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Thr[199] phosphorylation targets nucleophosmin to nuclear speckles and represses pre-mRNA processing

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Abstract **Nucleophosmin (NPM) is a multifunctional phosphoprotein, being involved in ribosome assembly, pre-ribosomal RNA processing, DNA duplication, nucleocytoplasmic protein trafficking, and centrosome duplication. NPM is phosphorylated by several kinases, including nuclear kinase II, casein kinase 2, Polo-like kinase 1 and cyclin-dependent kinases (CDK1 and 2), and these phosphorylations modulate the activity and function of NPM. We have previously identified Thr[199] as the major phosphorylation site of NPM mediated by CDK2/cyclin E (and A), and this phosphorylation is involved in the regulation of centrosome duplication. In this study, we further examined the effect of CDK2-mediated phosphorylation of NPM by using the antibody that specifically recognizes NPM phosphorylated on Thr[199]. We found that the phospho-Thr[199] NPM localized to dynamic sub-nuclear structures known as nuclear speckles, which are believed to be the sites of storage and/or assembly of pre-mRNA splicing factors. Phosphorylation on Thr[199] by CDK2/cyclin E (and A) targets NPM to nuclear speckles, and enhances the RNA-binding activity of NPM. Moreover, phospho-Thr[199] NPM, but not unphosphorylated NPM, effectively represses pre-mRNA splicing. These findings indicate the involvement of NPM in the regulation of pre-mRNA processing, and its activity is controlled by CDK2-mediated phosphorylation on Thr[199].**

Keywords: Nucleophosmin; mRNA splicing; CDK2; Cyclin E; Nuclear speckles; Splicing factor

1. Introduction

Nucleophosmin (NPM), also known as B23, NO38 or numatrin, is a multifunctional phosphoprotein, and has been implicated in a wide variety of cellular events, including ribosome assembly and pre-ribosomal RNA processing in nucleolus [1–4], DNA duplication [5–7], nucleocytoplasmic protein trafficking through directly binding to the nuclear localization signals (NLS) of the target proteins [8–11], and centrosome duplication [12,13]. In addition, NPM has been shown to possess interesting properties, including RNA-binding and molecular chaperoning activities [14–17]. NPM is phosphorylated by several different kinases, including casein kinase 2 (CK2), nuclear kinase II, Polo-like kinase 1 (PLK1) and cyclin-dependent kinases (CDK1/cyclin B, CDK2/cyclin E, and CDK2/cyclin A) [12,13,17–22]. Phosphorylation by CK2 increases NPM's affinity to the NLS sequences derived from the SV40 large T antigen and the HIV Rev protein [10,11] as well as to modulate its molecular chaperoning activity, especially for its interaction with target proteins [21]. Phosphorylation of NPM on Ser[4] by PLK1 has been shown to play a role in numeral homeostasis of centrosomes as well as cytokinesis [22]. Phosphorylation by CDK2/cyclin E (and A) on Thr[199] of NPM is critical for the regulation of centrosome duplication by affecting its binding affinity to centrosomes [13].

It has been shown that NPM binds and alters the secondary structure of RNA [15,17]. Moreover, the RNA-binding activity of NPM is controlled by phosphorylation. For instance, CDK1/cyclin B phosphorylates NPM on several residues, which results in a decrease in the RNA-binding affinity of NPM [17]. These observations suggest the role of NPM in RNA transcription, metabolism, and/or processing. Indeed, NPM was co-purified from HeLa cell nuclear extracts with general splicing activator RNPS1, which physically interacts with serine/arginine-rich (SR) splicing factors, pinin, human Tra2β, and CK2 to regulate splicing in vivo [23–25], suggesting its potential association with pre-mRNA splicing. Pre-mRNA splicing occurs in a macromolecular complex known as the spliceosome, which consists of five small nuclear ribonuclear particles (snRNPs) and a large number of non-snRNP protein splicing factors (reviewed in [26,27]). The phosphorylation state of splicing factors appears to be critical for at least two events during the pre-mRNA splicing process; (1) spliceosome formation and (2) selection of pre-mRNA splice sites (reviewed in [28]). Pre-mRNA splicing factors are mostly confined to 20–50 irregularly shaped nuclear speckles within the nucleoplasm, which are believed to be the sites of storage and/or assembly of pre-mRNA splicing factors (reviewed in [29]). Ultrastructural studies have revealed that the nuclear speckles consist of interchromatin granule clusters (IGCs) and perichromatin fibrils (PFs) (reviewed in [30,31]). IGCs are composed of particles measuring 20–25 nm in diameter, and they contain numerous factors that are involved in mRNA synthesis and processing. IGC constituents include small nuclear ribonucleoprotein particles (snRNPs), SR splicing factors, and hyperphosphorylated form of the large subunit of RNA polymerase II [32]. Despite the presence of splicing factors in IGCs, pre-mRNA processing does not occur within these structures, but rather at PFs, which are found at the periphery of or at some distance away from IGCs [33].

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In this communication, we exploited the biological significance of the Thr[199] phosphorylation of NPM in cellular event(s) other than centrosome duplication by the use of the antibody that specifically recognizes NPM phosphorylated on Thr[199] (phospho-Thr[199] NPM). We found that phospho-Thr[199] NPM localizes at nuclear speckles. As expected from the cell cycle phase-specific activation of CDK2/cyclin E as well as CDK2/cyclin A, appearance of phospho-Thr[199] NPM occurs in a cell cycle-dependent manner. Moreover, CDK2/cyclin E-mediated phosphorylation of NPM significantly enhances its RNA-binding affinity, and represses pre-mRNA splicing in vitro. These findings suggest the involvement of NPM in the regulation of pre-mRNA processing, which is modulated by CDK2/cyclin E (and A)-mediated phosphorylation on Thr[199].

2. Materials and methods

2.1. Cell and transfection

Wild-type mouse skin fibroblasts (MSFs) were prepared from abdominal skins of an 8-week-old C57L male mouse, and maintained in complete medium [DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml)] in an atmosphere containing 10% CO_2. Plasmid transfection was performed using Fugene 6 reagent (Roche).

2.2. Antibodies

Anti-pan NPM mouse monoclonal antibody is a gift from Dr. P.K. Chan (Baylor College of Medicine). Anti-phospho-Thr[199] rabbit polyclonal antibody (Cell Signaling Technology) was generated against a synthetic phospho-peptide around Thr[199] of human NPM. The antibodies were purified by protein A and affinity chromatography. Anti-cyclin E (M-20), anti-cyclin A (C-19), anti-cyclin B (SC-55), anti-hnRNP I and anti-SF2/ASF antibodies were purchased from Santa Cruz Biotechnology. Anti-FLAG (M2) and anti-α-tubulin (DM1A) antibodies were purchased from Sigma Immunochemicals. Anti-T7-tag antibody was purchased from Novagen. Generation of mouse anti-SC35 antibody was previously described [34].

2.3. Immunoblot analysis

Cells were washed three times with PBS and lysed in SDS/NP-40 lysis buffer [1% SDS, 1% NP-40, 50 mM Tris (pH 8.0), 150 mM NaCl, 4 mM Pefabloc SC (Roche), 2 μg/ml leupeptin, 2 μg/ml aprotinin]. The lysates were boiled for 5 min, and cleared by a 10 min centrifugation at 20 000 × *g* at 4 °C. The supernatant was denatured at 95 °C for 5 min in sample buffer [2% SDS, 10% glycerol, 60 mM Tris (pH 6.8), 5% β-mercaptoethanol, 0.01% bromophenol blue]. Samples were resolved by SDS–PAGE, and transferred onto Immobilon-P sheets (Millipore). The blots were incubated in blocking buffer [5% (wt/vol) nonfat dry milk in Tris-buffered saline (TBS) + Tween 20 (TBS-T)] for 1 h at room temperature. The blots were incubated with primary antibody for overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The antibody-antigen complex was visualized by ECL chemiluminescence (Amersham Biosciences).

2.4. Indirect immunofluorescence

Cells grown on coverslips were fixed with 10% formalin/10% methanol for 20 min at room temperature. The cells were permeabilized with 1% NP-40 in PBS for 5 min, followed by incubation with blocking solution [10% normal goat serum in PBS] for 1 h. Cells were then probed with primary antibodies for 1 h, and antibody-antigen complexes were detected with either Alexa Fluor 488- or Alexa Fluor 594-conjugated goat secondary antibody (Molecular Probes) by incubation for 1 h at room temperature. The coverslips were washed three times with PBS after each incubation, and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Immunostained cells were examined under a fluorescence microscope (Zeiss Axioplan 2 Imaging, 60× objective lens) or confocal microscope (Zeiss LSM510, 63× objective lens).

2.5. In vitro kinase assay

GST, GST-NPM/wt, or GST-NPM/T199A were incubated with baculovirally purified active CDK2/cyclin E [35] or CK2 (New England Biolabs). The enzymatic activities of both CDK2/cyclin E and CK2 were confirmed by in vitro kinase reactions in the presence of [γ-^{32}P]ATP using GST-NPM/wt as a substrate. The in vitro kinase reactions were performed in 10 mM PIPES buffer in the presence of ATP at 32 °C for 30 min. The reaction samples were resolved by SDS–PAGE.

2.6. Alkaline phosphatase treatment

Cells grown on coverslips were fixed with 100% methanol for 10 min at −20 °C. Cells were then air-dried, and re-hydrated in PBS for 10 min at room temperature. Cells were incubated in the solution [100 mM glycine (pH 10.4)] containing 10 units of alkaline phosphatase type IV (Sigma) for 2 h at 37 °C. The control cells were incubated in the solution without alkaline phosphatase.

2.7. RNA binding assay

The assay was performed as previously described [17]. Briefly, RNA was extracted from MSFs using TRIZOL (Gibco BRL). GST-NPM proteins were subjected to an in vitro kinase assay with CDK2/cyclin E, and mixed with RNA for 30 min at room temperature. The samples were loaded onto 15–40% sucrose gradient [20 mM Tris (pH 7.4), 50 mM NaCl, 0.5 mM PMSF, and 1 mM dithiothreitol], centrifuged at 39 000 rpm for 4 h, and fractions were collected from the bottom. The fractions were resolved in 10% SDS–PAGE for immunoblot analysis, and on 1% agarose-formaldehyde gel electrophoresis for Northern blot analysis using ^{32}P-labeled 18S rRNA DNA probe (Ambion).

2.8. Northwestern analysis

Bacterially purified GST, GST-NPM/wt, GST-NPM/T199A, or GST-NPM/T199D proteins were phosphorylated with CDK2/cyclin E, and re-purified. The proteins were separated by SDS–PAGE and transferred to a membrane. The membrane was blocked with 5% non-fat dry milk in RNA-binding buffer (RBB) [20 mM Tris–HCl (pH 7.5), 60 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol] containing 2 μg/ml yeast tRNA for 1 h, and then incubated in the RBB containing 0.25% non-fat dry milk, 2 μg/ml yeast tRNA, 1 U/ml RNase inhibitor, and ^{32}P-labeled β-globin pre-mRNA for 16 h. The membrane was washed, air-dried, and autoradiographed.

2.9. In vitro splicing assay

The m^{7}GpppG-capped ^{32}P-labeled pre-mRNA was made by run-off transcription of linearized β-globin template DNA with SP6 RNA polymerase, which was used as a substrate for in vitro splicing assay in HeLa cell nuclear extract [36]. Bacterially purified GST, GST-NPM/wt, GST-NPM/T199A, or GST-NPM/T199D proteins were phosphorylated with CDK2/cyclin E, and re-purified. The mock or CDK2/cyclin E phosphorylated GST, GST-NPM/wt, GST-NPM/T199A, or GST-NPM/T199D was added to splicing reactions (in 25 μl) with 20 fmol of ^{32}P-labeled β-globin pre-mRNA and incubated at 30 °C for 2–3 h. The spliced products were analyzed by denaturing 5.5% PAGE and autoradiography.

3. Results

3.1. NPM phosphorylated on Thr[199] localizes in a speckled pattern in the nuclei

NPM is phosphorylated on Thr[199] primarily by CDK2/cyclin E and A, and this phosphorylation is important in the regulation of centrosome duplication [13]. Since centrosome duplication occurs in coordination with other cell cycle events, and NPM is involved in a variety of cellular events, it is possible that CDK2-mediated phosphorylation of NPM may affect more than one cellular event. To test this possibility, we obtained an antibody, which was generated against phospho-Thr[199] of NPM. We first tested the specificity of the anti-phospho-Thr[199] NPM antibody. The bacterially purified wild-type