J. Biochem. **133**, 719–724 (2003)
DOI: 10.1093/jb/mvg092

Apoptosis-Signal Regulating Kinase-1 Is Involved in the Low Potassium–Induced Activation of p38 Mitogen-Activated Protein Kinase and c-Jun in Cultured Cerebellar Granule Neurons

Satoru Yamagishi[1,*], Masashi Yamada[1], Hisatsugu Koshimizu[1], Satomi Takai[1], Hiroshi Hatanaka[1], Kohsuke Takeda[2], Hidenori Ichijo[2], Koji Shimoke[3] and Toshihiko Ikeuchi[3]

[1]Division of Protein Biosynthesis, Institute for Protein Research, Osaka University, Suita, Osaka; [2]Cell Signaling, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549; [3]Laboratory of Neurobiology, Faculty of Engineering and High Technology Research Center, Kansai University, 3-3-35 Yamate, Suita, Osaka 564-8680

Previously, we reported that p38, which belongs to the mitogen-activated protein kinase (MAPK) superfamily, has an important role in the induction of apoptosis of cultured cerebellar granule neurons. However, the molecular mechanisms upstream of p38 activation remain unclear. Apoptosis signal-regulating kinase-1 (ASK1), a MAPK kinase kinase (MAPKKK) protein, is known to activate both c-Jun N-terminal kinase (JNK) and p38 via MAPK kinase (MKK) 4/7 and MKK3/6, respectively. Here, we examined whether ASK1 is involved in the activation of p38 in the low potassium (LK)-induced apoptosis of cerebellar granule neurons. We found that ASK1 was activated after a change to LK medium. In addition, the expression of ASK1-KM, a dominant-negative form of ASK1, using an adenovirus system was found to inhibit the activation of p38 and c-Jun and to prevent apoptosis. On the other hand, the expression of ASK1-ΔN, a constitutively active form of ASK1, activated p38 and c-Jun, but not JNK, another possible downstream target of ASK1. Furthermore, we examined the relationship between phosphatidylinositol 3-kinase (PI3-K) and ASK1. The addition of LY294002, a specific inhibitor of PI3-K, enhanced the ASK1 activity. These results indicate that ASK1 works downstream of PI3-K to regulate the p38–c-Jun pathway and apoptosis in cultured cerebellar granule neurons.

Key words: apoptosis signal-regulating kinase-1, c-Jun, low potassium, LY294002, p38, phosphatidylinositol 3-kinase.

Abbreviations: ASK1, apoptosis-signal regulating kinase1; HK, high potassium; JNK, c-Jun N-terminal kinase; LK, low potassium; MAPK, mitogen-activated protein kinase; MAPKKK, MAPK kinase kinase; MKK, MAPK kinase; MOI, multiplicity of infection; PI3-K, phosphatidylinositol 3-kinase.

Programmed cell death is an active process that occurs during both normal maturation of the nervous system and under pathological situations such as neurodegenerative diseases (*1–3*). A major type of programmed cell death is apoptosis with chromatin and cytoplasmic condensation and fragmentation (*4*). Culture systems with cerebellar granule neurons are widely used as models for studying neuronal apoptosis. Cerebellar granule cells from early postnatal rats can be cultured and maintained in serum-containing medium by elevating the extracellular potassium level (26 mM, high K^+; HK) (*5*) or by adding a low concentration of N-metyl-D-asparatate (NMDA) (*6*). NMDA and HK-stimulated depolarizations are presumed to mimic endogenous excitatory activity (*7*). After the maturation, a change to medium containing 5 mM potassium (low K^+; LK) induces neuronal apoptosis. In addition, this culture system provides a large homogeneous neuronal population. Therefore, these neurons are widely used as a primary cell culture system to investigate the biochemical and molecular mechanisms underlying neuronal apoptosis in the CNS.

Members of the mitogen-activated protein kinase (MAPK) family are involved in the signal transduction of apoptosis as well as in cell growth and differentiation (*8*, *9*). Two different MAPK cascades that converge on c-Jun N-terminal kinase (JNK; also known as SAPK, stress-activated protein kinase) and p38 are preferentially activated by cytotoxic stresses such as UV radiation, X-rays, heat shock and osmotic shock, and by extracellular cytokines such as tumor necrosis factor and interleukin-1 (*10–12*). Apoptosis signal-regulating kinase 1 (ASK1) is a ubiquitously expressed serine/threonine MAP kinase kinase kinase (MAPKKK) that activates the MKK4/7-JNK and MKK3/MKK6-p38 signaling cascades (*13*). The overexpression of ASK1 in epithelial cells under low serum conditions induces apoptosis, and in ovarian cancer cells, the expression of a kinase-inactive mutant of ASK1 inhibits microtubule-interfering agent-induced apoptosis, suggesting that ASK1 plays an essential role in the mechanism of stress-induced apoptosis (*13–15*). In addition, although the moderate expression of a constitutively active form of ASK1 induces neuronal differentiation in naive PC12 cells (*16*), overexpression of constitu-

*To whom correspondence should be addressed at the present address: Department of Anatomy and Neuroscience, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871. Tel: +81-6-6879-3221, Fax: +81-6-6879-3229, E-mail: yamagishi@anat2.med.osaka-u.ac.jp

 © 2003 The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

tively active ASK1 induces apoptosis in nerve growth factor-differentiated PC12 cells and primary cultured rat superior cervical ganglion (SCG) neurons (*17*). In cultured cerebellar granule neurons, p38, but not JNK, is important for the phosphorylation of c-Jun and for the induction of apoptosis by lowering the potassium level (*18*). However, the pathway upstream of p38 in the LK-induced apoptosis of cerebellar granule neurons is still unknown.

Here, we report that ASK1 is involved in the mechanisms to activate the p38-c-Jun pathway in LK-induced apoptosis of cultured cerebellar granule neurons. The expression of constitutive-active ASK1 using an adenovirus system was found to enhance the phosphorylation of both p38 and c-Jun, but not JNK. On the other hand, overexpression of dominant negative ASK1 attenuated the p38 and c-Jun phosphorylation in cerebellar granule neurons cultured in LK medium. Furthermore, the expression of dominant-negative ASK1 prevented LK-induced apoptosis. These results indicate that ASK1 plays a pivotal role in the activation of the p38–c-Jun pathway in the LK-induced apoptosis of cultured cerebellar granule neurons.

EXPERIMENTAL PROCEDURE

Cell Culture—Primary cultures of dissociated cerebellar granule neurons were prepared from the cerebella of postnatal day 9 (P9) rats (Wister ST, both sexes) as described previously (*19–22*). Briefly, cells were gently dissociated with a plastic pipette after digestion with papain (90 U/ml, Worthington) at 37°C. The cells were then cultured in medium consisting of 5% precolostrum newborn calf serum (Mitsubishi Kasei), 5% heat-inactivated horse serum (55°C, 30 min; Life Technologies) and of 90% a 1:1 mixture of Dulbecco's modified Eagle's (DME) medium and Ham's F12 medium containing 15 mM HEPES buffer, pH 7.4, 30 nM selenium and 1.9 mg/ml of sodium bicarbonate, at a final cell density of 5×10^5 cells/cm^2 on a polyethyleneimine-coated surface in ϕ6 cm dishes (21 cm^2 culture surface area, Sumitomo Bakelite). After culture for 1 day in a humidified CO2 (5%) incubator, the medium was changed to 26 mM potassium-containing (high K^+; HK) minimum essential medium (MEM) supplemented with 5% heat-inactivated horse serum and 1 mM cytosine arabinoside (AraC). MEM was supplemented with 2.2 mg/ml glucose and 2.2 mg/ml sodium bicarbonate. HK-MEM was prepared by increasing the $KHCO_3$ concentration from the normal low value of 5.4 to 26 mM, with the omission of a corresponding concentration of $NaHCO_3$. After 4 days in culture in a 10% CO_2 incubator, the medium was changed to serum-free 5.4 mM potassium-containing (low K^+; LK) MEM or HK-MEM. The assays described below were then performed.

Infection of Recombinant Adenoviruses—Recombinant adenoviruses encoding HA-tagged ASK1-KM, ASK1-ΔN, and β-galactosidase (LacZ) were prepared as described previously (*14*). They were propagated in HEK 293 cells and purified by cesium gradient centrifugation. The viral titer was determined by plaque-forming assay on HEK 293 cells (*23*). After 4 days maturation, cerebellar granule neurons were infected with recombinant adenoviruses in HK-MEM supplemented with 5% HS and 1 μM Ara C. After incubation with adenoviruses for 24 h, the viruses were deprived. One day after the deprivation, nearly 100% infection by recombinant adenoviruses of cerebellar granule neurons was achieved at a multiplicity of infection (MOI) of 100 as determined by anti-HA antibody staining (data not shown).

Assay of Neuronal Survival—Neuronal survival was determined by MTT assay according to the original procedure (*24*) with some modifications (*25*). Briefly, the tetrazolium salt MTT [3-(4,5-dimethyl-2-thizolyl)-2,5-diphenyl-2*H* tetrazolium bromide] was added to cultures to a final concentration of 1 mg/ml. After incubation for 2 h at 37°C, the assay was stopped by adding an 80% volume of lysis buffer (10% SDS in 50% N,N-dimethyl formamide, pH 4.7). Following overnight incubation at 37°C, the absorbance was measured spectrophotometrically at 570 nm. The percent survival was defined as [Abs.(experimental – blank)/Abs.(control – blank)] $\times$ 100; the blank value was taken from wells without cells.

In Vitro Kinase Assay—Lysates were prepared from cerebellar granule neurons cultured for 0, 1, 3, 5, and 7 h after a change to LK medium, using Triton lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA (pH 8.0), 10 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylarsine oxide (PAO), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were then clarified by centrifugation, and the supernatants were immunoprecipitated overnight with anti-ASK1 antibody. Protein G-Sepharose (10-μl gel) was then added and the mixtures were rotated at 4°C for 1 h. The immune-complexes were pelleted by centrifugation at 10,000 $\times g$ for 1 min at 4°C and then washed twice with Triton lysis buffer and twice with wash buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 2 mM DTT, and 1 mM PMSF. The ASK1 kinase reaction was carried out for 10 min at 30°C with the immunoprecipitate and 2 μg of GST-MKK6 (human) as a substrate in kinase buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM MgCl, 0.3 μCi [γ-^{32}P] ATP and 25 μM ATP. After SDS-PAGE, the incorporation of ^{32}P into GST-MKK6 was visualized using a Fuji BAS2000 image analyzer.

Immunoblotting—Cells were lysed in SDS lysis buffer containing 1% SDS, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8.0), 10 mM NaF, 2 mM Na_3VO_4, 0.5 mM PAO, and 1 mM PMSF. The lysates were boiled for 3 min, then clarified by ultracentrifugation at 60,000 $\times g$ for 30 min at 8°C. The protein concentration was determined using a BCA protein assay kit (PIERCE), and then 10 μg aliquots of protein were resolved by electrophoresis in 10% SDS–polyacrylamide gels. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corp.) in 0.1 M Tris base, 0.192 M glycine and 20% methanol using a semi-dry electrophoretic transfer system. The membranes were blocked with 0.1% Tween 20/Tris-buffered saline (T-TBS) containing 5% nonfat dried milk at room temperature for 1 h. Membranes were probed with 1:10,000 anti-ASK1 antibody, 1:500 anti-phospho-JNK antibody, 1:500 anti-JNK antibody, 1:500 anti-phospho-p38 antibody, 1:500 anti-p38 antibody, 1:500 anti-phospho-c-Jun antibody and 1:1000 anti-c-Jun antibody in T-TBS containing 1 or 5% nonfat dried milk at room temperature for 1 h. After three washes with T-TBS, the membranes were incubated with horseradish peroxidase-

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

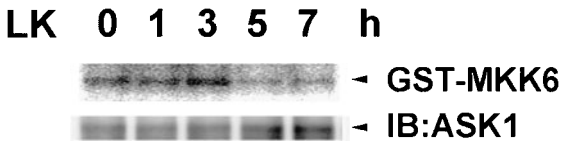

Fig. 1. **ASK1 activation during LK-induced apoptosis of cultured cerebellar granule neurons.** After the cells had matured for 4 days, lysates were prepared in Triton-X lysis buffer from granule neurons cultured for 0, 1, 3, 5, and 7 h in serum-free LK-MEM. ASK1 was immunoprecipitated from the lysates with anti-ASK1 antibody. The in vitro kinase reaction was carried out for 10 min at 30°C with the ASK1 immunoprecipitate and 2 μg of GST-MKK6 as a substrate in kinase buffer. After SDS-PAGE, the ^{32}P incorporated into GST-MKK6 was visualized using a Fuji BAS2000 image analyzer (upper). The same immunoprecipitates were immunoblotted with anti-ASK1 antibody (bottom). IB, immunoblot.

conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoReserch Laboratories) diluted 1:1500 with T-TBS containing 1% nonfat dried milk at room temperature for 1 h. The membranes were then washed at least 4 times with T-TBS, and visualized using an enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech) or Immunostar (Wako).

Antibodies and Reagents—Anti-ASK1 antibody (DAV) was prepared as described previously (*14*). Anti-phospho-p38, anti–phospho-JNK, anti-JNK and anti-phospho-c-Jun antibodies were purchased from New England Biolabs. Anti-p38 and anti-c-Jun antibodies were obtained from Santa Cruz Biotechnology. LY294002 was obtained from Calbiochem and dissolved at 10 mM in dimethyl sulfoxide (DMSO) as a stock solution.

RESULTS

ASK1 Is Activated in LK-Induced Apoptosis of Cultured Cerebellar Granule Neurons—ASK1, which works upstream of stress-responsive protein kinases such as JNK and p38, is a key protein in induction of apoptosis in various types of cells (*13–15*). We have reported that the activation of p38 is an important event in the LK-induced apoptosis of cultured cerebellar granule neurons (*18*). We thought that ASK1 might also be involved in the activation of p38 in LK-induced apoptosis. As a first step, we examined ASK1 activity during LK-induced apoptosis of cultured cerebellar granule neurons. The lysates for immunoprecipitation with anti-ASK1 antibodies (DAV) were prepared from cultured granule neurons at 0, 1, 3, 5, and 7 h after the change to LK medium. The immunoprecipitates were subjected to *in vitro* kinase assay using GST-MKK6 as a substrate (Fig. 1). At 3 h after the change to LK medium, the ASK1 activity was increased, but it declined to the basal level within 5 h. This result implies that ASK1 is involved in the LK-induced apoptosis of cultured granule neurons. ASK1 may regulate p38 activity during apoptosis.

Dominant-Negative ASK1 Prevents the LK-Induced Activation of p38 and c-Jun, but Not JNK, and Cell Death of Cultured Cerebellar Granule Neurons—Next, we examined whether the activated ASK1 is involved in the induction of LK-induced apoptosis in cultured cerebellar granule neurons. After maturation in HK medium for 4

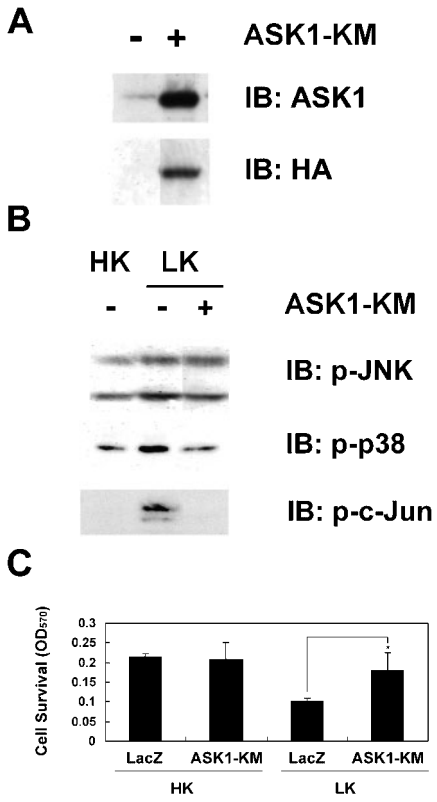

Fig. 2. **Effect of dominant-negative ASK1 on the phosphorylation of JNK, p38 and c-Jun and on cell death.** (A) Cerebellar granule neurons were infected with or without recombinant adenoviruses expressing ASK1-KM at a MOI of 100. One day after infection, cell lysates were prepared using SDS lysis buffer. The same lysates were immunoblotted with anti-ASK1 and anti-HA antibody. IB, immunoblot. (B) Cerebellar granule neurons were infected with or without recombinant adenoviruses expressing ASK1-KM at a MOI of 100. One day after the infection, the medium was switched to serum-free LK- or HK-MEM. At 0 and 3 h after the medium change, lysates were prepared in SDS lysis buffer. The lysates were immunoblotted with anti-phospho-p38, anti-phospho-JNK and anti-phospho-c-Jun antibodies. The blots shown came from the same lysates. IB, immunoblot. (C) One day after infection with recombinant adenoviruses encoding LacZ or ASK1-KM at a MOI of 100, the medium was changed to LK-MEM. After medium change, the cells were cultured for 24 h, then cell survival was quantified by MTT assay. Data represent means ± SD ($n = 4$). Statistical analysis was performed with Student's *t*-test. $^*p < 0.05$

days, dominant-negative ASK1 (ASK1-KM) or β-galactosidase (LacZ) as a control was expressed in cultured cerebellar granule neurons using the adenovirus expression system. First, we checked the expression of these exogenous proteins in cultured granule neurons. Cultured granule neurons were infected with recombinant adenoviruses expressing ASK1-KM or LacZ tagged with hemagglutinin (HA) epitope, and then stained with anti-HA antibody 24 h after infection. At a MOI of 100, all of the cultured neurons were positive for anti-HA antibody staining (data not shown). In Western blot analysis using anti-ASK1 and anti-HA antibodies, a high level of ASK1-KM expression was detected one day after infection at a MOI of 100, as compared with endogenous ASK1 (Fig. 2A).

In this system, we examined the effects of ASK1-KM on the phosphorylation of JNK, p38 and c-Jun in the LK-

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

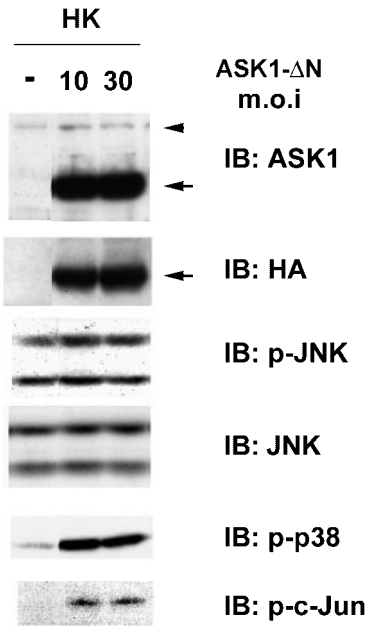

Fig. 3. **Activation of p38 and c-Jun, but not JNK, by the expression of constitutive-active ASK1.** Cerebellar granule neurons were infected with or without recombinant adenoviruses expressing HA-tagged ASK1-ΔN at MOI of 0, 10, and 30. One day after infection, lysates were prepared using SDS lysis buffer. The lysates were immunoblotted with anti-ASK1, anti-HA, anti-phospho-JNK, anti-JNK, anti-phospho-p38, and anti-phospho-c-Jun antibodies. The blots shown came from the same lysates. The arrowhead indicates endogenous ASK1 and arrows indicate exogenous ASK1-ΔN. IB, immunoblot.

induced apoptosis of cultured cerebellar granule neurons (Fig. 2B). Three hours after potassium deprivation, an increase in p38 phosphorylation was detected as described in our previous report (*18*). The ectopic expression of ASK1-KM at a MOI of 100 inhibited the phosphorylation of p38, however, the phosphorylation of JNK was not affected by the exogenous expression of ASK1-KM. In our previous report, we showed that p38 regulates the phosphorylation and activation of c-Jun in cultured cerebellar granule neurons (*18*). Immunoblot analysis using anti-phospho-c-Jun antibody showed that the increase in c-Jun phosphorylation during LK-induced apoptosis was also blocked by the ectopic expression of ASK1-KM. These results suggest that ASK1 is specifically involved in the phosphorylation of p38 and c-Jun, but not JNK, in the LK-induced apoptosis of cultured cerebellar granule neurons. Next, we examined whether LK-induced apoptosis is inhibited by the expression of ASK1-KM (Fig. 2C). After infection with ASK1-KM or LacZ adenovirus at a MOI of 100, apoptosis was induced by lowering the potassium concentration. Twenty four hours after the induction of apoptosis, cell survival was quantified by MTT assay. Only 46.5% of LacZ-expressing cells survived in LK medium, as compared with HK medium. On the other hand, the expression of ASK1-KM, a dominant negative form of ASK1, markedly prevented LK-induced apoptosis (86.2%). This result shows that ASK1 plays a pivotal role in the induction of apoptosis by lowering potassium concentration.

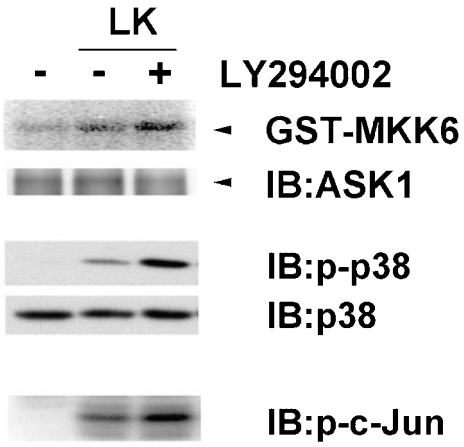

Fig. 4. **LY294002 enhances ASK1 activation in LK-induced apoptosis of cultured cerebellar granule neurons.** After maturation for 4 days, lysates were prepared in Triton-X lysis buffer from granule neurons cultured for 0 and 3 h in serum-free LK-MEM with or without 10 μM LY294002. ASK1 was immunoprecipitated with anti-ASK1 antibody. The in vitro kinase reaction was carried out for 10 min at 30°C with the ASK1 immunoprecipitate and 2 μg of GST-MKK6 as a substrate in kinase buffer. After SDS-PAGE, the ^{32}P incorporated into GST-MKK6 was visualized using a Fuji BAS2000 image analyzer (upper panel). The same immunoprecipitates were immunoblotted with anti-ASK1. The lysates prepared as above were immunoblotted with anti-phospho-p38, anti-p38 and anti–phospho-c-Jun antibodies. IB, immunoblot.

Constitutive-Active ASK1 Activates p38 and c-Jun, but Not JNK, in Cultured Cerebellar Granule Neurons—To examine whether the activation of ASK1 is sufficient to induce the activation of p38 and c-Jun in cultured cerebellar granule neurons, we used an adenovirus expressing an N-terminal-deleted constitutive-active ASK1 (ASK1-ΔN) (Fig. 3). The expression level of ASK1-ΔN was determined by Western blotting analysis with anti-ASK1 and anti-HA antibodies. The level of exogenous ASK1-ΔN protein is much higher than endogenous ASK1at MOI of 10 and 30. Then, the same samples were immunoblotted with anti-phospho-p38 antibody. As a result, even in cultured granule neurons in HK medium, ASK1-ΔN expression induced a remarkable phosphorylation of p38. However, the phosphorylation of JNK was not affected even by a high level of expression of ASK1-ΔN. Next, we investigated c-Jun phosphorylation by immunoblotting with anti-phospho-c-Jun antibody. Phosphorylation of c-Jun was increased by infection with ASK1-ΔN adenovirus at MOI of 10 and 30, despite the high potassium conditions. These results indicate that the activation of ASK1 is sufficient to activate p38 and c-Jun, but not JNK, in cultured cerebellar granule neurons.

ASK1 Is Regulated by the PI3-K Pathway—Recently, we found that BDNF and IGF-1 inhibit p38 and c-Jun activation *via* the PI3-K pathway in cultured cerebellar granule neurons (Yamagishi *et al*., unpublished data). It is an intriguing question whether PI3-K inhibits the activation of ASK1 upstream of p38. To answer this question, we examined the effect of LY294002, a specific inhibitor of PI3-K, on the activity of ASK1 in cultured granule neurons (Fig. 4). An *in vitro* kinase assay was carried out using GST-MKK6 as a substrate. The activation of ASK1

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

caused by lowering the potassium concentration was further increased by the addition of LY294002. In addition, LY294002 also enhanced the phosphorylation of p38 and c-Jun. These results suggest that PI3-K negatively regulates ASK1, leading to an attenuation of the activation of p38 and c-Jun in cultured cerebellar granule neurons.

DISCUSSION

In cultured cerebellar granule neurons, the activation of c-Jun plays an important role in the induction of apoptosis by lowering the potassium concentration (*26*). In our previous report, we showed that p38 directly phosphorylates c-Jun in the LK-induced apoptosis of cerebellar granule neurons. In an attempt to identify a mediator of p38 activation in the LK-induced apoptosis of cultured cerebellar granule neurons, we examined the role of ASK1. ASK1 has been reported to be involved in several apoptotic processes (*13–15*). ASK1 belongs to the MAPKKK family and activates the JNK and p38 pathways via MKK4/7 and MKK3/6, respectively. In the present study, we found that ASK1 regulates the p38 activity in LK-induced apoptosis of cultured cerebellar granule neurons.

Cells are continuously exposed to multiple opposing "death" and "survival" triggers. Akt is a key molecule that switches between cell survival and death in various ways. For example, Akt phosphorylates and inactivates two pro-apoptotic molecules, BAD and FKHR, promoting cell survival (*27*, *28*). The withdrawal of survival factors leads to the inactivation of Akt, resulting in the dephosphorylation of BAD and release of cytochrome C from mitochondria, or in the activation of FKHR followed by the expression of pro-apoptotic proteins such as Fas (*28*). Yamaguchi *et al*. have reported that Akt inactivation causes Bax translocation to mitochondria, leading to mitochondrial dysfunction (*29*). Recently, Kim *et al*. reported that the N-terminal Ser^{83} of ASK1 is phosphorylated by Akt, resulting in the inactivation of ASK1 (*30*). As shown in Fig. 4, LY294002, a specific inhibitor of PI3-K, enhances the ASK1 activity in cultured cerebellar granule neurons. LY294002 also enhances the phosphorylation of p38 and c-Jun. Furthermore, we found that BDNF and IGF-1, both known to activate PI3-K, inhibited the activation of ASK1 as well as p38 and c-Jun in LK-induced apoptosis (Yamagishi *et al*., unpublished data). These findings indicate that the PI3-K-Akt pathway negatively regulates ASK1, preventing the activation of p38 and c-Jun in cultured cerebellar granule neurons.

c-Jun is a very important molecule for the induction of apoptosis in superior cervical ganglia (SCG), pheochromocytoma PC12 cells, and cerebellar granule neurons (*26*, *31*). However, the upstream regulators of c-Jun are distinct among these cells. In SCG and PC12 cells, JNK phosphorylates c-Jun after the withdrawal of NGF. Kanamoto *et al*. have reported that ASK1 plays an important role in the activation of JNK downstream of cdc42 in the NGF withdrawal-stimulated apoptosis of SCG neurons and NGF-differentiated PC12 cells (*17*). On the other hand, in cultured cerebellar granule neurons, p38 rather than JNK phosphorylates c-Jun when the potassium concentration is lowered. The expression of constitutive-active ASK1-ΔN increases the level of p38 phosphorylation, but not that of JNK phosphorylation in cultured cerebellar granule neurons (Fig. 3). These results indicate that ASK1 regulates the activation of p38, but not JNK, in cultured cerebellar granule neurons.

ATF2 is well known as a substrate of p38 and JNK (*32–24*). It is possible that p38 phosphorylates not only c-Jun but also ATF2 during LK-induced apoptosis. We performed Western blotting with anti-phospho ATF2 antibody (data not shown). At 3 h after the change to LK medium, the phosphorylation of ATF2 increased as well as that of c-Jun. However, the phosphorylation of ATF2 was not inhibited by the expression of dominant-negative ASK1-KM. Furthermore, constitutively active ASK1-ΔN did not enhance the ATF2 phosphorylation (data not shown). Taken together, ATF2 is unlikely to work downstream of ASK1 and p38 in cultured cerebellar granule neurons. The LK-induced increase in the phosphorylation of ATF2 may be mediated by kinases other than p38 in cultured cerebellar granule neurons. The MEF2 family is another possible substrate for p38. Mammalian MEF2 proteins are encoded by four genes (MEF2A, MEF2B, MEF2C, and MEF2D). Recently, Li *et al*. reported that MEF2A and 2D, but not MEF2B and MEF2C, are phosphorylated and degraded in the LK-induced apoptosis of cultured cerebellar granule neurons (*35*). p38 may also phosphorylate MEF2A and 2D, although further study is required.

Recently, Coffey *et al*. have reported that JNK 2/3 are activated after the change to LK medium, and play an important role in the phosphorylation of c-Jun. It may be possible that ASK1 regulates not only p38 but also JNK 2/3 (*36*). However, we could detect the activation of p38, but not JNK, in the case of the overexpression of constitutive active ASK1 (Fig 3). In our previous paper, we showed that the phosphorylation of c-Jun is blocked by the inhibition of p38 (*18*). This suggests that p38 is important in ASK1-mediated c-Jun phosphorylation, but further experiments are needed to clarify this inconsistent phenomenon.

We thank Prof. A. Ogura of the Graduate School of Science, Osaka University for discussions and encouragement. This work was financially supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan and by the Kansai University Special Research Fund, 2002. S.Y. is a research fellow of the Japan Society for Promotion of Science.

REFERENCES

1. Clarke, P.G. (1990) Developmental cell death: morphological diversity and multiple mechanisms. *Anat. Embryol. (Berl)* **181**, 195–213
2. Henderson, C.E. (1996) Programmed cell death in the developing nervous system. *Neuron* **17**, 579–585
3. Pettmann, B. and Henderson, C.E. (1998) Neuronal cell death. *Neuron* **20**, 633–647
4. Nagata, S. (2000) Apoptotic DNA fragmentation. *Exp. Cell Res.* **256**, 12–18
5. Gallo, V., Kingsbury, A., Balazs, R., and Jorgensen, O.S. (1987) The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. *J. Neurosci.* **7**, 2203–2213
6. Balazs, R., Jorgensen, O.S., and Hack, N. (1988) N-methyl-D-aspartate promotes the survival of cerebellar granule cells in culture. *Neuroscience* **27**, 437–451

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012

7. Burgoyne, R.D., Graham, M.E., and Cambray-Deakin, M. (1993) Neurotrophic effects of NMDA receptor activation on developing cerebellar granule cells. *J. Neurocytol.* **22**, 689–695
8. Nishida, E. and Gotoh, Y. (1993) The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.* **18**, 128–131
9. Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol. Rev.* **79**, 143–180
10. Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J., and Greenberg, M.E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270**, 1326–1331
11. Kyriakis, J.M. and Avruch, J. (1996) Protein kinase cascades activated by stress and inflammatory cytokines. *Bioessays* **18**, 567–577
12. Ichijo, H. (1999) From receptors to stress-activated MAP kinases. *Oncogene* **18**, 6087–6093
13. Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* **275**, 90–94
14. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J.* **17**, 2596–2606
15. Wang, T.H., Popp, D.M., Wang, H.S., Saitoh, M., Mural, J.G., Henley, D.C., Ichijo, H., and Wimalasena, J. (1999) Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun N-terminal kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. *J. Biol. Chem.* **274**, 8208–8216
16. Takeda, K., Hatai, T., Hamazaki, T.S., Nishitoh, H., Saitoh, M., and Ichijo, H. (2000) Apoptosis signal-regulating kinase 1 (ASK1) induces neuronal differentiation and survival of PC12 cells. *J. Biol. Chem.* **275**, 9805–9813
17. Kanamoto, T., Mota, M., Takeda, K., Rubin, L.L., Miyazono, K., Ichijo, H., and Bazenet, C.E. (2000) Role of apoptosis signal-regulating kinase in regulation of the c-Jun N-terminal kinase pathway and apoptosis in sympathetic neurons. *Mol. Cell. Biol.* **20**, 196–204
18. Yamagishi, S., Yamada, M., Ishikawa, Y., Matsumoto, T., Ikeuchi, T., and Hatanaka, H. (2001) p38 mitogen-activated protein kinase regulates low potassium-induced c-Jun phosphorylation and apoptosis in cultured cerebellar granule neurons. *J. Biol. Chem.* **276**, 5129–5133
19. Hatanaka, H., Tsukui, H., and Nihonmatsu, I. (1988) Developmental change in the nerve growth factor action from induction of choline acetyltransferase to promotion of cell survival in cultured basal forebrain cholinergic neurons from postnatal rats. *Brain Res.* **467**, 85–95
20. Levi, G., Aloisi, F., Ciotti, M.T., and Gallo, V. (1984) Autoradiographic localization and depolarization-induced release of acidic amino acids in differentiating cerebellar granule cell cultures. *Brain Res.* **290**, 77–86
21. Shimoke, K., Yamada, M., Ikeuchi, T., and Hatanaka, H. (1998) Synthetic lipid products of PI3-kinase which are added to culture medium prevent low K+-induced apoptosis of cerebellar granule neurons via Akt kinase activation. *FEBS Lett.* **437**, 221–224
22. Matsumoto, T., Numakawa, T., Adachi, N., Yokomaku, D., Yamagishi, S., Takei, N., and Hatanaka, H. (2001) Brain-derived neurotrophic factor enhances depolarization-evoked glutamate release in cultured cortical neurons. *J. Neurochem.* **79**, 522–530
23. Kanegae, Y., Makimura, M., and Saito, I. (1994) A simple and efficient method for purification of infectious recombinant adenovirus *Jpn. J. Med. Sci. Biol.* **47**, 157–166
24. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55–63
25. Hansen, M.B., Nielsen, S.E., and Berg, K. (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods* **119**, 203–210
26. Watson, A., Eilers, A., Lallemand, D., Kyriakis, J., Rubin, L.L., and Ham, J. (1998) Phosphorylation of c-Jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons. *J. Neurosci.* **18**, 751–762
27. Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M.E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**, 231–241
28. Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857–868
29. Yamaguchi, H. and Wang, H.G. (2001) The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. *Oncogene* **20**, 7779–7786
30. Kim, A.H., Khursigara, G., Sun, X., Franke, T.F., and Chao, M.V. (2001) Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. *Mol. Cell. Biol.* **21**, 893–901
31. Eilers, A., Whitfield, J., Babij, C., Rubin, L.L., and Ham, J. (1998) Role of the Jun kinase pathway in the regulation of c-Jun expression and apoptosis in sympathetic neurons. *J. Neurosci.* **18**, 1713–1724
32. Gupta, S., Campbell, D., Derijard, B., and Davis, R.J. (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* **267**, 389–393
33. van Dam, H., Wilhelm, D., Herr, I., Steffen, A., Herrlich, P., and Angel, P. (1995) ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *EMBO J.* **14**, 1798–1811
34. Livingstone, C., Patel, G., and Jones, N. (1995) ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *EMBO J.* **14**, 1785–1797
35. Li, M., Linseman, D.A., Allen, M.P., Meintzer, M.K., Wang, X., Laessig, T., Wierman, M.E., and Heidenreich, K.A. (2001) Myocyte enhancer factor 2A and 2D undergo phosphorylation and caspase-mediated degradation during apoptosis of rat cerebellar granule neurons. *J. Neurosci.* **21**, 6544–6552
36. Coffey, E.T., Smiciene, G., Hongisto, V., Cao, J., Brecht, S., Herdegen, T., and Courtney, M.J. (2002) c-Jun N-terminal protein kinase (JNK) 2/3 is specifically activated by stress, mediating c-Jun activation, in the presence of constitutive JNK1 activity in cerebellar neurons. *J. Neurosci.* **22**, 4335–4345

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 29, 2012