Biochemical and Biophysical Research Communications 496 (2018) 1222–1228

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Intracellular localization and binding partners of death associated protein kinase-related apoptosis-inducing protein kinase 1

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ARTICLE INFO

Article history:
Received 12 January 2018
Accepted 29 January 2018
Available online 31 January 2018

ABSTRACT

Death associated protein kinase (DAPK)-related apoptosis-inducing protein kinase (DRAK)-1 is a positive apoptosis regulator. However, the molecular mechanisms underlying the DRAK1-mediated apoptotic pathway remain unclear. In this study, we demonstrated the intracellular localization and binding partners of DRAK1. In human osteosarcoma cell line U2OS cells, DRAK1 was mainly localized in the nucleus and translocated outside the nucleus through Ser^{395} phosphorylation by protein kinase C. In the nucleus, DRAK1 associated with tumor suppressor p53 and positively regulated p53 transcriptional activity in response to DNA-damaging agent cisplatin. On the other hand, DRAK1 interacted with the mitochondrial inner-membrane protein, adenine nucleotide translocase (ANT)-2, an anti-apoptotic oncoprotein, outside the nucleus. These findings suggest that DRAK1 translocates in response to stimuli and induces apoptosis through its interaction with specific binding partners, p53 and/or ANT2.

1. Introduction

Death associated protein kinase (DAPK)-related apoptosis-inducing protein kinase (DRAK) 1 is a member of the DAPK family comprising five types: DAPK1-DAPK3, DRAK1, and DRAK2 [1]. These family members have the highest homology in their Ser/Thr kinase domains at the N terminus, whereas their C-terminal regions largely differ and contain unique domains. DAPK1, which is the most studied of the family members, has a long sequence at its C terminus, comprising a calmodulin-binding domain, eight ankyrin-binding repeats, a ROC-COR domain, and a C-terminal death domain. These unique domains affect its function and localization through interaction with several protein partners [2,3]. For example, the death domain mediates an interaction with ERK, and ERK causes an increase in DAPK1 activity by phosphorylating DAPK1 [2]. Similarly, many studies report significant relationships between specific binding proteins and the physiological functions of the other DAPK family members [1,4]. However, although DRAK1 has been known to be exclusively localized in the nucleus and induce apoptosis [5], the molecular mechanism of the DRAK1-mediated apoptotic signaling pathway, including its binding partners, remains to be elucidated.

A recent study showed that DRAK1 is a direct target gene of tumor suppressor p53 [6]. p53 is activated by various cell stresses, such as DNA damage and activated oncogenes, thereby inducing the transcription of genes that promote cell death and growth arrest (e.g., p21, the proapoptotic protein Bax) and inhibiting the transcription of several cell-survival genes (e.g., survivin, the anti-apoptotic protein Bcl-2) [7]. DRAK1 was upregulated by cisplatin, a DNA-damaging agent in various cell lines in a p53-dependent manner, and p53 directly bound to an upstream element in *DRAK1* [6]. In response to DNA damage, p53 function is regulated by phosphorylation at multiple sites. Casein kinase 2 (CK2) phosphorylates p53 at Ser^{392}, thereby enhancing DNA binding ability [8]. In addition, DAPK1 phosphorylates p53 at Ser^{23} (Ser^{23} in mice and Ser^{20} in humans), and p53 transcriptionally regulates apoptosis-promoting genes, such as *Bax* [3].

Proapoptotic proteins Bax and Bad can regulate the channel activity of mitochondrial adenine nucleotide translocases (ANTs) that control the mitochondrial apoptosis pathway [9,10]. The ANT

Abbreviations: DAPK, death associated protein kinase; DRAK, DAPK-related apoptosis-inducing protein kinase; ANT, adenine nucleotide translocase; VDAC, voltage-dependent anion channel; NLS, nuclear localization signal; PKC, protein kinase C; PMA, phorbol myristate acetate.

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https://doi.org/10.1016/j.bbrc.2018.01.175
0006-291X/

family comprises four members, ANT1-ANT4, which are abundant in the inner mitochondrial membrane, catalyze the exchange of ADP/ATP, and regulate mitochondrial membrane permeability together with a voltage-dependent anion channel (VDAC) [9,11]. The expressions of ANT isoforms differ depending on the cell type. ANT1 is abundantly expressed in terminally differentiated cells, whereas ANT2 is highly expressed in undifferentiated cells with proliferating ability. Importantly, ANT2 expression is higher than that of ANT1 in several types of human cancer cells. ANT1 plays a proapoptotic role, whereas ANT2 functions as an antiapoptotic factor in cancer cells.

In this study, we investigated the mechanism of intracellular localization of DRAK1. DRAK1 was localized in the nucleus by two nuclear localization signal (NLS) sequences, and translocated outside the nucleus in response to stimuli that activate protein kinase C (PKC). Furthermore, we identified p53 and ANT2 as novel DRAK1-interacting partners. DRAK1 positively regulated p53 transcriptional activity in cisplatin-treated human osteosarcoma. These findings suggest that subcellular localization of DRAK1 through interaction with specific binding partners may be important for DRAK1 signaling.

2. Materials and methods

2.1. Antibodies

Antibody against DRAK1 was purchased from Novus Biologicals. Anti-p53, anti-phospho-p53 (Ser-392), and anti-phospho-(Ser) PKC substrate antibodies were from Cell Signaling Technology, and anti-GST and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were from Wako. Anti-Strep antibody was purchased from Qiagen, and anti-FLAG antibody was from Sigma-Aldrich.

2.2. Plasmid construction

Full-length human DRAK1, DRAK2, and ANT2 cDNAs were cloned by PCR using specific primers. The DRAK1 and DRAK2 cDNAs were subcloned into a mammalian expression vector, pEXPR-IBA105 (N-terminal Strep tag) (IBA). The ANT2 cDNA was inserted in-frame into pCMV-3Tag-3A vector (C-terminal 3x FLAG tag) (Agilent Technologies). Site-directed mutagenesis was performed using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa).

2.3. Cell culture, transfection, and RNA interference

U2OS, HEK293T, and COS-7 cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfection and RNA interference were performed using Lipofectamine 2000 (Thermo Fisher Scientific). The synthetic siRNA oligonucleotide for DRAK1 was purchased from Sigma (ID# SASI_Hs01_00136992). MISSION siRNA Universal Negative Control #1 (Sigma) was used as the negative control. Cisplatin was dissolved in 100% N,N-dimethyl formamide (DMF), and the final concentration of DMF in the culture medium was 0.1%.

2.4. Immunofluorescence analysis

Immunofluorescence analysis was performed as previously described [12]. In brief, U2OS cells were transfected with various expression plasmids in the presence or absence of mitochondria-targeted mKeima-Red expression plasmid, pMT-mKeima-Red (MBL). After 24 h, cells were fixed, permeabilized, and then blocked in 5% BSA. Cells were incubated with anti-Strep or anti-GST antibodies, followed by incubation with anti-IgG directly conjugated to AlexaFluor 488 or 555 (Thermo Fisher Scientific). Fluorescent images were obtained using an IN Cell Analyzer 6000 system (GE Healthcare).

2.5. Protein identification by MALDI-TOF MS

HEK293T cells were transfected with Strep-DRAK1 or DRAK2 expression plasmids. After 24 h, the cells were scraped in cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After centrifugation, the supernatants were incubated with Strep-Tactin Sepharose (IBA). After the beads were washed, the bound proteins were eluted with SDS-loading buffer and resolved by 12% SDS-PAGE, followed by silver staining. The subsequent protocol has previously been described [4].

2.6. Pull-down assay

HEK293T and COS-7 cells transfected with N-terminal Strep-tagged or GST-fused DRAK1 together with C-terminal FLAG-tagged ANT2 were scraped in lysis buffer, and cell lysates were incubated with Strep-Tactin or Glutathione Sepharose (GE Healthcare) overnight at 4 °C by rotation. The bound proteins were analyzed by immunoblot analysis using anti-FLAG antibody.

2.7. Luciferase reporter assay

U2OS cells were plated at a density of 2×10^4 cells in a 24-well plate. After 24 h, the cells were transfected with negative control siRNA or DRAK1 siRNA. Six hours later, cells were transfected with pGL4.38 [luc2P/p53 RE/Hygro] (Promega), which contains two tandem repeats of a p53 response element, together with pCMV-β-gal for 24 h. After treatment with 30 μM cisplatin for 24 h, the cells were lysed with cell culture lysis reagent (Promega) and subjected to luciferase reporter assay. Luciferase and β-galactosidase activities were measured as previously described [13].

2.8. 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. Subcellular localization of DRAK1 in U2OS cells

A previous study demonstrated that DRAK1 is exclusively localized in the nucleus of COS-7 cells [5]. However, a recent report showed that subcellular localization of DRAK1 is dependent on cell type, and that DRAK1 is localized in the cytoplasm but not the nucleus in head and neck squamous cell carcinoma cell lines [14]. Therefore, we first examined the subcellular localization of the N-terminal Strep-tagged DRAK1 wild type (Strep-DRAK1-WT) in human osteosarcoma cell line U2OS cells. Immunostaining analysis revealed that Strep-DRAK1-WT was localized mainly in the nucleus and only slightly localized in the cytoplasm in U2OS cells (Fig. 1B (i)). Similar results were obtained in HeLa cells (data not shown). Since DRAK1 was localized mainly in the nucleus, we examined whether the NLS sequence exists in the DRAK1 amino acid sequence. DRAK1 contains two patches of basic residues (Arg or