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Cyclophilin A regulates JNK/p38-MAPK signaling through its physical interaction with ASK1

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ABSTRACT

Cyclophilin A (CypA), a member of the immunophilin family, is predominantly localized in the cytoplasm. The peptidylprolyl isomerase (PPIase) activity of CypA has been demonstrated to be involved in diverse cellular processes, including intracellular protein trafficking, mitochondrial function, pre-mRNA processing, and maintenance of multiprotein complex stability. In this study, we have demonstrated that CypA regulates apoptosis signaling-regulating kinase 1 (ASK1) through its direct binding. ASK1 is a member of MAPK kinase kinase (MAP3K) family, and selectively activates both JNK and p38 MAPK pathways. Here, we also report that CypA negatively regulates phosphorylation of ASK1 at Ser966, and that CypA reduces ASK1 and its downstream kinases of the JNK and p38 signaling. ASK1 is known to induce caspase-3 activation and apoptosis, and CypA inhibited ASK1-mediated apoptosis by decrease in caspase-3 activity under cellular stress conditions. Overall, we conclude that CypA negatively regulates ASK1 functions by its physical interaction with ASK1.

1. Introduction

Cyclophilins (Cyps) are found in various species. Cyps belongs to the immunophilin family, and there are seven major Cyps in human. They are CypA, CypB, CypC, CypD, CypE, Cyp40 and CypNK [1]. The Cyps were originally discovered as intracellular binding protein for cyclosporine A (CsA). They are abundant, ubiquitous, and highly conserved cytosolic protein [2]. They have a peptidyl-prolyl *cis*–-transisomerase (PPIase) activity [1,3]. CypA is known to possess multiple biological functions and affect regulation of signal transduction and gene expression [4]. It has been shown that CypA is associated with various proteins and regulate their functions. For instance, CypA binds to peroxiredoxins (Prx) and activates its peroxidase activity. It is still unclear how Cyp is related to various biological functions, although the importance of Cyp in many tissues was accumulated from a number of studies [5,6]. These studies provide a potent molecular mechanisms how CypA can play an anti-apoptotic roles in human cancers, inflammation, and oxidative stress.

Apoptosis signaling-regulating kinase 1(ASK1) is known as a Mitogen-activated protein (MAP) kinase kinase kinase (MAP3K). It is widely recognizes to activates the JNK and p38 MAPK pathways in response to various intra- and extracellular stresses, including oxidative stress, ER-stress, anti-cancer drug, calcium overload, apoptosis and inflammation [6]. Phosphorylation of a threonine residue within the kinase domain of ASK1 is essential for the activation of ASK1 [7]. The S966 phosphorylation site of ASK1 has been shown to occur through the trans-autophosphorylation under oxidative stress [8].

In this study, we report the novel mechanism of ASK1 inhibition by direct interaction with CypA. Our study demonstrates

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that CypA directly binds to ASK1 and negatively regulates its activity. CypA phosphorylates ASK1 at Ser966 to negatively regulate ASK1. In addition, CypA leads to a decrease in the downstream of JNK and p38 pathway. ASK1 activity was successfully prevented by CypA, leading to ASK1 dependent PARP and caspase-3 inactivation and reduction of cell death. Also, oxidative stress induced ASK1-JNK-p38 MAPK pathways and caspase-3 apoptotic pathway were negatively regulated through its physical interaction with CypA.

2. Materials and methods

2.1. Cell culture and reagents

HEK293 (Human embryonic kidney) cells and Chang (normal liver) cells were grown in Dulbecco's modified Eagle's medium (DMEM), 10% Fetal bovine serum (FBS), 1% penicillin-streptomycin, and HeLa (human cervix adenocarcinoma) was grown in Roswell Park Memorial Institute (RPMI) 1640 media, 10% Fetal bovine serum (FBS), 1% penicillin-streptomycin. These cell lines cultured in humidified air containing 5% CO_2 at 37 °C. DMEM, RPMI, FBS and penicillin-streptomycin were purchased from Hyclone Laboratories, Inc. (South Logan, UT, USA).

2.2. Plasmids and transient transfection

A cDNA encoding HA-CypA was subcloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) using BamHI and EcoRI restriction sites. HA-CypA R55A was subcloned into pcDNA3.1 plasmids (Invitrogen) using BamHI and EcoRI restriction sites. Flag-CypA was subcloned into pCMV-Tag2B using BamHI and XhoI restriction sites. GST-CypA was subcloned into pGEX-KG *Escherichia coli* using BamHI and EcoRI restriction sites. Encoding HA-tagged full-length ASK1 and Flag-tagged ASK1 deletion mutants (ΔN-ASK1 ($ASK1^{649-1375}$), ΔC-ASK1 ($ASK1^{1-936}$), or NT-ASK1 ($ASK1^{1-656}$)) were kindly provided by Dr. H. Ichijo (The University of Tokyo, Tokyo, Japan). Plasmid encoding HA-JNK, Flag-p38 were kindly provided by Eui-Ju Choi (Korea University, Seoul, Korea). These genes were transfected with X-tremeGENE™ HP DNA transfection reagent from Roche Diagnostics (Indianapolis, IN, USA).

2.3. Western blot analysis

HEK293 Cells were harvested and lysed in lysis buffer (50 mM Tris–HCl, pH 7.3, 150 mM NaCl, 1% NP40, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 0.01% protease inhibitor cocktail). Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). The samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Protein was then transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA), and incubated with indicated antibodies. Protein bands were visualized using ECL blotting detection reagents (Santa Cruz).

2.4. Co-immunoprecipitation assays

HEK293 cells were co-transfected and were harvested and lysed in lysis buffer. The cell lysates subjected to indicated antibodies and then incubated overnight at 4 °C. The lysate and antibody complex was incubated with protein A/G PLUS-agarose beads (Santa Cruz, CA, USA) for 1–2 h at 4 °C. The immune complex were then washed three times with 1x lysis buffer. Whole cellular lysates were subjected to SDS-PAGE and detected by western blot.

2.5. GST pull down assay

GST-CypA (WT), GST-CypA (R55A) were incubated at 37 °C and then induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG) to 1 mM for 3 h. The pellet was suspended and sonicated three times for 1 min by adding GST lysis buffer (1x PBS, 0.1% Triton X-100, 1 mM DTT, 10% Glycerol, 1 mg/ml lysozyme containing protease inhibitors). The supernatant was incubated overnight with glutathione-agarose beads (Sigma, Saint Louis, MI, USA) and the beads were washed three times with ice cold PBS. Glutathione-agarose beads was coupled with GST-CypA (WT),

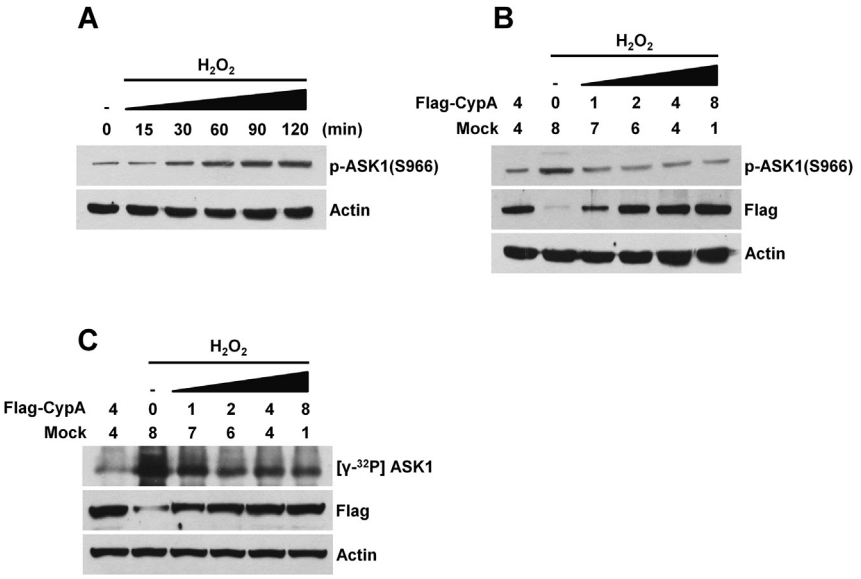

Fig. 1. ASK1 activity is regulated by CypA under oxidative stress. (A) Western blotting analysis of p-ASK1 using HEK293 cell lines exposed to H_2O_2 for the indicated time. (B) HEK293 cells transfected with the indicated amounts of Flag-CypA and exposed to H_2O_2 for 1 h treatment and followed by Western blotting. (C) HEK293 cells transfected with the indicated amounts of Flag-CypA and exposed to H_2O_2 for 1 h treatment and followed by ASK1 kinase assay. The data are an average of at least three independent experiments.