Biochemical and Biophysical Research Communications 468 (2015) 113–118

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Death-associated protein kinase 2 mediates nocodazole-induced apoptosis through interaction with tubulin

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ARTICLE INFO

Article history:
Received 10 October 2015
Accepted 28 October 2015
Available online 31 October 2015

Keywords:
DAPK2
Apoptosis
Tubulin
Nocodazole

ABSTRACT

Death-associated protein kinase 2 (DAPK2) is a positive regulator of apoptosis. Although we recently reported that 14-3-3 proteins inhibit DAPK2 activity and its subsequent apoptotic effects via binding to DAPK2, the molecular mechanisms underlying the DAPK2-mediated apoptotic pathway remain unclear. Therefore, we attempted to further identify DAPK2-interacting proteins using pull-down assays and mass spectrometry. The microtubule β-tubulin was identified as a novel DAPK2-binding protein in HeLa cells. Pull-down assays revealed that DAPK2 interacted with the α/β-tubulin heterodimer, and that the C-terminal region of DAPK2, which differs from that of other DAPK family members, was sufficient for the association with β-tubulin. Although the microtubule-depolymerizing agent nocodazole induced apoptosis in HeLa cells, the level of apoptosis was significantly decreased in the DAPK2 knockdown cells. Furthermore, we found that treatment with nocodazole resulted in an increased binding of DAPK2 to β-tubulin. These findings indicate that DAPK2 mediates nocodazole-induced apoptosis via binding to tubulin.

1. Introduction

The death-associated protein kinase (DAPK) family consists of five kinases, including DAPK1, DAPK2, and DAPK3, and plays an important role in cell death induced by a wide variety of apoptosis inducers, including interferon γ, tumor necrosis factor (TNF)-α, Fas ligand, and TNF-related apoptosis-inducing ligand [1–3]. The family possesses highly homologous N-terminal catalytic domains and diverse C-terminal domains. The variation in the C-terminal region suggests that the family members are controlled by different regulatory mechanisms and have distinct functions through different signaling pathways. DAPK1, the most well-studied member of the family, possesses a Ca^{2+}/calmodulin (CaM)-binding domain, eight ankyrin repeats, a cytoskeletal-binding domain, and a death domain at the C-terminal region. Binding of Ca^{2+}/CaM to the CaM-binding domain causes catalytic activation. On the other hand, the cytoskeletal-binding domain contributes to the subcellular localization of DAPK1 and consequently affects its physiological function, including the disruption of integrin signaling, promotion of anoikis, and suppression of cell motility [4,5]. DAPK3, also known as zipper-interacting protein kinase, contains two nuclear localization signals and a leucine zipper domain, which are involved in the nuclear localization and the interaction of DAPK3 with several transcriptional factors including activating transcription factor 4 and signal transducer and activator of transcription 3 [6]. Although DAPK2 shares a CaM-binding domain with DAPK1 and is activated by CaM in response to Ca^{2+} stimuli, its C-terminal region is short and does not include any other domains, such as the cytoskeletal-binding domain. Recently, we identified 14-3-3 proteins as the interaction partners of DAPK2 from human breast cancer MCF-7 cells [7]. 14-3-3 proteins bind to phosphorylated serine or threonine residues in defined consensus sequences. Interactions between 14-3-3 proteins and DAPK2 are dependent on the phosphorylation of Thr^{369} in the C-terminal region and effectively suppress DAPK2 kinase activity and DAPK2-induced apoptosis. However, the molecular mechanism of the DAPK2-mediated apoptotic signaling pathway remains to be elucidated.

Microtubules, which are composed of α/β-tubulin heterodimers, are a major component of the cytoskeleton. Microtubule dynamics (polymerization and depolymerization) are regulated by the

Abbreviations: DAPK, death-associated protein kinase; TNF, tumor necrosis factor; CaM, calmodulin; GST, glutathione S-transferase; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PARP, poly (ADP-ribose) polymerase.

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http://dx.doi.org/10.1016/j.bbrc.2015.10.151
0006-291X/

coordinated action of microtubule-stabilizing and -destabilizing factors and play an important role not only in cell migration and polarization but also in mitosis and the cell cycle, particularly the formation of the spindle apparatus. Antimicrotubule agents, including polymerizing (e.g., paclitaxel) and depolymerizing (e.g., vinca alkaloid and nocodazole) agents, interfere with these dynamic processes, resulting in apoptotic cell death. Therefore, several antimicrotubule agents are used as anticancer drugs [8]. Nocodazole is a well-known inhibitor of microtubule polymerization and arrests cell cycle progression at the G2/M-phase. Disruption of microtubules with nocodazole influences signal transduction events, such as the disruption of the complex between Smads and microtubules [9] and the phosphorylation of tubulin by cyclin-dependent kinase 1 [10].

In this study, we identified β-tubulin as a novel DAPK2-interacting partner using pull-down assays and mass spectrometry (MS). DAPK2 strongly interacted with tubulin during nocodazole-induced apoptosis. Furthermore, we found that DAPK2 knockdown resulted in a significant suppression of nocodazole-induced apoptotic cell death. These findings indicate that DAPK2 mediates nocodazole-induced apoptosis through interaction with tubulin.

2. Materials and methods

2.1. Antibodies

Antibody against DAPK2 was purchased from Millipore, and anti-Strep antibody was purchased from Qiagen. Anti-FLAG M2 antibody was purchased from Sigma. Anti-α-tubulin and anti-pan-14-3-3 antibodies were from Santa Cruz Biotechnology. Anti-PARP antibody and anti-14-3-3 ζ/δ antibodies were from Cell Signaling Technology, and anti-glutathione S-transferase (GST) and anti-β-tubulin antibodies were from Wako Pure Chemical Industries.

2.2. Plasmid construction

The expression plasmids pFLAG-DAPK2 and p105-DAPK2 encoding mouse DAPK2 are previously described [7,11]. Full-length human β-tubulin cDNA was cloned by PCR using specific primers. The PCR product was cloned into the TA-cloning vector pGEM-T Easy (Promega), and the inserted DNA sequences were confirmed by DNA sequencing. The full-length β-tubulin cDNA was subcloned into the mammalian expression vectors pFLAG-CMV-2 (Sigma) and pEXPR-IBA105 (IBA). Site-directed mutagenesis was performed using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa Bio) according to the manufacturer's instructions. The mutation was confirmed by DNA sequencing analysis.

2.3. Cell culture, transfection, and RNA interference

HeLa and HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2. Transfection and RNA interference were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The synthetic small interfering RNA (siRNA) oligonucleotide for DAPK2 was purchased from Sigma (ID# SASI_Hs01_00206405). MISSION siRNA Universal Negative Control #1 (Sigma) was used as the negative control. Nocodazole treatment was performed after 24 h of transfection or knockdown.

2.4. Protein identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

HeLa cells were transfected with p105-DAPK2. After 24 h, the cells were scraped in cell lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 10 mM leupeptin, and 10 mg/ml aprotinin). The cell extracts were centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatants were incubated with Strep-Tactin Sepharose (IBA) overnight at 4 °C. The beads were washed with wash buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, and 1 mM EDTA), and the bound proteins were eluted with 6× SDS-loading buffer. The eluates were resolved by 10% SDS-PAGE, followed by silver staining (Silver Staining MS Kit; Wako Pure Chemical Industries). The subsequent protocol has previously been described [7]. Briefly, the bands in the gel were cut off and destained. After the protein bands were reduced and alkylated by dithiothreitol and iodoacetamide, respectively, trypsin digestion was performed overnight at 37 °C. The trypsin-digested peptides were extracted and desalted using C18 ZipTip (Millipore). The samples were mixed with the matrix, and then spotted onto the AnchorChip sample target (Bruker Daltonics). The mass spectra of the peptides were acquired by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Autoflex Speed; Bruker Daltonics) and processed using FlexAnalysis 3.3 and Biotools 3.2 (Bruker Daltonics). The generated data were analyzed using the Mascot Server (Matrix Science).

2.5. Co-immunoprecipitation and pull-down assays

For the Strep or GST pull-down assays, HEK293T or HeLa cells were co-transfected with DAPK2 and β-tubulin. After 24 h, the cells were scraped in lysis buffer, and the cell lysates were incubated with Strep-Tactin or Glutathione Sepharose overnight at 4 °C. The bound proteins were analyzed by immunoblot analysis using anti-FLAG M2 antibody, anti-Strep antibody, and/or anti-GST antibody.

2.6. Apoptotic cell death assay

Apoptotic cell death was evaluated using Cell Death Detection ELISA (Roche) according to the manufacturer's instructions. After 24 h of nocodazole treatment, the cells were harvested with incubation buffer. After centrifugation, the cell lysates were added to a 96-well plate coated with anti-histone antibody. Following washing with wash buffer, a peroxidase-conjugated anti-DNA antibody was added to each well. After washing, 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid substrate solution for peroxidase was added to generate a colored reaction product. The absorbance of the samples was determined using the Infinite M200 plate reader (Tecan) at 405 nm.

2.7. Statistical analysis

All experiments were performed multiple times to confirm their reproducibility. One representative set of data was shown in the figures. The results were quantified using Image J software (NIH). Data were expressed as the mean ± standard error, and statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test using GraphPad Prism (GraphPad Software).

3. Results

3.1. Identification of β-tubulin as a novel binding partner to DAPK2

To understand the molecular mechanisms underlying DAPK2-induced apoptosis, we attempted to identify novel binding partners of DAPK2. We previously identified 14-3-3 proteins as the interaction partners of DAPK2 from human breast cancer MCF-7 cells by combined Strep pull-down assay and MALDI-TOF MS,