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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),

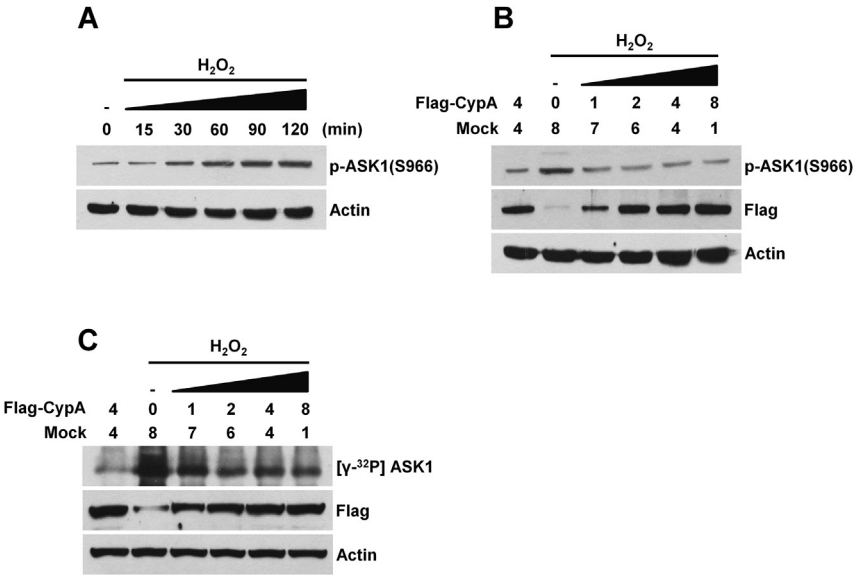

Fig. 1. ASK1 activity is regulated by CypA under oxidative stress. (A) Western blotting analysis of p-ASK1 using HEK293 cell lines exposed to H_2O_2 for the indicated time. (B) HEK293 cells transfected with the indicated amounts of Flag-CypA and exposed to H_2O_2 for 1 h treatment and followed by Western 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.

GST-CypA (R55A) mixed with GST binding buffer (20 mM Tris–HCl, PH 8.0, 125 mM $NaCl_2$, 0.5% NP40, 1 mM DTT and 10% Glycerol) at 4 °C overnight with shaking. Incubated beads were centrifuged and washed 1x PBS. The beads were subjected for western blotting.

2.6. Annexin V-FITC apoptosis assay

The cells were fixed and stained with Fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI). Annexin V-FITC assay was performed by using Annexin V-FITC apoptosis detection kit (BioBud, Seoul, Korea). HeLa Cells were plated at a density of 1×10^5 cells per dish on the 60 mm dish. Cells were transfected with HA-ASK1, HA-CypA and exposed to 1 mM H_2O_2 for 1 h. The cells were incubated with Annexin V-FITC and PI in the dark. The stained apoptotic cells were analyzed by flow cytometry (Beckman–Coulter, Brea, CA, USA).

2.7. Statistical analysis

All results were expressed as mean ± SD of three independent experiments. Statistical analyses were performed using the student two tailed *t-tests*. Values of $^{*}p < 0.05$, $^{**}p < 0.01$, and ns, not significant were considered statistically significant.

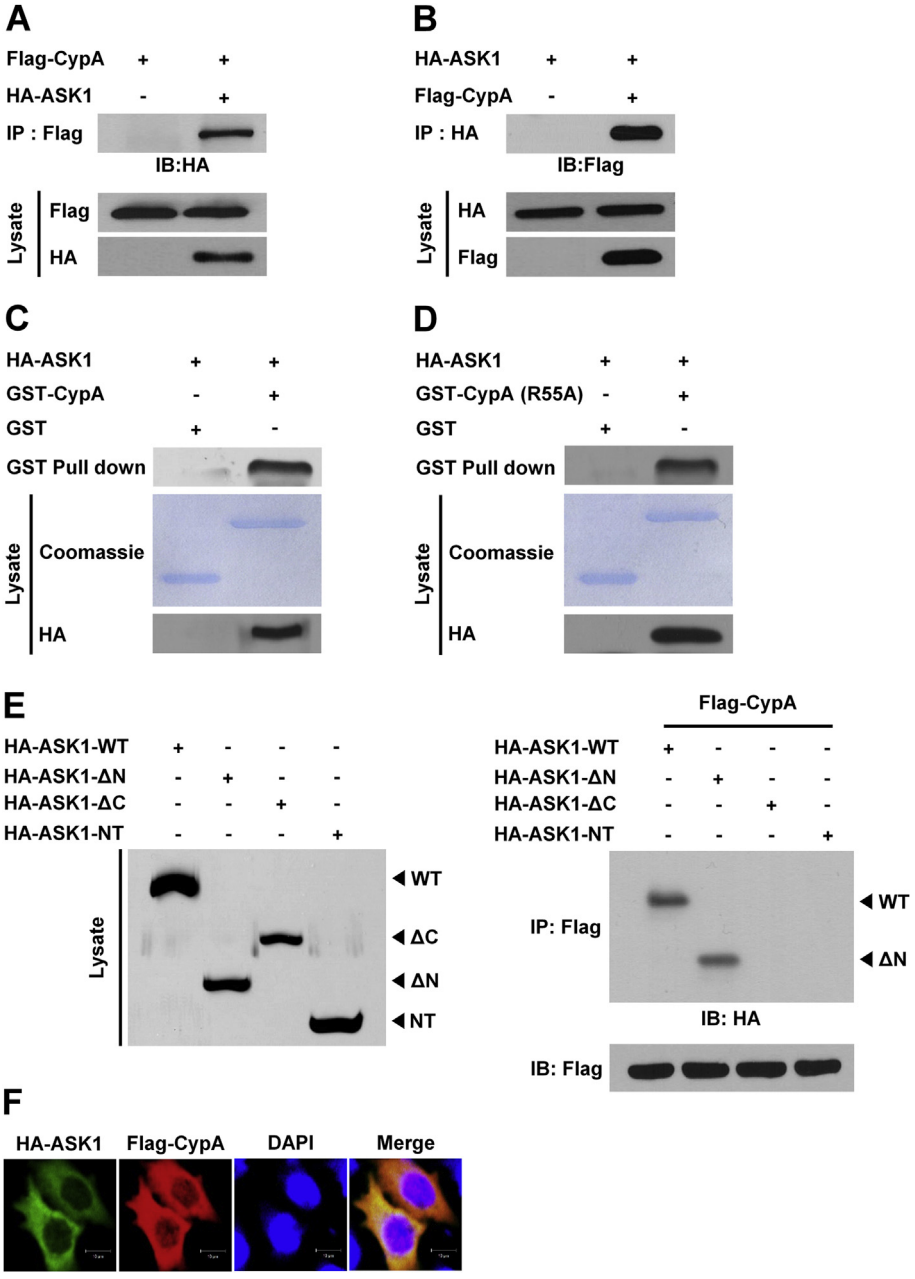

Fig. 2. CypA physically interacts with ASK1. (A) HEK293 cells lysates co-transfected with Flag-CypA and HA-ASK1 and immunoprecipitated with anti-Flag antibody. (B) HEK293 cells lysates co-transfected with Flag-CypA and HA-ASK1 and then immunoprecipitated with anti-HA antibody. (C) HEK293 cells lysates co-transfected with GST-CypA and HA-ASK1 and then subjected to GST pull down assay. (D) HEK293 cells lysates co-transfected with GST-CypA (R55A) and HA-ASK1 and then subjected to GST pull down assay. (E) Expression levels of truncated derivative of HA-ASK1 (ΔN-ASK1, ΔC-ASK1, and NT-ASK1) and monitored by Western blotting (left panel). HEK293 cells lysates co-transfected with Flag-CypA and several versions of truncated HA-ASK1 were immunoprecipitated with anti-Flag antibody. (F) Chang cells were transfected with HA-ASK1 and Flag-CypA. The cells were stained with anti-ASK1 (green), and anti-CypA (red), followed by nuclear staining with DAPI (blue). Cells were visualized by confocal microscopy. Bars, 10 μm. The values are shown as the mean ± SD of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. ASK1 phosphorylation is negatively regulated by CypA

Since ASK1 is known to be induced by oxidative stress [9], we first tried to confirm that treatment with H_2O_2 increases the activity of endogenous ASK1. We subjected HEK293 cells to oxidative stress (1 mM H_2O_2) for up to 120 min. Western blot analysis showed that phosphorylation (S966) level of endogenous ASK1 protein was increased rapidly after 15 min of exposure up to 120 min, whereas actin protein levels used as a control are constant (Fig. 1A). Next, HEK293 cells were transiently transfected with an expression vector encoding Flag-CypA, and then subjected to Western blot analysis. Interestingly, phosphorylation level of endogenous ASK1 protein was decreased by CypA transfection (Fig. 1B). In order to confirm the above results, we also monitored ASK1 Kinase activity. Fig. 1C showed that H_2O_2 treatment induces ASK1 kinase activity. However, ASK1 kinase activity was decreased by CypA expression. This result suggest that CypA expression inhibits endogenous ASK1 phosphorylation and ASK1 kinase activity.

3.2. CypA directly interacts with ASK1

We hypothesized that there is an interaction between ASK1 and CypA. In order to address the hypothesis, we investigated whether CypA physically interact with ASK1. HEK293 cells were co-

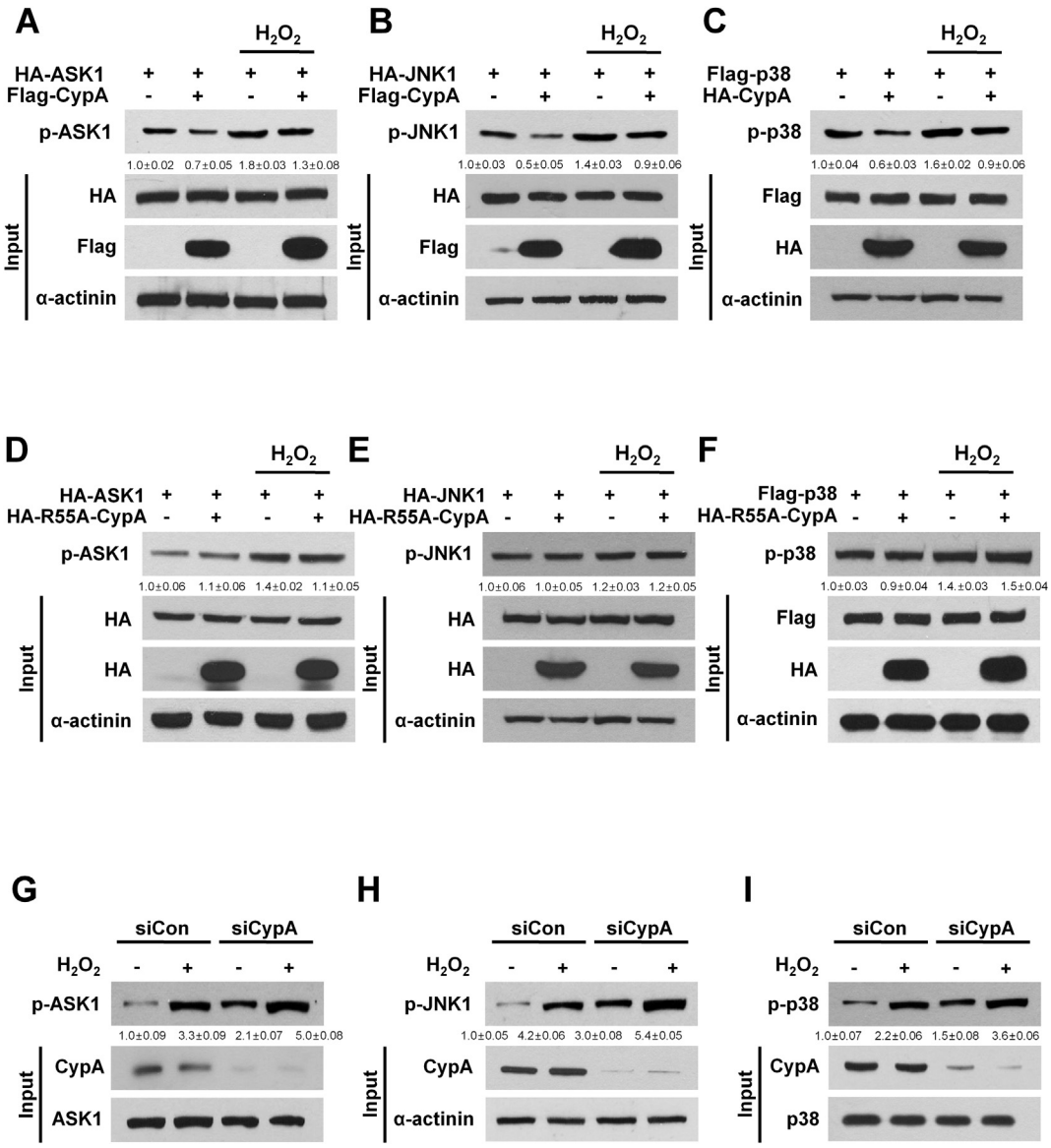

Fig. 3. CypA inhibits the phosphorylation level of ASK1. (A) HEK293 cells were transiently transfected with HA-ASK1 and Flag-CypA for 24 h. The cells were treated with 1 mM H_2O_2 for 1 h at 37 °C, and the cell lysates were subjected to Western blot analysis. (B) HEK293 cells were transiently transfected with HA-ASK1 and Flag-CypA for 24 h. The cell were treated with 1 mM H_2O_2 for 1 h at 37 °C, and the cell lysates were subjected to Western blot analysis. (C) HEK293 cells were transiently transfected with HA-ASK1 and Flag-CypA for 24 h. The cell were treated with 1 mM H_2O_2 for 1 h at 37 °C, and the cell lysates were subjected to Western blot analysis. (D) HEK293 cells were transiently transfected with HA-ASK1 and HA-CypA R55A for 24 h. The cell were treated with 1 mM H_2O_2 for 1 h at 37 °C, and the cell lysates were subjected to Western blot analysis. (E) HEK293 cells were transiently transfected with HA-JNK1 and HA-CypA R55A for 24 h. The cell were treated with 1 mM H_2O_2 for 1 h at 37 °C, the cell lysates were subjected to Western blot analysis. (F) HEK293 cells were transiently transfected with Flag-p38 and HA-CypA R55A for 24 h. The cell were treated with 1 mM H_2O_2 for 1 h at 37 °C, the cell lysates were subjected to Western blot analysis. (G, H, I) HEK293 cells were transfected a control siRNA or CypA-siRNA for 48 h at 37 °C. The cells were treated with 1 mM H_2O_2 for 1 h at 37 °C, and the cell lysates were assayed for ASK1, JNK and p38 phosphorylation levels. The data are an average of at least three independent experiments.

transfected with Flag tagged CypA (Flag-CypA) and HA tagged ASK1 (HA-ASK1) constructs, and subjected to immunoprecipitation analysis (Fig. 2A, B). Both immunoprecipitations using anti-HA and anti-Flag antibody showed the physical interaction between HA-ASK1 and Flag-CypA in HEK293 cells. The direct interaction between ASK1 and CypA was confirmed by GST pull down assays. GST-CypA fusion protein was incubated with HEK293 cell lysates overexpressing HA-ASK1. ASK1 proteins were detected in the GST CypA pull downs (Fig. 2C). Next, in order to address that PPIase activity is necessary for the physical interaction, GST pull down assay using ASK1 and CypA-R55A (PPIase-defective mutant) was carried out. ASK1 protein was also detected in the GST-CypA (R55A) pull downs, suggesting that the PPIase activity is not required for the physical interaction (Fig. 2D).

Furthermore, we attempted to map the region of ASK1 required for the interaction with CypA. A series of truncated mutants of ASK1 were subjected to the interaction analysis. Expression levels of full-length ASK1 (amino acids 1–1375), ΔN ($ASK1^{649-1375}$), ΔC ($ASK1^{1-936}$), and NT ($ASK1^{1-656}$) were monitored by Western blots (Fig. 2E). Immunoprecipitation assays with CypA showed that CypA strongly interacts with ΔN ($ASK1^{649-1375}$) in HEK293 cells as well as Full-length ASK1 (1–1375), suggesting that the C-terminal region of ASK1 is required for the interaction. Next, we monitored co-localization of CypA and ASK1. Confocal analysis showed that ASK1 was co-localized with CypA in the cell (Fig. 2F) [10,11]. The overall results show that ASK1 physically interacts with CypA in HEK293 cells.

3.3. CypA inhibits ASK1 signaling pathways

To determine the effects of CypA-ASK1 interaction on either ASK1 phosporylation or ASK1 downstream signaling pathway, HEK293 cells transiently co-transfected with HA-ASK1 and Flag-CypA were exposed to H_2O_2, followed by Western blot analysis. ASK1 has been shown to be phosphorylated under oxidative stress in Fig. 3A. However, the phosphorylation has been reduced significantly by CypA expression. Since ASK1 has been reported to activate JNK and p38 kinase, we monitored the level of phosphorylation of JNK and p38 under oxidative stress. As expected, CypA expression decreased the kinase activity of JNK and p38 (Fig. 3B and C). Interestingly, CypA-R55A did not affect the phosphorylation of ASK1, JNK and p38 (Fig. 3D, E, and F). In order to confirm these results, we monitored the effects of CypA knockdown on the phosphorylation of JNK and p38 [12]. As shown in Fig. 3G, H and I, CypA knockdown significantly activated the phosphorylation under oxidative stress, compared to the controls.

3.4. ASK1-mediated apoptosis is suppress by CypA

In this study, we have shown that ASK1 directly interacts with CypA, and CypA inhibits ASK1 signaling pathway [7,13]. We next observed whether ASK1-mediated apoptosis is inhibited by CypA. Fig. 4A showed that H_2O_2-induced activation of ASK1 results in PARP and caspase-3 cleavages [14]. However, overexpression of CypA reduced PARP and caspase-3 cleavages, suggesting that CypA

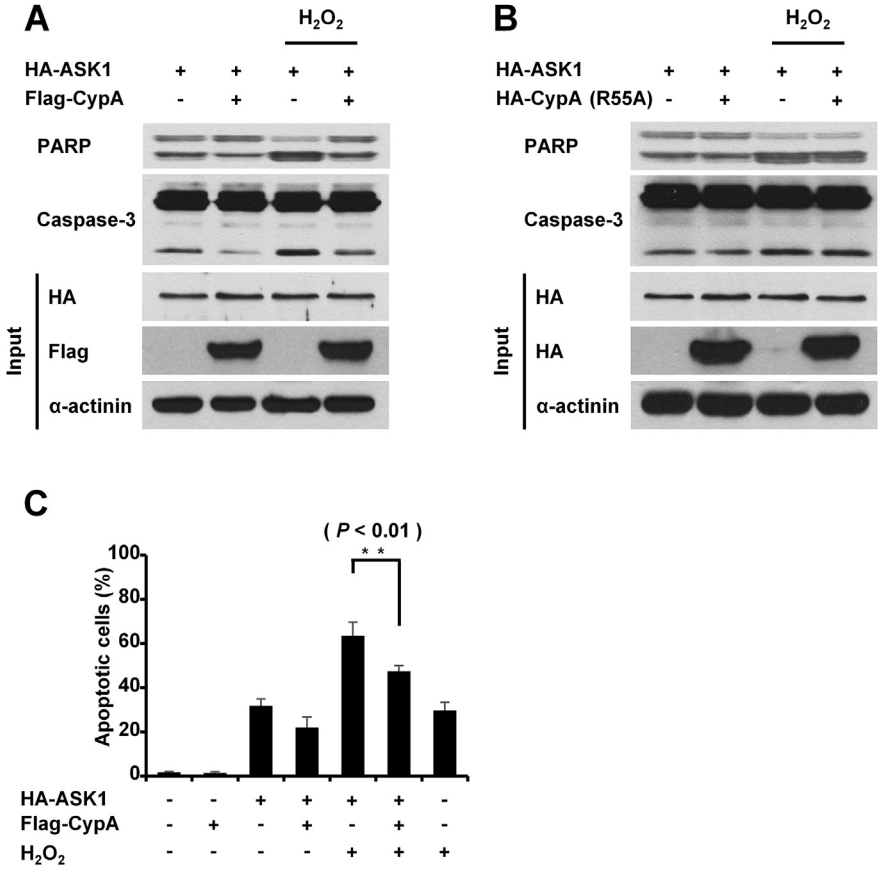

Fig. 4. CypA inhibits ASK1-induced apoptosis under oxidative stress. (A) HeLa cells were transiently transfected with HA-ASK1 and Flag-CypA for 24 h. The cells were treated with 1 mM H_2O_2 for 1 h at 37 °C, and the cell lysates were used for Western blot analysis with anti-PARP antibody and anti-caspase-3 antibody. (B) HeLa cells were transient transfected with HA-ASK1 and Flag-CypA (R55A) for 24 h. The cells were treated with 1 mM H_2O_2 for 1 h at 37 °C, and the cell lysates were used for Western blot analysis with anti-PARP antibody and anti-caspase-3 antibody. (C) HeLa cells were transient transfected with HA-ASK1 and Flag-CypA (R55A) for 24 h. The cells were treated with 1 mM H_2O_2 for 1 h at 37 °C, and the cells were stained with annexin V, followed by flow cytometry analysis. The bar graph represents an apoptotic cell percentages. The values are shown as the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, and ns, not significant.

inhibited H_2O_2-induced activation of ASK1. We next tested whether suppression of ASK1-mediated apoptosis by CypA requires its PPIase activity. Fig. 4B showed that PPIase-defective CypA-R55A failed to suppress H_2O_2-induced activation of ASK1. This results suggest an important role of PPIase activity of CypA in the ASK1-mediated apoptosis signal pathway.

In order to confirm our data, we finally evaluated ASK1-mediated apoptotic cell death in the HeLa cells transfected with CypA or not. The results showed that CypA significantly suppresses ASK1-induced apoptotic under oxidative stress (Fig. 4C). The overall data show that CypA interacts with ASK1 and negatively regulates ASK1-induced apoptosis signals.

4. Discussion

Oxidative stress is known to trigger several human diseases including diabetes, and Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS) [4,15]. Many attempts have been done to reduce or remove oxidative stress-induced cell death. Some studies have shown that CypA protects oxidative stress-induced apoptotic cell death [3]. However, the mechanism by which CypA inhibits oxidative stress-induced apoptosis was not clearly understood. Here, we demonstrated that pro-apoptotic protein ASK1 physically interacts with CypA and its phosphorylation level was reduced by CypA, suggesting that CypA interacts with pro-apoptotic molecules and inhibit the signal transduction pathway. Consistent with our data, CypA is reported to interact with Apoptosis Inducing Factor (AIF) to show cytoprotective functions [16].

A few molecules have been reported to interact with ASK1. Notch1 was shown to interact with ASK1 and inhibit ASK1 activation, thus resulting in suppression of ASK1 signal transduction pathway [17]. Also previous studies have demonstrated Caspase-activated DNase (CAD), anti-apoptotic protein, inhibitor (CIIA) that interacts with ASK1. CIIA functions as a natural antagonist against ASK1-mediated signaling and DNA fragmentation [18]. Our data indicate that CypA is a new binding partner of ASK1.

We have shown that ASK1 is regulated by CypA under oxidative stress. Overexpressed CypA interacted with ASK1 and reduced the phosphorylation level of ASK1. Moreover, the phosphorylation of JNK and p38 was also inhibited by overexpressed CypA. Thus, our findings suggest that CypA functions as a molecular switch regulating the oxidative stress-induced activation of ASK, and that CypA may play an important role in modulating ASK1 signaling pathway in oxidative stress-induced apoptosis [19,20]. Our data showed that the cytoprotective role of CypA seems to require PPIase activity, even though its exact mechanism remained to be elucidated. Future animal studies are needed to study in detail the relationship between CypA and ASK1 with regarding to the related diseases, providing the important insight about CypA as a therapeutic target in the related diseases.

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