization of PES1 using confocal microscopy. Data showed that down-regulation of B23 resulted in increased nucleoplasmic distribution of GFP-PES1 compared with the negative control [Fig. 3(A)]. The morphology of the nucleolus remained intact while knocking down B23, as shown by the differential interference contrast (DIC) images, thus ruling out the possibility that distribution of GFP-PES1 in the nucleoplasm was due to a disrupted nucleolus. Furthermore, we carried out a biochemical fractionation study on HeLa cells. Consistently, more endogenous PES1 molecules accumulated in the nucleoplasmic fraction (Np) while knocking down B23 [Fig. 3(C)]. Furthermore, we examined the cellular localization of the mutant PES1(322–588) which lost binding activity to B23. Confocal images showed that PES1(322–588) was not able to locate at the nucleolus while the full-length PES1 (aa 1–588) exhibited nucleolar localization (Fig. 4). Taken together, these results suggest that B23 might be an important factor that maintains PES1 in the nucleolus.

**Discussion**

In this study, we identified a novel protein–protein interaction between B23 and PES1 and showed that B23 is involved in the nucleolar localization of PES1. B23 has been proposed as a hub protein for many nucleolar targeting proteins [14]. Our results give a new evidence to further support the role of B23 in protein nucleolar targeting. A recent study demonstrated that depletion of B23 lead to distortion of nucleolar and nuclear structures with micronuclei formation [31], which highlighted the role of B23 in the maintenance of nucleolar and nuclear structure integrity. In our study we had a relatively low B23 knocking-down efficiency (approximately 50%, Fig. 3(B)), in which the amount of B23 remained in the cells was enough to maintain the integrity of nucleolar and nuclear structure, as shown by the DIC images [Fig. 3(A)]. Therefore, the nucleoplasmic accumulation of PES1 after B23 down-regulation in our study was not simply a consequence of nucleolar distortion.

PES1 is predominantly localized in the nucleolus and forms a stable complex with Bop1 and WDR12 named as PeBoW complex, which plays a critical role in pre-rRNA processing and 60S ribosomal subunit maturation [18]. Studies on the interdependence of the three protein in controlling nucleolar localization of each protein revealed that transport of Bop1 from the cytoplasm to the nucleolus is PES1 dependent, while PES1 could migrate to the nucleolus independently of Bop1 [19]. Our current findings suggest additional information to the nucleolar localization of PES1, which might be B23 dependent. Therefore, it is tempting to predict that B23 may be involved in the formation of PeBoW complex in the nucleolus for ribosome biogenesis.

Though we have demonstrated that B23 could regulate the nucleolar localization of PES1, there still remain...
some other possible physiological implications for their interaction. Ribosome biogenesis is a coordinated process involving many different proteins acting at various stages from early rDNA transcription, followed by pre-rRNA processing, ribosome subunit assembly and nuclear export of the ribosome [32]. B23 has been reported to participate in several steps of ribosome biogenesis including assembly of pre-ribosomal particles [33,34] and processing of the 32S rRNA precursor into 28S rRNA [4]. In addition, PES1 has also been implicated in the maturation process of 28S rRNA [17,19,25,30]. Whether B23 and PES1 participate in the ribosome biogenesis in a specific protein complex should be further studied. Moreover, both B23 and PES1 have been implicated in various cancers [1,2,22,23]. On the basis of their physical interaction, it would be of particular interest to investigate whether these two proteins promote cancer development in the same signaling pathway.

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