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NEK2 phosphorylation antagonizes the microtubule stabilizing activity of centrobin

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ABSTRACT

Centrobin was initially identified as a centrosome protein for centriole duplication. Centrobin is also detected outside the centrosome and involved in other cellular functions, such as spindle assembly. We previously reported that centrobin is a substrate of both NEK2 and PLK1, but it is not clear what functional properties of centrobin are regulated by two kinases. Here, we report that centrobin is involved in cell spreading, migration and microtubule stabilization in interphase cells. The NEK2-depleted cells looked spread with well-developed microtubule networks and migrated faster than the control cells. The microtubule stability in NEK2-depleted cells was higher than the control cells. However, the opposite was the case in centrobin-depleted cells. The opposite outcomes in NEK2- and centrobin-depleted cells suggest that NEK2 antagonizes biological functions of centrobin. We identified NEK2 phosphorylation sites within centrobin, which is distinct from the PLK1 phosphorylation sites. In fact, the phospho-resistant mutant of centrobin against NEK2 stabilized microtubule networks in vivo. Based on the results, we propose that NEK2 phosphorylation antagonizes the microtubule stabilizing activity of centrobin. Centrobin is a novel example that NEK2 and PLK1 independently phosphorylate a substrate and result in opposite outcomes in substrate function.

1. Introduction

An interphase cell includes a mixed population of microtubules. Some microtubules show dynamic instability with a short half-life, while the other microtubules are stable with a long half-life [1]. Stable microtubules are frequently modified by acetylation, and become resistant to depolymerizing agents such as nocodazole [2]. A significant change in microtubule dynamics is accompanied when a cell enters mitosis [3,4]. Most microtubules in mitotic cells are short and dynamic to function as spindles. Phosphorylation is one of key regulatory mechanisms for microtubule dynamics during the cell cycle [5].

NEK2 is a serine/threonine kinase whose activity oscillates during the cell cycle [6]. The kinase activity of NEK2 is high at S and G2 phase and low at M and G1 phase [7]. An augmented activity of NEK2 at G2 phase contributes to centrosome separation by phosphorylating C-NAP1 and rootletin, components of inter-centriolar linker proteins [8–10]. Nek2 proteins are also localized in both the nucleus and cytoplasm throughout the cell cycle, and exhibited dynamic changes in distribution, depending on the cell cycle stage [11]. Nek2 is associated with chromosomes from prophase to metaphase and then is dissociated upon entering into anaphase [11,12]. This dynamic distribution of NEK2 implies its involvement in multiple cellular functions during the cell cycle [11]. For example, NEK2 is located at the kinetochore of mitotic cells and regulates signaling of the spindle assembly checkpoint through the HEC1 phosphorylation or interaction with MAD1 [13–15]. NEK2 is also critical for bipolar spindle pole formation in acentrosomal mouse oocytes and early embryos [16,17]. Currently available data consistently support that NEK2 as well as other NEK kinases coordinates microtubule-dependent processes in both inside and outside the centrosome [6].

Centrobin was originally identified as a daughter centriole-associated protein [18]. Depletion of centrobin results in centrosomes with one or no centriole, demonstrating that centrobin is required for centriole duplication [18]. It was proposed that centrobin facilitates the elongation and stability of centrioles via its interaction with tubulins [19]. Centrobin is also detected outside the centrosome and involved in other cellular functions [20]. Centrobin-depleted cells show a range of spindle abnormalities including unfocused poles that are not associated with centrosomes, S-shaped spindles and mini spindles [20,21]. In fact, centrobin is associated with microtubules and promotes microtubule polymerization and stabilization in vitro [22].

We previously observed that centrobin is a substrate of both NEK2 and PLK1 [20,22]. However, it is not clear what functional properties of centrobin are regulated by two kinases. In this study, we elucidate biological functions of centrobin in cell spreading and migration. Furthermore, we investigated how NEK2 regulates the microtubule stabilizing functions of centrobin.

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2. Materials and methods

2.1. Antibodies, transfection and RNA interference

α-Tubulin (Sigma), acetylated α-tubulin (Sigma), γ-tubulin (Sigma), HA (Sigma), FLAG (Sigma) and NEK2 (BD bioscience) antibodies were used according to the manufacturer's instruction. Centrobin antibodies were used as previously described [20]. Transient transfection of plasmid DNA was performed using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instruction. For RNA interference, siRNAs specific to *NEK2* (5′-GGCAAATTCAGGCGAATTC-3′), *NEK2*-3′UTR (5′-GCTGTAGTGTTGAATACTT-3′) and *centrobin* (5′-GGATGGTTCTAAGCATATC-3′) were purchased from ST Pharm and transfected into the cells using Lipofectamine RNAi max (Invitrogen) according to the manufacturer′s instruction. Non-specific control siRNA (5′-AAGTAGCCGAGCTTCGATTGC-3′) was also used.

2.2. Cell culture and stable cell lines

293T, HeLa and tet-on HeLa cells were cultured in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% FBS. U2OS cells were cultured in McCoy's 5A media supplemented with 10% FBS. RPE1 cells were cultured in DMEM F12 supplemented with 10% FBS. Stable tet-on HeLa cell lines were generated with Lenti-X HT packaging system (Clontech) according to manufacturer's instruction using pLVX-IRES-Puro vector which is substituted its original promoter with tet-responsive promoter.

2.3. Immunoblot and immunoprecipitation

For immunoblot analysis, protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a nitrocellulose membrane. The membrane was incubated with a primary antibody for 2 h after blocking with 5% skim milk in 0.1% TBST (Tris-buffered saline TBS with 0.1% Triton X-100) for 30 min, and incubated with a horseradish peroxidase-conjugated secondary antibody for 30 min after washing three times with 0.1% TBST. And then, the membrane was incubated with the ECL solution after washing three times with 0.1% TBST, and exposed to an X-ray film. For immunoprecipitation, the cells were lysed with the NP40 buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40) with protease inhibitors for 20 min on ice and centrifuged with 15,000*g* for 20 min at 4 °C. The supernatant was incubated with specific antibodies for 2 h, followed by protein A Sepharose (Amersham Pharmacia) for 2 h at 4 °C.

2.4. Immunocytochemistry and image processing

For immunochemistry, the cells cultured on coverslip were fixed with cold methanol for 10 min after washing with phosphate-buffered saline (PBS). The fixed cells were blocked with 5% bovine serum albumin (BSA) in 0.1% PBST (PBS with 0.1% Triton X-100), incubated with the primary antibodies for 1 h, washed with 0.1% PBST three times, and incubated with secondary antibodies for 30 min. And then, the cells were washed three times with 0.1% PBST, incubated with DAPI solution to stain DNA, and the coverslip was mounted on a glass slide. The immunostained cells were observed using fluorescence microscope with a CCD (Qicam Fast 1394; Qimaging) camera and processed with ImagePro 5.0 (Media Cybernetics, Inc.) software.

2.5. Nocodazole-resistance assay

Nocodazole-resistance assay was performed as previously described with a slight modification [23]. In brief, the cells cultured on coverslip were treated with 2 mM thymidine for 16 h to synchronize cell cycle and incubated with 2 μM nocodazole for the last 30–60 min at 37 °C. And then, the cells were rinsed twice in PEM buffer (100 mM PIPES [pH 6.9], 1 mM EGTA, 2 mM $MgCl_2$), incubated 1 min at 37 °C with 0.2% Triton X-100 in PEM to remove monomeric tubulin, rinsed again twice in PEM buffer, and fixed with cold methanol. After fixation, the cells were subjected to immunocytochemistry using acetylated α-tubulin antibody. To quantitative analysis, the cells were scored for the presence of more than 10 acetylated microtubules per cell.

2.6. In vitro kinase assay

For the preparation of NEK2 kinases, 293T cells transfected with wild-type NEK2 (*pNEK2RHA1*) or kinase-dead NEK2 (*pNEK2KHA5*) expression vectors were lysed with NP40 lysis buffer and subjected to immunoprecipitation with an antibody against the HA tag. The immunoprecipitates were washed twice with lysis buffer and once with kinase buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 1 g/ml heparin). The centrobin substrates were prepared from bacterially expressed fusion proteins. Kinase reactions were carried out for 30 min at 30 °C in kinase buffer supplemented with 5 μM ATP, 1 mM dithiothreitol and 5 μCi [γ-^{32}P]ATP in a total volume of 20 μM. The reactions were stopped by adding 2× SDS sample buffer and heating for 5 min at 95 °C. Protein samples were subjected to SDS–PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was exposed to a BAS plate or an X-ray film to obtain an autoradiograph image, and then stained with Coomassie brilliant blue solution.

2.7. Cell migration and cell spreading

For cell migration assay, cells were grown to subconfluency and treated with 10 μg/ml Mitomycin C (Sigma) for 3 h to arrest cell proliferation. And then, a wound track was introduced by scraping the cell monolayer with a yellow pipette tip. After rinsing with PBS, the cells were cultured in growth medium for a further 24 h and the recovery area was measured. For cell spreading assay, cells were treated with 2 mM thymidine for 16 h to synchronize cell cycle, and the cell area was measured. For measurement of the area, phase-contrast images were analyzed using ImagePro 5.0 software.

3. Results

3.1. NEK2 antagonizes centrobin functions in cell spreading and migration

We previously reported that centrobin is a substrate of NEK2 [20]. To have an insight into the functional outcomes of NEK2 phosphorylation on centrobin, we compared the knockdown phenotypes of NEK2 and centrobin in cell morphology. The results showed that the NEK2-depleted HeLa, U2OS and RPE1 cells looked spread with well-developed microtubule networks (Fig. 1A). On the other hand, the centrobin-depleted cells appeared shrunk with disrupted microtubule networks (Fig. 1A).

Next, we examined the cell migration ability of the NEK2- or centrobin-depleted cells. The results showed that the NEK2-depleted cells migrated faster than the control cells but the opposite was the case in centrobin-depleted cells (Fig. 1B). The opposite outcomes in NEK2- and centrobin-depleted cells suggest that NEK2 antagonizes biological functions of centrobin.