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# Dyrk1A Positively Stimulates ASK1-JNK Signaling Pathway during Apoptotic Cell Death

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## ABSTRACT

Dual-specificity tyrosine (Y)-phosphorylation-regulated protein kinase 1A (Dyrk1A) is the mammalian homologue of *Drosophila melanogaster* minibrain and its human gene is mapped to the Down syndrome critical region of chromosome 21. Dyrk1A phosphorylates several transcription factors, including NFAT and CREB and a number of cytosolic proteins such as APP, tau, and $\alpha$-synuclein. Although Dyrk1A is involved in the control of cell growth and postembryonic neurogenesis, its potential role during cell death and signaling pathway is not clearly understood. In the present study, we show that Dyrk1A is activated under the condition of apoptotic cell death. In addition, Dyrk1A is coupled to JNK1 activation, and directly interacts with apoptosis signal-regulating kinase 1 (ASK1). Moreover, Dyrk1A positively regulates ASK1-mediated JNK1-signaling, and appears to directly phosphorylate ASK1. These data indicate that Dyrk1A regulates cell death through facilitating ASK1-mediated signaling events.

**Key words:** ASK1, cell death, Dyrk1A, JNK, signal transduction

## INTRODUCTION

Dual-specificity tyrosine (Y)-phosphorylation-regulated protein kinase 1A (Dyrk1A) is firstly identified as Minibrain in *Drosophila* (Tejedor et al., 1995). *Dyrk1A* is mapped within the Down syndrome (DS) critical region (a 4-megabase region containing 60~100 genes between markers D21S17 and ETS2) of chromosome 21, which is associated with a variety of DS abnormalities, including mental retardation (Smith et al., 1997). Transgenic mice carrying extra copies of Dyrk1A exhibit neurodevelopmental delays, motor abnormalities, and cognitive deficits (Altafaj et al., 2001). Dyrk1A knockout mice also show a general delay in fetal development and embryonic lethality, indicating the vital and non-redundant biological functions of Dyrk1A (Fotaki et al., 2002).

Diverse types of proteins have been identified as the substrates of Dyrk1A, including transcription factors, including NF-AT and Forkhead (Woods et al., 2001; Arron et al., 2006; Gwack et al., 2006), several endocytosis and synaptic vesicle recycling proteins, such as dynamin 1 and amphiphysin 1 (Chen-Hwang et al., 2002; Murakami et al., 2006), and cytosolic proteins, such as APP and tau (Ryoo et al., 2007; Ryoo et al., 2008), implying that Dyrk1A participates in various biological responses. Regarding to the functional role of Dyrk1A, active Dyrk1A phosphorylates the transcription factor cAMP response element (CRE)-binding protein (CREB), which subsequently leads to the stimulation of CRE-

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mediated gene transcription during neuronal differentiation (Yang et al., 2001). Yak1p, the *Saccharomyces cerevisiae* homolog of Dyrk1A, was identified as a functional antagonist of the RAS/protein kinase A pathway and has been characterized as a negative regulator of growth (Holtzman et al., 1993; Raths et al., 1993). Thus, it is suggested that Dyrk1A is a key regulator of neuronal cell growth and differentiation.

Recently, Dyrk1A appears to be involved in the pathogenesis of several neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease, and Huntington's disease (HD). For example, Dyrk1A phosphorylates $\alpha$-synuclein and regulates its inclusion formation, and potentially affecting neuronal cell viability (Kim et al., 2006). In addition, up-regulation of Dyrk1A in immortalized hippocampal progenitor H19-7 cells causes AD-like pathogenesis through the formation of tau inclusion and the generation of $\beta$-amyloid fragment (Park et al., 2007). However, the exact role of Dyrk1A and its signal transduction leading to cell death is not clearly elucidated yet.

Apoptosis signal-regulating kinase 1 (ASK1), a member of the mitogen-activated protein kinase kinase kinase family, is composed of an inhibitory N-terminal domain, a kinase domain, and a C-terminal regulatory domain (Ichijo et al., 1997). ASK1 can promote apoptosis in response to common pro-apoptotic stresses, such as oxidative stress (Song et al., 2002), death receptor ligands (Nishitoh et al., 1998), and endoplasmic reticulum stress (Nishitoh et al., 2002). ASK1 also phosphorylates and activates both p38 and JNK pathways (Ichijo et al., 1997). The mechanism of ASK1 activation is positively regulated by its binding proteins such as TNF receptor-ssociated factors 2/6 (Noguchi et al., 2005) and Daxx (Chang et al., 1998). On the other hand, several cellular proteins, including thioredoxin (Saitoh et al., 1998), Hsp90 (Zhang et al., 2005), and 14-3-3 (Zhang et al., 1999), have been reported to interact with ASK1 and inhibit ASK1 activity.

In the present study, we investigated whether and how Dyrk1A becomes activated during cell death. We found that Dyrk1A is linked to JNK signaling pathway and acts as the upstream kinase of JNK. Additionally, Dyrk1A interacts with and positively regulates ASK1 under various cell death conditions. These finding suggest that Dyrk1A plays an important role in ASK1-mediated transmission of cell death signals.

## MATERIALS AND METHODS

### *Materials*

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), LipofectAMINE PLUS reagent, anti-Xpress, horseradish peroxidase-conjugated anti-rabbit, and anti-mouse IgGs were purchased from Invitrogen (Carlsbad, CA, USA). Enhanced chemiluminescence (ECL) reagents and [$\gamma$-$^{32}$P]ATP were purchased from Perkin-Elmer Life and Analytical Sciences (Waltham, MA, USA). Glutathione-Sepharose 4B and Protein A-Sepharose were obtained from Amersham Biosciences (Piscataway, NJ, USA). Anti-phosphoASK1 (Thr-845), anti-phosphoJNK, and anti-Dyrk1A antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-HA, anti-ASK1, anti-JNK, anti-Hsp90, mouse immunoglobulins (IgGs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Dyrk1A antibody was purchased from Abnova Corporation (Taipei City, Taiwan). Anti-phosphotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-Flag antibodies and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mammalian constructs encoding wild-type and kinase-inactive rat Dyrk1A tagged with hemagglutinin (HA) (pSVL-HA-Dyrk1A WT and K188R) were a kind gift from W. Becker (Institut fur Pharmakologie und Toxikologie, Universitatsklinikum der RWTH, Germany). Plasmids encoding 6xHis-Xpress-tagged wild-type and K188R mutant Dyrk1A (pcDNA4/HisMax-Dyrk1A WT and K188R) were generated as described previously (Park et al., 2007). Constructs encoding HA-tagged forms of ASK1, ASK1$\Delta$N, ASK1$\Delta$C, ASK1-NT, and Flag-ASK1 was kindly provided by E.J. Choi (Korea University, Seoul, Korea).

### *Cell culture and preparation of cell lysates*

Human embryonic kidney 293 (HEK293) cells were maintained in DMEM containing 10% FBS and 100 unit/ml penicillin-streptomycin. The cells were transfected with various expression vectors using

LipofectAMINE PLUS reagent, according to the manufacturer's protocols. In order to prepare cell lysates, the cells were rinsed twice with ice-cold phosphate-buffered saline, and then solubilized in lysis buffer (50 mM Tris, pH 7.5, containing 1.0% Nonidet P-40, 150 mM NaCl, 10% glycerol, 1 mM $Na_3VO_4$, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml aprotinin, 1 mM EGTA, 1 mM EDTA,10 mM NaF, and 0.2 mM phenylmethylsulfonyl fluoride). The cells were scraped, and the supernatants were collected after centrifugation for 20 min at 14,000xg at 4°C.

***Immunoprecipitation and immunoblot assay***

One microgram of suitable antibodies was incubated with 0.5 to 1 mg of cell extracts prepared in cell lysis buffer overnight at 4°C. Fifty microliter of a 1 : 1 suspension of Protein A-Sepharose beads was added and incubated for 2 h at 4°C with gentle rotation. After beads were pelleted and washed extensively with cell lysis buffer, the immunocomplexes were dissociated by boiling in SDS-PAGE sample buffer, separated onto SDS-PAGE gel, and transferred to a nitrocellulose membrane (Millipore, Japan). Membranes were then blocked in TBST buffer (20 mM Tris, pH 7.5, 137 mM NaCl, and 0.1% Tween 20) plus 5% nonfat dry milk for 1 h at room temperature, and incubated overnight at 4°C in TBST buffer with 3% nonfat dry milk and the appropriate primary antibodies. Membranes were washed three times in TBST, and then incubated with appropriate secondary IgG-coupled horseradish peroxidase antibodies for 1 h at room temperature. The membranes were washed three times with TBST and visualized with ECL reagent.

***In vitro kinase assay***

After cells were harvested and lysed in lysis buffer, the protein extracts (~500 $\mu$g) were incubated with appropriate antibody for overnight at 4°C. The immunocomplexes were mixed with protein A-Sepharose beads, and washed three times in lysis buffer. For JNK assay, the kinase reaction was carried out at 30°C for 30 min in 20 $\mu$l of kinase buffer (25 mM HEPES, pH 7.4, 25 mM glycerophosphate, 25 mM $MgCl_2$, 100 $\mu$M sodium orthovanadate) containing 10 $\mu$M ATP, 5 $\mu$Ci of [$\gamma$-$^{32}$P] ATP, and 5 $\mu$g GST-c-Jun as a substrate. For Dyrk1A kinase assay, immunocomplexes were resuspended in the kinase reaction buffer (20 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$) containing 10 $\mu$M ATP, 5 $\mu$Ci [$\gamma$-$^{32}$P]ATP, and incubated for 15 min at 30°C in the presence of 5 $\mu$g of GST-ASK1 as a substrate. The reactions were stopped by adding SDS-sample buffer, and analyzed by SDS-PAGE followed by autoradiography.

## RESULTS

***Dyrk1A becomes activated by toxic stimuli, such as TNF$\alpha$ and hydrogen peroxide***

In a previous report we have shown that the addition of etoposide to embryonic hippocampal H19-7 cells causes the activation of Dyrk1A, which facilitates the interaction between Hip-1 and caspase-3 and then leads to the cell death (Kang et al., 2005). In addition to etoposide, we firstly examined whether Dyrk1A becomes activated upon the stimulation with other toxic stimuli, such as tumor necrosis factor-$\alpha$ (TNF$\alpha$) and hydrogen peroxide ($H_2O_2$). After HEK293 cells were incubated with hydrogen peroxide or TNF$\alpha$, cell lysates were subjected to immunoprecipitation with anti-Dyrk1A, followed by immunoblotting with anti-phosphotyrosine. As shown in Fig. 1, the auto-phosphorylation of Dyrk1A was significantly increased upon the exposure to these two stimuli, reaching the maximum value of Dyrk1A activity at 2 hr and sustained thereafter until 4 hr of post-stimuli treatment. The quantification of the phospho-Dyrk1A bands revealed a 14.92- and 1.81-fold increase of Dyrk1A activity in response to TNF$\alpha$ and $H_2O_2$, respectively (Fig. 1). These results suggest that Dyrk1A becomes activated in response to toxic stimuli.

***Dyrk1A stimulates JNK activity***

Based on the finding that intracellular JNK signaling pathway is activated by exposure with pro-inflammatory cytokines and cellular stress stimuli, such as etoposide and $H_2O_2$ (Ip and Davis, 1998), we next examined whether Dyrk1A is linked to JNK signaling pathway, and if it occurs, how they interact together in a biochemical and functional mode. After HEK293 cells were mock-transfected or transfected with Xpress-Dyrk1A for 24 hr followed by treatment with etoposide, cell lysates were immunoblotted with anti-phosphoJNK. As shown in

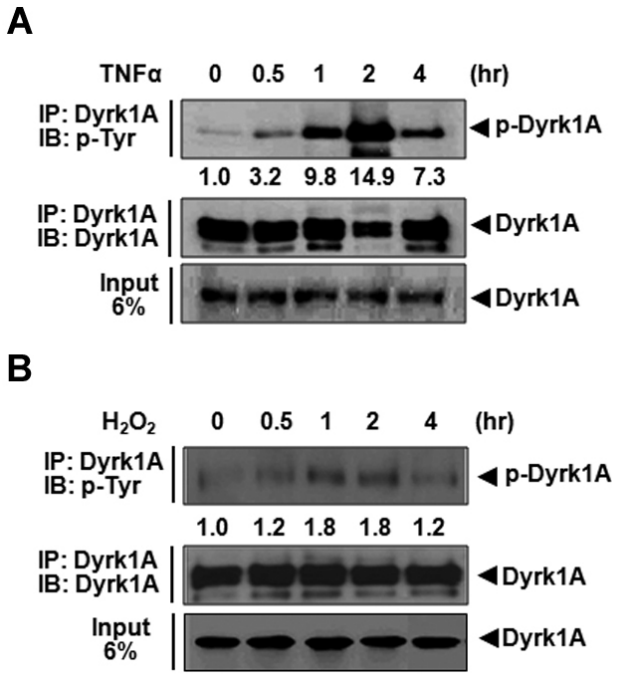


**Fig. 1.** Effect of TNF$\alpha$ and hydrogen peroxide on Dyrk1A activation. Where indicated, HEK293 cells were incubated with either TNF$\alpha$ (100 ng/ml; A) or $H_2O_2$ (1 mM; B) for the indicated times. Cell lysates were subjected to immunoprecipitation with anti-Dyrk1A, and the levels of Dyrk1A and active Dyrk1A were analyzed by immunoblot using anti-Dyrk1A or anti-phosphotyrosine IgGs.

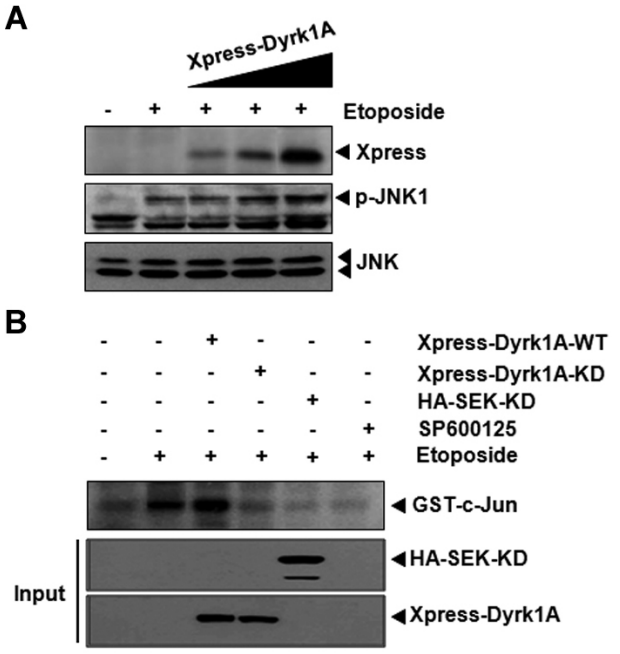


**Fig. 2.** Dyrk1A potentiates JNK activation. (A) HEK293 cells were either mock-transfected or transfected with Xpress-Dyrk1A in a dose-dependent manner for 24 hr, as indicated. Then the cells were stimulated with etoposide (50 $\mu$M) for 1 hr, and cell lysates were subjected to immunoblot with anti-Xpress, anti-phosphoJNK, and anti-JNK IgGs. (B) Where indicated, HEK293 cells were transfected for 24 h with a plasmid encoding Xpress-tagged wild type Dyrk1A (Xpress-Dyrk1A-WT) or kinase-defective K188R mutant (Xpress-Dyrk1A-KD), or HA-tagged kinase-inactive SEK mutant. Then, the cells were preincubated with SP600125 (20 $\mu$M) for 24 hr, and treated with etoposide (50 $\mu$M) for 1 hr, as indicated. After cells were immunoprecipitated with anti-JNK antibodies, the JNK activity of immunocomplexes was examined by *in vitro* kinase assay using GST-c-Jun as a substrate. To check the proper expression of plasmid, cell lysates were immunoblotted with anti-Xpress or anti-HA antibodies.

Fig. 2A, while the addition of etoposide caused JNK activation, transfection of wild type Dyrk1A led to a synergistic increase in the level of phospho-JNK in a dose-dependent manner. To further check whether Dyrk1A causes the JNK activation, *in vitro* kinase assay was performed. HEK293 cells were transfected a plasmid encoding either Xpress-tagged wild type Dyrk1A (Xpress-Dyrk1A) or its kinase-deficient mutant (Xpress-Dyrk1A-K188R), or HA-tagged kinase-defective SEK1, the upstream activator of JNK (HA-SEK mutant), and/or pre-incubated with JNK inhibitor SP600125 for 24 hr. The cells were then left unstimulated or treated with etoposide for 1 hr, and the total cell lysates were immunoprecipitated with anti-JNK followed by *in vitro* kinase assay by using bacterial recombinant c-Jun fused to GST as a substrate. As shown in Fig. 2B, the phosphorylation of GST-c-Jun was enhanced upon the stimulation with etoposide. Furthermore, the expression of Dyrk1A resulted in an enhancement of phosphorylated c-Jun levels, compared with the cells stimulated by etoposide alone (Fig. 2B). Meanwhile, marked reduction of phospho-c-Jun levels was observed under the inhibition of Dyrk1A arising from the transfection of kinase-deficient Dyrk1A mutant. These results suggest that Dyrk1A activates JNK signaling pathway. This finding was further supported by the observation that the levels of phospho-c-Jun were decreased by JNK inhibitor (SP600125) or kinase-deficient SEK mutant type (Fig. 2B), which is present upstream component of apoptotic JNK pathway.

### *Dyrk1A binds to ASK1, which is enhanced by toxic stimuli*

To further characterize how Dyrk1A is linked to JNK pathway, we examined the possibility that Dyrk1A directly interacts with JNK. After HEK293

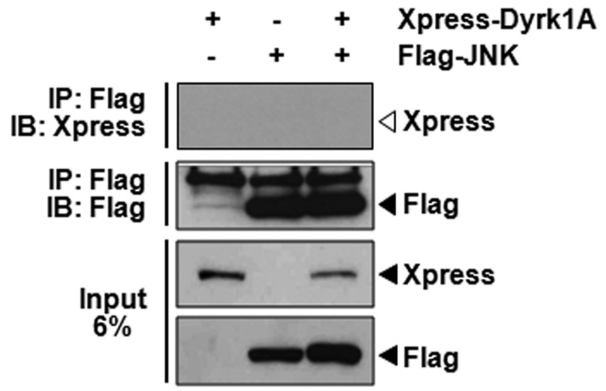


**Fig. 3.** Dyrk1A does not interact with ASK1. After HEK293 cells were transfected for 24 hr with Xpress-Dyrk1A or/and Flag-JNK, cell lysates were subjected to immunoprecipitation with anti-Flag, followed by immunoblot with anti-Xpress or anti-Flag IgGs. As a control, cell lysates were also immunoblotted with anti-Xpress and anti-Flag antiserum.

cells were transfected with Xpress-Dyrk1A or/and Flag-JNK alone or together, cell lysates were immunoprecipitated with anti-Flag followed by immunoblotting with anti-Xpress. As shown in Fig. 3, the anti-Flag-JNK immunoprecipitates did not include Xpress-tagged Dyrk1A, suggesting that Dyrk1A does not bind with JNK directly, whereas it is related to JNK signaling pathway.

Like as other MAP kinase family member, ERK and p38, JNK-mediated signaling pathway consists of three components, such as upstream MAP3K (ASK1), MAP2K (MKK4/7 or SEK1), and JNK/SAPK, (JNK1 and JNK2) (Kanamoto et al., 2000). Next we examined whether Dyrk1A interacts with ASK1. After HEK293 cells were transfected with HA-ASK1

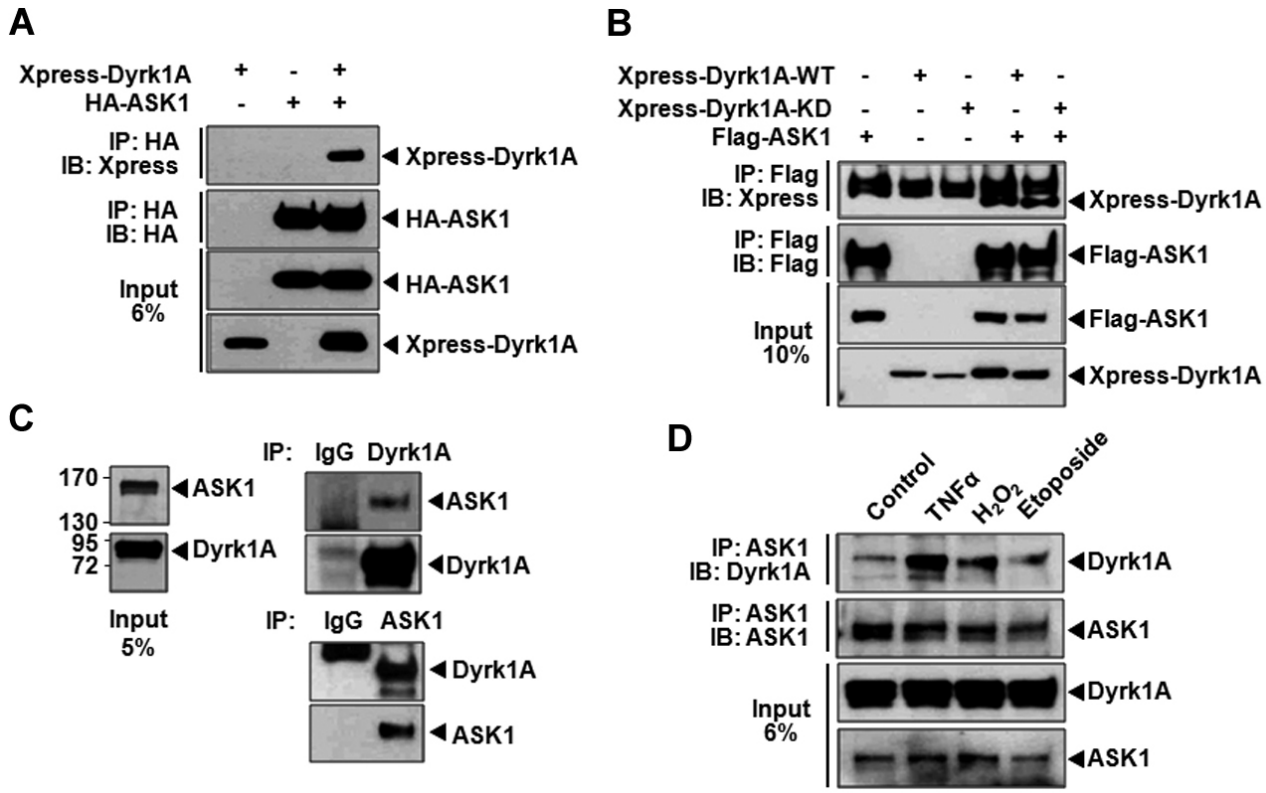


**Fig. 4.** Dyrk1A binds to ASK1. (A) HEK293 cells were transfected for 24 h with Xpress-Dyrk1A or/and HA-ASK1. Cell lysates were subjected to immunoprecipitation with anti-HA, and then immunoblot assay was performed with anti-Xpress or anti-HA antibodies. To examine the proper expression of plasmid, cell lysates were immunoblotted with anti-HA and anti-Xpress IgGs. (B) After HEK293 cells were transfected for 24 h with Flag-ASK1 alone or together with either Xpress-tagged wild type (WT) or kinase-defective Dyrk1A (KD), cell lysates were subjected to immunoprecipitation with anti-Flag, followed by immunoblot assay with anti-Xpress antibodies. The proper expression of plasmid was examined by immunoblot assay with anti-Xpress or anti-Flag antibodies. (C) HEK293 cell lysates were subjected to immunoprecipitation with mouse anti-Dyrk1A or preimmune mouse anti-IgG, followed by immunoblot with anti-ASK1 IgG. The cell lysates were also immunoprecipitated with mouse anti-ASK1 or preimmune mouse anti-IgG, followed by the immunoblot with anti-Dyrk1A antiserum. The levels of endogenous proteins were measured by immunoblotting with anti-Dyrk1A and anti-ASK1 antibodies, as indicated. (D) Where indicated, HEK293 cells were treated with $H_2O_2$ (1 mM), TNF$\alpha$ (100 ng/ml), or etoposide (100 $\mu$M) for 30 min. Cell lysates were subjected to immunoprecipitation with anti-ASK1, and then immunoblot assay was performed by using anti-Dyrk1A or anti-ASK1 antiserum.

or/and Xpress-Dyrk1A alone or together, cell lysates were immunoprecipitated with anti-HA and immunoblotted with anti-Xpress. As shown in Fig. 4A, when the cells were co-transfected with Dyrk1A and ASK1, Dyrk1A binds to ASK1. We further investigated whether the kinase activity of Dyrk1A is necessary for its binding to ASK1. As shown in Fig. 4B, HEK293 cells were transfected with a plasmid encoding either Xpress-tagged wild type or kinase-defective Dyrk1A alone or together with Flag-ASK1. Co-immunoprecipitation assay revealed that ASK1 interacts with kinase-deficient Dyrk1A as well as with wild type (Fig. 4B). These data suggest that the kinase activity of Dyrk1A appears not to be required for the interaction between Dyrk1A and ASK1.

Next we examined whether the mutual interaction between endogenous Dyrk1A and endogenous ASK1 still occurs. After HEK293 cell lysates were immunoprecipitated with mouse anti-Dyrk1A, followed by immunoblotting with anti-ASK1 and anti-Dyrk1A, Dyrk1A well interacts with ASK1 endogenously (Fig. 4C). Immunoprecipitation of the same lysates with mouse monoclonal anti-ASK1 antibody with followed by immunoblotting with anti-Dyrk1A in a reverse order produced the same binding band (Fig. 4C). As a control, the immunoprecipitation with mouse preimmune anti-IgG produced no band with anti-Dyrk1A or anti-ASK1, confirming the validity of assay (Fig. 4C). This data confirms that the mutual interaction between ASK1 and Dyrk1A is not an artifact arising from the DNA transfection, and still occurs inside cells.

To examine whether the interaction between Dyrk1A and ASK1 is being affected by toxic stimuli, including $H_2O_2$, TNF$\alpha$, or etoposide, HEK293 cells were incubated with each stimuli for 1 hr. When cell lysates were immunoprecipitated with anti-ASK1, followed by immunoblotting with anti-Dyrk1A, the binding of Dyrk1A to ASK1 was significantly increased by the exposure with these toxic stimuli (Fig. 4D). While the addition of TNF$\alpha$ caused the most remarkable increased binding of Dyrk1A to ASK1, $H_2O_2$ and etoposide had less effect (Fig. 4D). These results suggest that toxic stimuli facilitate interaction between Dyrk1A and ASK1.

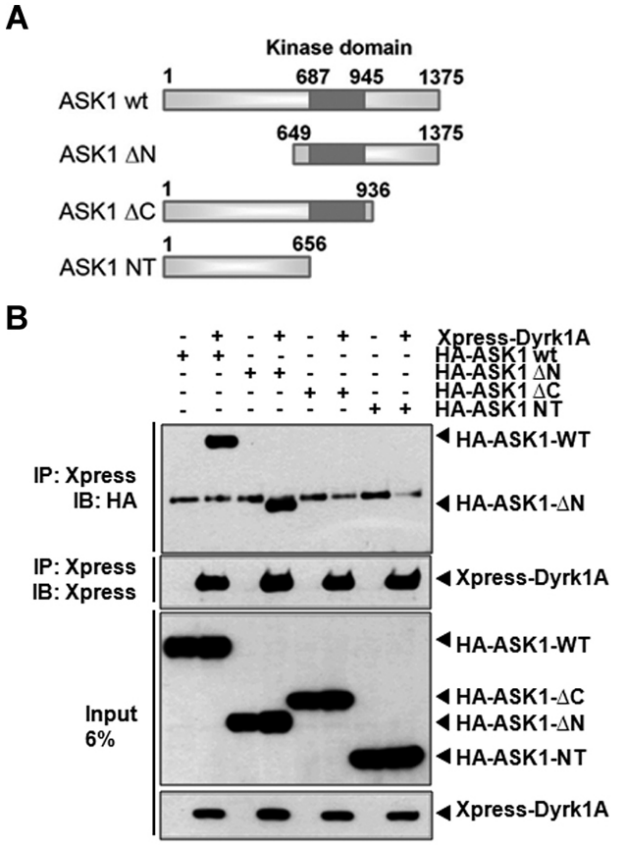


**Fig. 5.** Dyrk1A binds the C-terminal region of ASK1. (A) The diagram of full length ASK1 and its deletion mutants. (B) Where indicated, HEK293 cells were transfected for 24 hr with Xpress-Dyrk1A or/and either HA-tagged intact (ASK1 wt) or deletion ASK1 mutants, such as ASK1-$\Delta$N, ASK1-$\Delta$C, or ASK1-NT. Cell lysates were subjected to immunoprecipitation with anti-Xpress, and then immunoblot assay was performed by using anti-HA or anti-Xpress IgGs. Cell lysates were also immunoblotted with anti-HA and anti-Xpress.

***Dyrk1A binds to the C-terminal domain of ASK1***

To identify the specific Dyrk1A-binding region within ASK1, several ASK1 constructs encoding its deletion fragments, such as HA-tagged wild type ASK1, ASK1-$\Delta$N, ASK1-$\Delta$C, and ASK1-NT, were generated (Fig. 5A). After HEK293 cells were transfected with expression plasmid encoding HA-tagged wild type ASK1, ASK1$\Delta$N, ASK1$\Delta$C, or ASK1-NT alone or together with Xpress-Dyrk1A, cell lysates were then subjected to immunoprecipitation with anti-HA. Immunoblotting of anti-HA-ASK1 complexes with anti-Xpress revealed that Dyrk1A still binds to ASK1-$\Delta$N as well as the full length of ASK1 (Fig. 5B). However, Dyrk1A did not interact with ASK1-$\Delta$C and ASK1-NT (Fig. 5B). These results suggest that the C-terminal domain of

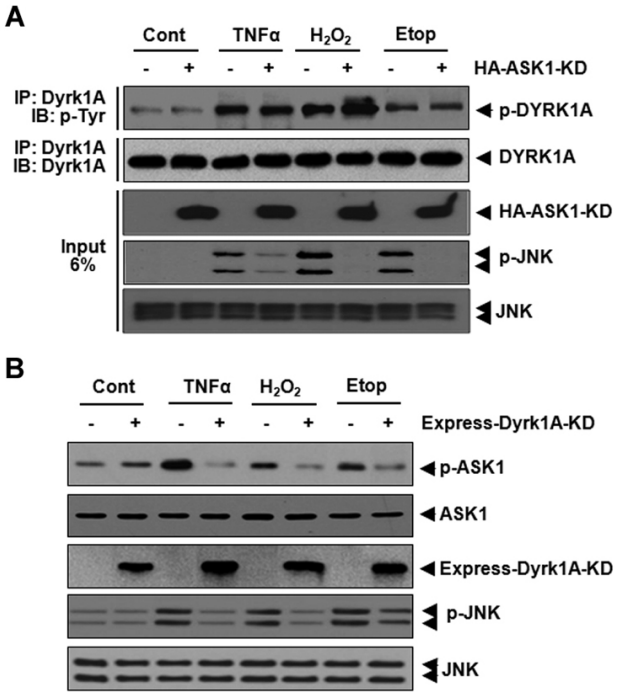


**Fig. 6.** Dyrk1A positively stimulates ASK1 and JNK activity. (A) Where indicated, HEK293 cells were mock-transfected or transfected for 24 hr with HA-tagged kinase-defective ASK1. Then, the cells were left un-stimulated or treated with TNF$\alpha$ (100 ng/ml), $H_2O_2$ (1 mM), or etoposide (100 $\mu$M) for 1 hr. After cell lysates were immunoprecipitated with anti-Dyrk1A, the levels of Dyrk1A and active Dyrk1A in the immunocomplexes were analyzed by immunoblot with anti-Dyrk1A and anti-phosphotyrosine antiserum. Toxin-induced JNK activation and the proper expression of HA-ASK1 were examined by immunoblotting of cell lysates with anti-HA, anti-phosphoJNK, or anti-JNK antibodies. (B) After HEK293 cells were mock-transfected or transfected with Xpress-tagged kinase-defective Dyrk1A for 24 h, the cells were left untreated or with TNF$\alpha$ (100 ng/ml), $H_2O_2$ (1 mM), or etoposide (100 $\mu$M) for 1 hr, as indicated. The activation of ASK1 was examined by immunoblot assay using anti-phospho ASK1 (Thr-845). The levels of HA-ASK1, JNK, and active JNK in cell lysates were also immunoblotted with anti-Xpress, anti-JNK, or anti-phosphoJNK antibodies, respectively.

ASK1 spanning 937~1145$^{th}$ amino acid is critical for the interaction with Dyrk1A.

### *Dyrk1A lies on the upstream of ASK1 in toxin-induced JNK signal transduction*

Based on the finding that Dyrk1A is linked to JNK signaling pathway and interacts with ASK1, we next examined how Dyrk1A is linked to ASK1 and/or JNK signaling cascades. To firstly determine whether the activation of Dyrk1A is mediated by ASK1, HEK293 cells were mock-transfected or transfected with Flag-tagged kinase defective ASK1 for 24 hr. After treatment with TNF$\alpha$, cell lysates were then immunoprecipitated with anti-Dyrk1A, followed by immunoblotting with anti-phosphotyrosine. As shown in Fig. 6A, the exposure of TNF$\alpha$ caused the activation of Dyrk1A as well as JNK, and the presence of kinase-defective ASK1 resulted in the significant reduction of JNK activity, suggesting that ASK1 is present upstream of JNK. Unlike to JNK, the levels of phospho-Dyrk1A levels were not changed remarkably by kinase-inactive ASK1, compared to the cells in the absence of ASK1 mutants (Fig. 6A). When the same experiments were performed under the condition of $H_2O_2$- and etoposide-induced cell death, the similar activation pattern of JNK and Dyrk1A was observed in HEK293 cells, whereas these two stimuli caused much less activation of Dyrk1A than TNF$\alpha$ (Fig. 6A). However, the presence of kinase-defective ASK1 did not affect the activation of Dyrk1A induced by $H_2O_2$ and etoposide (Fig. 6A). Taken together, these results suggest that ASK1 does not affect Dyrk1A activity.

We next examined whether Dyrk1A acts as an upstream regulator of ASK1-JNK signaling. After HEK293 cells were co-transfected with HA-ASK1 alone or together with kinase-defective Xpress-Dyrk1A-K188R, the activation pattern of ASK1 and JNK under the condition of toxin-induced cell death was determined by immunoblot assay with anti-phospho ASK1 and anti-phospho JNK. As shown Fig. 6B, when the cells were co-transfected with ASK1 plus kinase-defective Dyrk1A, the activation of ASK1 and JNK was significantly inhibited by TNF$\alpha$, $H_2O_2$, and etoposide, compared with the cells transfected with ASK1 alone. These data indicated that Dyrk1A as a upstream regulator positively modulates ASK1-JNK signaling during toxin-induced cell death.

To examine Dyrk1A directly phosphorylates ASK1, *in vitro* kinase assay was performed. After transfected with a plasmid encoding Xpress-Dyrk1A for 24 h, HEK293 cells were stimulated with etoposide. Then, cell lysates were immunoprecipitated with anti-Xpress antiserum, followed by *in vitro* kinase assay by using bacterial recombinant ASK1$^{750\text{-}1013}$ or ASK1$^{1014\text{-}1374}$ fragment fused to GST as a substrate. As shown in Fig. 7, both of these two ASK1 fragments were well phosphorylated by Dyrk1A. These results suggest that Dyrk1A directly phosphorylates the C-terminal domain of ASK1.

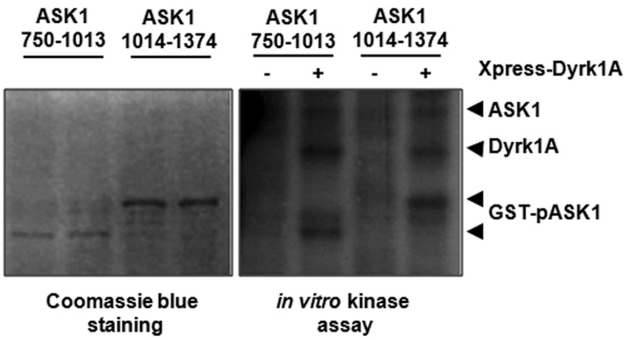


**Fig. 7.** Dyrk1A directly phosphorylates the C-terminal domain of ASK1. Where indicated, HEK293 cells were mock-transfected or transfected with Xpress-Dyrk1A for 24 h. Then, the cell lysates were immunoprecipitated with anti-Xpress, and the immunocomplexes was subjected to *in vitro* kinase assay with GST-tagged recombinant ASK1 fragments, such as GST-ASK1$^{750\text{-}1013}$ or GST-ASK1$^{1014\text{-}1375}$ as a substrate.

## DISCUSSION

Dyrk1A activity is involved in cell proliferation and differentiation (Dierssen and de Lagran, 2006). For example, while Ras-dependent signaling is required for promoting or maintaining neuronal differentiation, Dyrk1A modulates ERK activation by interacting with Ras, B-Raf, and MEK1 and by facilitating the formation of a Ras/B-Raf/MEK1 multi-protein complex (Kelly and Rahmani, 2005). Recently, many studies implicated a potential role of Dyrk1A during cell death. For example, Dyrk1A caused the formation of abnormal protein aggregates through the phosphorylation of $\alpha$-synuclein, APP, and tau (Kim et al., 2006; Park et al., 2007; Ryoo et al., 2008). Furthermore, these cells show a marked increase of apoptotic cell death (Park et al., 2007), indicating that the overexpression of Dyrk1A induces cell death. Moreover, Dyrk1A seems to participate in the pathogenesis of Huntington disease by modulating the interaction of toxic huntingtin and Hip-1 in hiipocampal neuronal cells (Kang et al., 2005). However, the functional role of Dyrk1A during cell death and its signal transduction pathway have not been elucidated yet. Presently, we demonstrated that the activation of Dyrk1A occurs during toxin-induced cell death, including TNF$\alpha$, hydrogen peroxide, and etoposide. In addition, while the activation of Dyrk1A is linked to and positively stimulates JNK-signaling, Dyrk1A did not directly bind to JNK.

Pro-apoptotic ASK1 mediates the induction of apoptosis under the stimulation of diverse stress through the activation of JNK signaling pathway. This hypothesis was confirmed by the current finding. We demonstrated that Dyrk1A physically interacts with ASK1 and enhances ASK1 and acts as a positive regulator of ASK1. Moreover, Dyrk1A appears to directly phosphorylate the C-terminal domain of ASK1. Similar to Dyrk1A, several proteins were reported to positively regulate ASK1 activity. For example, TRAF family and Gemin5 promoted the homo-oligomerization of ASK1 (Nishitoh et al., 1998; Kim et al., 2007), and CaMKII induces the phosphorylation of ASK1 in $Ca^{2+}$-influx condition (Takeda et al., 2004). Further study is required how Dyrk1A positively regulates ASK1.

The Dyrk family consists of five mammalian members (Dyrk1A, Dyrk1B, Dyrk2, Dyrk3, and Dyrk4). Among these family members, Dyrk1A is the only member located on chromosome 21. Dyrk1A contains multiple domains, including a nuclear localization signal at the N-terminus, a kinase domain, a PEST domain for protein degradation, a 13-consecutive-histidine repeat, and an S/T rich region. Even though Dyrk1A has a nuclear localization signal and 13 histidine repeat for nuclear speckle targeting, Dyrk1A has been detected in the soma and dendrites of neurons. Therefore, it is not surprising that Dyrk1A substrates comprise both nuclear and cytosolic proteins, including transcriptional factors (CREB, NFAT, STAT3, FKHR, Gli1), splicing factors (cyclin L2, SF2, SF3), a translation factor (eIF2B$\varepsilon$), and synaptic proteins (dynamin I, amphiphysin I, synaptojanin I). Expanding the substrate diversity and pleiotropic roles for Dyrk1A, the current finding demonstrates that Dyrk1A interacts with and phosphorylates other cytosolic protein kinase, ASK1.

Although Dyrk1A is linked to JNK signal transduction pathway during cell death, the direct effect of Dyrk1A on cell viability or cell death/ survival was not assessed yet. The current finding that Dyrk1A enhances the activities of ASK1 and JNK1, it could act as a pro-apoptotic player. This speculation was further supported by the previous reports. For example, the activation of Dyrk1A has been reported in several neurodegenerative diseases, such as DS and AD. For example, Ts65Dn mouse, a well-

known mouse model of DS with abnormal 1.5-fold accumulation of Dyrk1A, exhibits hippocampal hypocellularity in early development and adult (Lorenzi and Reeves, 2006), and increased mitochondrial superoxide level (Schuchmann and Heinemann, 2000). Besides, the susceptibility of cell death has been increased in various types of cells from DS patients (Busciglio and Yankner, 1995; Roat et al., 2007). Furthermore, the cells overexpressing Dyrk1A exhibited abnormal processing of several AD-related pathogenic proteins (Park et al., 2007). Like to Dyrk1A, ASK1 activity also contributes to the cell death in the neurodegenerative diseases, including AD and HD. The accumulation of intracellular aggregates induced cellular stress, which subsequently leads to neuronal cell death in an ASK1-dependent manner in HD (Nishitoh et al., 2002). ROS-induced activation of ASK1 is also a key mechanism for $\beta$-amyloid-induced neurotoxicity in AD (Hashimoto et al., 2003; Kadowaki et al., 2005). Based on these previous reports, it would be interesting to examine whether and how Dyrk1A leads to cell death through ASK1-JNK signal transduction.

In conclusion, the current study demonstrated that Dyrk1A positively regulates ASK1-JNK activation during toxin-induced cell death.

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