dp5/HRK Is a c-Jun Target Gene and Required for Apoptosis Induced by Potassium Deprivation in Cerebellar Granule Neurons*

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In cerebellar granule neurons, a BH3-only Bcl-2 family member, death protein 5/harakiri, is up-regulated in a JNK-dependent manner during apoptosis induced by potassium deprivation. However, it is not clear whether c-Jun is directly involved in the induction of dp5. Here, we showed that the up-regulation of dp5, but not fas ligand and bim, after potassium deprivation was suppressed by the expression of a dominant negative form of c-Jun. Deletion analysis of the 5’-flanking sequence of the dp5 gene revealed that a major responsive element responsible for the induction by potassium deprivation is an ATF binding site located at −116 to −109 relative to the transcriptional start site. Mutation of this site completely abolished promoter activation. Furthermore, a gel shift assay showed that a specific complex containing c-Jun and ATF2 recognized this site and increased in potassium-deprived cerebellar granule neurons. Chromatin immunoprecipitation demonstrated that c-Jun was able to bind to this site in vivo. Finally, we demonstrated that knockdown of Dp5 by small interfering RNA rescued neurons from potassium deprivation-induced apoptosis. Taken together, these results suggest that dp5 is a target gene of c-Jun and plays a critical role in potassium deprivation-induced apoptosis in cerebellar granule neurons.

The Bcl-2 family proteins can be divided into three major subgroups (1). Antiapoptotic proteins, such as Bcl-2, Bcl-XL, and Mcl-1, typically share four conserved motifs termed Bcl-2 homology (BH)3 domains and inhibit mitochondrial cytochrome c release and apoptosis. Multidomain proapoptotic proteins, the second subgroup, such as Bax, Bak, and Bok, typically have three BH domains but promote cytochrome c release and apoptosis. The third, and the most structurally diverse subgroup, is the BH3-only proteins, including Dp5/HRK (death protein 5/harakiri), Bim (Bcl2-interacting mediator of cell death), Bid, Bad, Puma, and Noxa, which share the BH3 domain. The BH3-only proteins are critical initiators of apoptosis. Upon challenge, BH3-only proteins translocate to mitochondria and promote the chromec release by neutralizing the antiapoptotic action of Bcl-2 family members. BH3-only proteins are stringently regulated at the transcriptional and post-translational levels during apoptosis, such as Dp5, Bim, and Puma, depending on the cell type and apoptotic stimulus (2–6).

Among the BH3-only proteins, Dp5 is of particular interest to studies of apoptosis in the nervous system. In rodents, the expression of Dp5 is largely restricted to and is developmentally regulated in the nervous system (2, 7). Dp5 is the first found BH3-only protein to be induced by NGF deprivation in sympathetic neurons (2). dp5 is highly homologous to the human gene harakiri (HRK) cloned by a two-hybrid screen with Bcl-2 and Bcl-XL (3). As well as being induced in NGF-deprived sympathetic neurons, the induction of dp5 is also observed in cerebellar granule neurons (CGNs) deprived of potassium, cortical neurons exposed to toxic concentrations of amyloid-β protein, retinal ganglion cells of axotomized rat retinas, and axotomized postnatal mouse motoneurons. Overexpression of Dp5 in sympathetic neurons or CGNs induces apoptosis in a Bax-dependent manner, and this effect can be attenuated by co-expression of antiapoptotic Bcl-2. Deletion of dp5 delays sympathetic neuron apoptosis triggered by NGF withdrawal and rescues motoneurons from axotomy-induced apoptosis. These studies suggest that Dp5 plays a critical role in neuronal apoptosis.

Studies from several laboratories have demonstrated that JNK is involved in dp5 up-regulation during neuronal injury or apoptosis. In sympathetic neurons deprived of NGF (8), CGNs deprived of potassium (8), cortical neurons exposed to amyloid-β protein (9), and spinal cord injury triggered by trauma (10), dp5 is induced in a manner that is clearly JNK-dependent. Although JNK has been shown to be involved in dp5 up-regulation, the mechanism of how JNK regulates dp5 expression is not clarified.
c-Jun Targets dp5

In the present study, we used recombinant adenoviruses that express a dominant negative form of c-Jun (FLAG-Δ169) to identify the potential targets of c-Jun in neuronal apoptosis. We showed that c-Jun mediates the induction of dp5, but not that of fas ligand (fasL) and bim during potassium deprivation-induced apoptosis in CGNs. In addition, we identified an ATF site located at −116 to −109 of the dp5 promoter that was required for its activation. Furthermore, a gel shift assay showed that a specific complex containing c-Jun and ATF2 recognized this site, and the association of c-Jun/ATF2 complex was significantly increased in potassium-deprived CGNs. Chromatin immunoprecipitation (ChIP) demonstrated that c-Jun was able to bind to this site, and the association of c-Jun/ATF2 complex was significantly increased in potassium-deprived CGNs. Chromatin immunoprecipitation (ChIP) demonstrated that c-Jun was able to bind to this site in vivo. Finally, knockdown of Dp5 protects CGNs from apoptosis. Taken together, our results suggest that dp5 is a c-Jun target gene and required for apoptosis induced by potassium deprivation in CGNs.

EXPERIMENTAL PROCEDURES

Neuronal Culture and Potassium Deprivation—Rat CGNs were prepared from 7–8-day-old Sprague-Dawley rat pups (15–19 g) as described previously (11, 12). Briefly, neurons were dissociated from freshly dissected cerebella by mechanical disruption in the presence of trypsin and DNase and then seeded at a density of 1.5 × 10⁶ cells/ml in basal modified Eagle’s medium containing 10% fetal bovine serum and 25 mM KCl (25 K medium). Cytosine arabinoside (10 μM) was added 24 h after seeding to limit the growth of nonneuronal cells. For potassium deprivation, experiments were performed as described previously (11, 12). Briefly, cells cultured for 7 days (DIV7) in medium containing 25 K + 5 were switched into serum-free medium containing 25 or 5 mM KCl (25 K or 5 K) in the presence or absence of the inhibitors SP600125 (Calbiochem) or CEP11004 (a kind gift from Cephalon Inc.). Cells that did not receive inhibitors received Me2SO as a control. The final concentration of Me2SO was less than 0.1%.

Western Blotting—Western blotting analysis was performed as described in detail previously (11, 12). Briefly, protein lysates prepared from neurons were separated by a 10% polyacrylamide gel, transferred to polyvinylidene difluoride membrane and subjected to immunoblotting with polyclonal antibodies against phospho-JNK (Thr183/Tyr185), JNK, phospho-c-Jun (Ser73) (Cell Signaling Technology; diluted 1:1000), and c-Jun reverse primer 5′-GACCGACGTCGCCG-3′ and reverse primer 5′-CTCGAGATCTAGCGTCGCCGCA-3′. The full length of the −1568 to +81 fragment of dp5 promoter containing the mutated ATF site was obtained by overlap extension PCR. First, two DNA fragments with a sequence encoding FLAG-Δ169 were also constructed by PCR using dp5 (−1568/−81)-luc as the template and the common reverse primer as mentioned above. Forward primers are as follows: 5′-ATCTTTTTCTATACACCGCAA-3′ for dp5 (−356/−356) and 5′-GAGGCTCCGAGCACC-3′ for dp5 (−270/−81)-luc; 5′-CGGCGCGATGATGAA-3′ for dp5 (−125/−81)-luc; and 5′-CCCCCTCACCACATGTGACACT-3′ for dp5 (−85/−81)-luc. The mutated ATF site (TTACATCA/GGACATCG) (13) was introduced into dp5 (−1568/−81)-luc by overlap extension PCR. First, two DNA fragments with a 19-bp overlap (GAGGGGGGACATCTGGCCGCA-3′) containing the mutated ATF site were synthesized by PCR using dp5 (−1568/−81)-luc as template with forward primer 5′-GATA-GGTACACCATCTAGCTAGCATGTGACATTATTA-3′ and reverse primer 5′-GAGGCGGACATCTGCAGCA-3′ and with forward primer 5′-CGGCGATGATGATGAA-3′ and reverse primer 5′-CTCGAGATCTAGCGTCGCCGCA-3′. The full length of the −1568 to +81 fragment of dp5 promoter containing the mutated ATF site was obtained by mixing the two DNA fragments produced from the first-step PCR as template in the second PCR with the outermost primers. The second PCR was carried out at 94°C for 1 min and then 25 cycles of denaturing at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 3 min. All PCR products were subcloned into the Nhel and HindIII sites of pGL3-Basic. The sequence encoding FLAG-Δ169 was amplified from the Ad-FLAG-Δ169 by PCR and subcloned into pcDNA3 between XhoI and HindIII following the ATG and 8-amino acid FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys). Specific primers are as follows: forward, 5′-ATTACTCGAGATGACTAC-AAGGACGA-3′; reverse, 5′-ATTACTCGAGATGACTAC-AAGGACGA-3′; and reverse, 5′-ATTACTCGAGATGACTAC-AAGGACGA-3′; and reverse, 5′-ATTACTCGAGATGACTAC-AAGGACGA-3′.

Q-PCR—Total RNA was extracted and isolated from CGNs using TRIzol reagent (Invitrogen) as described previously (12). First strand cDNA was synthesized from 1 μg of mRNA by using SuperscriptIII reverse transcriptase (Invitrogen) and oligo(dT) as primers. Q-PCR was performed in triplicate on an ABI Prism 7000 sequence detection system using ABI Sybr Green PCR mixture as described by the manufacturer. PCR cycling conditions were as follows: initial denaturation at 95°C for 5–10 min followed by 40 cycles of 95°C for 30 s, 1 min of annealing (annealing temperature adapted for the specific primer set used), and 1 min of extension at 72°C. Fluorescence data were collected during the annealing stage of amplification. Specificity of the amplification was verified by melt curve analysis. Cycle threshold (Ct) values were calculated using identical threshold values for all experiments. β-Actin was used as control and for normalization. Relative RNA expression was calculated using the formula ratio = 2^{ΔC_{t} (ref−target)}.

Data shown represent the mean and S.E. of three separate experiments. The second PCR was carried out at 94°C for 1 min and then 25 cycles of denaturing at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 3 min. All PCR products were subcloned into the Nhel and HindIII sites of pGL3-Basic. The sequence encoding FLAG-Δ169 was amplified from the Ad-FLAG-Δ169 by PCR and subcloned into pcDNA3 between XhoI and HindIII following the ATG and 8-amino acid FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys). Specific primers are as follows: forward, 5′-ATTACTCGAGATGACTAC-AAGGACGA-3′; reverse, 5′-ATTACTCGAGATGACTAC-AAGGACGA-3′; and reverse, 5′-ATTACTCGAGATGACTAC-AAGGACGA-3′.
TTGCAACTGCT-3’. All of the constructs were confirmed by DNA sequencing.

CGNs were transfected using the calcium phosphate co-precipitation method as described previously (11, 12). Plasmids transfected contained 2 μg of various reporter plasmids, 9 μg of expression plasmids or pcDNA3-based vectors, and 100 ng of Renilla luciferase reporter plasmid, pCMV-RL (Promega). Neurons were kept in conditioned medium after transfection for 12 h and then switched to the serum-free medium containing 25 or 5 mM KCl for 12 h. The levels of *firefly* luciferase activity were normalized to Renilla luciferase activity, as we reported previously (12).

**Adenovirus Infection**—The recombinant adenoviruses, Ad-GFP and Ad-FLAG-Δ169 (12, 14), were purified and used as described previously (12). DIV5 neurons were infected with Ad-GFP or Ad-FLAG-Δ169 at a multiplicity of infection (MOI) of 100 for 36 h. To test the efficiency of adenovirus-mediated gene transfer, CGNs were infected at DIV5 with 100 MOI of Ad-FLAG-Δ169, the cells were fixed 36 h after infection and stained with FLAG antibody and with the DNA dye bisbenzimide (Hoechst 33258) (5 μg/ml) to visualize nuclear morphology. Fluorescent images were captured by using a fluorescence microscope equipped with a CCD camera.

**Gel Mobility Shift Assay**—Nuclear extracts were prepared as described in detail previously (15). Synthetic oligonucleotide probes spanning the ATF binding site (forward, 5′-AGCTCGGATGAGTAAACCCTGCATCGGATGAGTACCCCAAGTTTCGCTCTGC-3’; reverse, 5′-GATCGGGGTAGCTCGGACGATGTCCCCCCGATC-3’), as well as those bearing mutated ATF site (forward, 5′-AGCTCGGACGATGTCCCCCCGATC-3′; reverse, 5′-GATCGGGGTAGCTCGGACGATGTCCCCCCGATC-3′) were annealed and labeled with γ-32P (PerkinElmer Life Sciences) by using T4 polynucleotide kinase. 32P-Labeled probes were incubated with 5 μg of nuclear proteins in a 20-μl DNA binding reaction buffer. For supershift, 1 μg of c-Jun antibody (catalog number sc-187x; Santa Cruz Biotechnology) was preincubated with γ-32P-labeled probes, overnight at room temperature and were terminated with glycerol. The binding reaction was resolved by 4% polyacrylamide gel and exposed to photography.

**ChIP Assay**—ChIP was performed using the ChIP assay kit (Upstate Cell Signaling Solutions) according to the manufacturer’s instructions. Approximately 4.5 × 107 cerebral granule neurons were used in each treatment. 4 h after they were switched to the serum-free medium containing 25 or 5 mM KCl, neurons were cross-linked by the addition of 1% formaldehyde for 10 min at room temperature and were terminated with glycine (final concentration of 0.125 M). Neurons were harvested and incubated in 600 μl of SDS lysis buffer containing protease inhibitors for 10 min on ice. Chromatins were sonicated to yield fragments of about 0.5 kb in length. After sonication, the lysate was centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was diluted in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM HCl, pH 8.1, 167 mM NaCl, and protease inhibitors). After it was precleared with protein G-agarose, 5% of the supernatant was saved as input DNA. 2 μg of rabbit c-Jun antibody (catalog number sc-1694x; Santa Cruz Biotechnology) or rabbit normal IgG (Sigma) was added to the supernatant and incubated overnight at 4 °C with rotation. After wash, immune complexes were eluted with elution buffer (1% SDS, 0.1 M NaHCO3, and 200 mM NaCl). A part of the captured immunocomplex was subjected to Western blotting with another mouse c-Jun antibody (610327; BD Biosciences Pharmingen) to detect whether captured chromatin contained c-Jun. Cross-linking was reversed by heating at 65 °C overnight. RNA was degraded with RNase A for 30 min, and protein was degraded with proteinase K for 2 h. DNA was purified using the EZChIP polypropylene spin column and subjected to PCR amplification using the primers spanning the ATF site on the dp5 promoter (forward, 5′-AGGTTAAAAGTTACCTCTCCGC-3’; reverse, 5′-ACCCCAATTTGCCGCTGC-3’; c-Jun promoter spanning the TRE-jun2 site (forward, 5′-CTAGACAGACCAACACAAGAC-3’; reverse, 5′-GCTCAGGGATGAGTGTA-3’).

**RNA Interference**—The BS/U6 vector was kindly provided by Dr. Cress W. Douglas (University of South Florida College of Medicine) (16). To ensure the specificity, multiplicity controls were employed (17). Two Dp5 small hairpin RNAs (shRNAs), shdp5a and shdp5b, targeting the 19-nucleotide AGAGAAACG-GGGATGTCATT (938–956) or GACGGAGCGTGATTT-CTAA (1386–1404) in the 3′-untranslated region of *dp5* mRNA (NCBI accession number NM_057130) were designed (18). The targeted regions showed no significant homology with any other genes by BLAST searches. The *shRNA* expression cassettes containing sense loop (TTCAGAGAGAAACG-GGGATGTCATT) were inserted downstream of the U6 promoter. For ectopic Dp5 expression, the intact coding sequence of dp5 with its 3′-untranslated region containing the *shRNA* targeting sites was amplified from the genome of Sprague-Dawley rats and inserted into pCMV3×FLAG (Sigma). All constructs were confirmed by sequencing.

To knock down the expression of FLAG-dp5, 0.5 μg of FLAG-dp5 and 5 μg of shdp5a or shdp5b were co-transfected into 293A cells by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. 48 h after transfection, cell lysates were harvested and processed for Western blotting with FLAG antibody to detect the expression of FLAG-dp5 protein.

DIV5 CGNs were transfected with BS/U6 vector, shdp5a, or shdp5b together with pCMV-EGFP by the calcium phosphate co-precipitation method (11). EGFP was used to mark the transfected cells. 48 h after transfection, neurons were switched to medium containing 25 or 5 mM KCl for 12 h. Neurons were stained with Hoechst 33258 to visualize nuclear morphology and propidium iodide (PI) to detect membrane damage. Apoptosis was quantified by scoring the percentage of EGFP-positive neuron population with pyknotic nuclei or with PI-positive cells. Unbiased counting cells (>600 for each group) were scored blindly without knowledge of their previous treatment.

**RESULTS**

*JNK Mediates the Up-regulation of dp5 during Apoptosis Induced by Potassium Deprivation in CGNs*—To determine whether potassium deprivation evokes the activation of JNK/c-Jun in CGNs, DIV7 neurons maintained in 25 K+5 were switched to 25 K or 5 K medium for various durations of time (0.5, 1, 2, and 4 h), and then processed for Western blotting with antibodies against phospho-JNK and phospho-c-Jun. Consistent with previous studies (12, 19), potassium deprivation led to
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An increase in the level of phospho-JNK starting at 0.5 h without detectable changes in total JNK level (Fig. 1A). As observed in Fig. 1A and consistent with previous results (12), potassium deprivation also resulted in a significant increase in levels of c-Jun phosphorylation starting at 0.5 h and lasting for up to 4 h, which paralleled the increase in level of JNK phosphorylation. c-Jun is a autoregulated immediate early gene, and our results clearly showed that the c-Jun protein level is significantly increased at 0.5 h. These results suggested that JNK and c-Jun are activated in the early period of apoptosis induced by potassium deprivation, as we reported previously (12). To test whether the observed potassium deprivation-evoked increase of JNK/c-Jun activity was accompanied by the induction of dp5, a time course experiment was carried out in which dp5 mRNA levels were measured through Q-PCR analysis at different times (0.5, 1, 2, and 4 h). The dp5 mRNA increased significantly, starting at ~1 h after the time point (0.5 h) at which JNK/c-Jun activation was detected after potassium deprivation (Fig. 1B).

To clarify whether JNK activity is required for dp5 up-regulation, we first used a JNK inhibitor, SP600125 (12, 20). Treatment of CGNs with SP600125 inhibited the induction of dp5 mRNA by ~62% (Fig. 1C). As a further test of specificity, SP600125 was replaced by CEP11004, a mixed lineage kinase inhibitor that inhibits the JNK pathway but not JNK itself. Treatment with CEP11004 led to an over 65% reduction of dp5 mRNA (Fig. 1C). These results suggest that JNK is involved in dp5 up-regulation. The incomplete inhibition of dp5 induction by SP600125 or CEP11004 was not due to their inefficiency to inhibit JNK, because the induction of c-jun mRNA, a result of JNK activation, was completely inhibited in both inhibitor-treated cultures (Fig. 1C). Consistent with a previous study (8), these results indicate that JNK partially mediates the up-regulation of dp5 by potassium deprivation in CGNs.

**Dominant Negative c-Jun, FLAG-Δ169, Attenuates the Up-regulation of dp5**—The facts that c-Jun, a downstream transcription factor of JNK, was activated after potassium deprivation (12, 19, 21) preceding the up-regulation of dp5 and that inhibition of the JNK/c-Jun pathway attenuated dp5 induction (Fig. 1B) raise the possibility that the activation of c-Jun is required for the up-regulation of dp5. To identify the c-Jun-dependent events in the neuronal apoptosis and investigate whether c-Jun is involved in dp5 up-regulation, we used the recombinant adenoviruses expressing FLAG-Δ169 (Ad-FLAG-Δ169) (12, 14, 22), a c-Jun dominant negative mutant, to inhibit c-Jun activity. Without the transactivation domain, it prevents endogenous c-Jun complex from activating target genes by exhausting functional c-Jun and occupying c-Jun binding sites. To verify that FLAG-Δ169 encoded by the adenoviruses was expressed in infected CGNs, the cells were fixed 36 h after infection and stained with FLAG antibody and with the DNA dye Hoechst 33258 to visualize nuclear morphology. Fig. 2A showed representative immunofluorescence pictures of neurons infected with Ad-FLAG-Δ169 at an MOI of 100, and ~70–80% of the CGNs were found to express FLAG-Δ169. c-Jun is a nuclear protein, and FLAG-Δ169 was also localized exclusively in the nucleus. We also detected the expression of Ad-Δ169 by Western blotting with FLAG antibody (Fig. 2A).

In order to test whether c-Jun mediates the induction of dp5 mRNA by potassium deprivation observed here, the CGNs were infected at DIV5 with 100 MOI of Ad-FLAG-Δ169 or Ad-GFP and then were maintained in 25 K+ + S for 36 h. The infected neurons were switched to medium containing 25 or 5 mM KCl for 4 h. RNA was isolated, and Q-PCR experiments were performed using primers specific for c-jun, dp5, bim, fasL, or β-actin. Potassium deprivation led to a significant increase in c-jun, dp5, bim, and fasL mRNA level in cells infected with Ad-GFP. It is well characterized that c-jun is an autoregulated gene, its protein c-Jun binds to the TRE-jun1 and TRE-jun2 sites on its own promoter to promote its expression (23). Results showed that Ad-FLAG-Δ169 almost completely inhibi-
To our surprise, we did not observe any effect of Ad-FLAG on apoptosis of CGNs (12), here we demonstrated that Ad-FLAG-bim, which was in agreement with the results of SP600125 and potassium. However, it has been reported that the fasL gene. Crucially, the increase in level of negative inhibitor of c-Jun-dependent events in infected CGNs. These results indicate that the deletion mutants from gene. All deletion constructs tested had a common 3′ sequence responsible for the activation of dp5 gene is regulated by c-Jun at the transcriptional level, we first tested a DNA fragment spanning from −1568 to +81 of the dp5 5′-flanking region relative to the transcription start site. This fragment was fused to the promoterless firefly luciferase gene of pGL3-Basic vector to generate a dp5 (−1568/+81)-luc reporter. Promoter activity was assessed by measuring luciferase activities in transfected CGNs. The levels of luciferase activity were normalized to Renilla luciferase activity. As shown in Fig. 2C, this 1.6-kb construct expressed a high level of luciferase activity (20-fold more than pGL3-Basic; data not shown), indicating that it contains sequences important for dp5 transcription. Next, we observed a significant increase in the activity of dp5 (−1568/+81)-luc reporter approximately 3-fold after potassium deprivation (Fig. 2C), indicating that dp5 is transcriptionally up-regulated after potassium deprivation. To determine the role of c-Jun in the induction of dp5, we tested whether inhibition of c-Jun activity by co-transfection with a plasmid encoding FLAG-Δ169 suppresses the activation of the dp5 promoter. We found that the induction in dp5 promoter activity by potassium deprivation was reduced by ~70% by FLAG-Δ169 (Fig. 2C).

These results indicated that the dp5 (−1568/+81)-luc reporter behaves in a similar manner to the endogenous dp5 gene with respect to regulation by potassium deprivation and c-Jun transcription factors. The dp5 (−1568/+81)-luc reporter could, therefore, be used to further investigate the involvement of c-Jun transcription factor in dp5 promoter activation after potassium deprivation.

An ATF Site on the dp5 Promoter Is Required for Its Activation Induced by Potassium Deprivation—To determine the minimal sequence responsible for the activation of dp5 promoter by potassium deprivation, we next analyzed a series of reporter constructs containing various lengths of the dp5 5′-flanking regions (from −1568 to +81) upstream of the firefly luciferase gene. All deletion constructs tested had a common 3′-end. These constructs were transfected into CGNs, and their inducibilities were assayed by potassium deprivation. Fig. 3A shows that the deletion mutants from −1568 to −125 were as active as c-Jun mediates up-regulation of dp5 but not that of bim or fasL in CGNs deprived of potassium.
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**A**

![Diagram A](image1)

**B**

![Diagram B](image2)

**FIGURE 3.** An ATF site located at −116 to −109 of the dp5 promoter is necessary for dp5 promoter activation induced by potassium deprivation. A, structures of dp5 5′ sequential deletion constructs are shown. dp5 promoter fragments of different length but with the same 3′-end were cloned into pGL3-Basic. The ATF sites are represented by filled black circles. DIV6 neurons were co-transfected with reporter plasmids and pCMV-RL for 12 h. Then neurons were placed in 25 or 5 mM KCl medium for 10 h. The data represent ± S.E. of three separate experiments. B, mutation of the ATF sites abolished activation of the dp5 promoter. The wild type ATF site, TTACATCA, was mutated to GGACATCG (Mut), which is represented by a cross. DIV6 neurons were transfected with dp5 promoter reporter containing the WT or Mut ATF site as in A. The levels of luciferase activity were normalized to Renilla luciferase activity.

the original construct in responding to potassium depletion. In contrast, further deletion to −85 nearly abolished the activation. The region from −125 to −85 of the dp5 promoter contains a putative ATF site at positions −116 to −109, TTACATCA, that has only one single nucleotide mismatch with a well known c-Jun/ATF2 DNA binding sequence, TTACCTCA, on the promoter of the c-Jun gene termed TRE-jun2 (25). Also, this putative ATF site is conserved between the rat, mouse, and human dp5 gene. The TRE-jun2 sequence preferentially binds c-Jun/ATF2 heterodimers (26). To determine the role of this putative ATF site in dp5 promoter activation, we next introduced mutations (TTACATCA to GGACATCG) into the ATF site in the −1568/+81 construct and assayed its inducibility. These mutations abolished the increase in promoter activity observed in the wild type construct following potassium deprivation (Fig. 3B). Thus, the ATF site is crucial for the transcriptional activation of dp5 gene by potassium deprivation.

c-Jun Binds Directly to the ATF Site on the dp5 Promoter—To determine whether c-Jun binds directly to the dp5 promoter at the ATF site at positions −116 to −109, gel mobility shift assays were performed using an end-labeled oligonucleotide containing the wild-type or mutated ATF site (designated wt ATF and mut ATF, respectively) in the dp5 promoter (Fig. 4). Nuclear extracts were prepared from CGNs placed in medium containing 25 or 5 mM KCl for 4 h. Extract from CGNs deprived of potassium exhibited markedly increase in intensity of the complex compared with that from CGNs maintained in 25 K medium. When this analysis was performed with the mutated ATF site probe (Fig. 4, lanes 6 and 7), the specific DNA-protein complex completely disappeared, suggesting that the complex formed was specific. Next, we examined the composition of the DNA-protein complex by supershift assay. As shown in Fig. 4, the addition of c-Jun antibody to the binding reactions produced a strong supershifted band with concomitant reduction of the specific complex (Fig. 4, lane 3, *). In light of the fact that there is a high similarity between the ATF site on the dp5 promoter and the TRE-jun2 site on the c-jun promoter, we reasoned that ATF2 could be a component of the complex binding to the ATF site. As we expect, AT2 antibody also competed the complex and furthermore caused a supershifted band (Fig. 4, lane 4, †), indicating that ATF2 also binds to the ATF site. As expected, normal rabbit IgG did not compete the complex (Fig. 4, lane 5). Taken together, these results indicate that c-Jun binds directly to the ATF site on the dp5 promoter in conjunction with ATF2, and these factors are induced to increase the binding following potassium deprivation in CGNs.

To confirm that c-Jun binds directly to the dp5 promoter in vivo, we performed a ChIP assay in CGNs maintained in medium containing 25 or 5 mM KCl for 4 h. Chromatin was
Knockdown of Dp5 Protects Neurons from Apoptosis Induced by Potassium Deprivation—Our results with dominant negative c-Jun inhibiting c-Jun activity clearly showed that there was a significant reduction of c-jun mRNA, c-Jun target dp5 (Fig. 2C), and neuronal apoptosis (12) following potassium deprivation. In addition, our result consistently demonstrated that dp5 is sufficient to induce apoptosis, since overexpression of Dp5 led to a significant reduction in survival of CGNs, even in the presence of survival signal,4 which is in agreement with a previous study (8). Although this is so, there is no evidence that the induction of endogenous dp5 by c-Jun is directly inducing apoptosis in CGNs following potassium deprivation. Thus, we performed further gene silencing experiments using small interfering RNAs specific for rat Dp5. We designed two shRNAs (shdp5a and shdp5b) targeting the 3′- untranslated region of the dp5 transcript. In addition, we constructed an expression plasmid, FLAG-dp5, encoding the intact coding sequence of dp5 and its 3′- untranslated region containing the target sites for shRNAs. We first examined whether Dp5 shRNAs specifically reduce the expression of the ectopic dp5 gene when expressed in 293 cells. 293 cells were transfected with shdp5a or shdp5b together with FLAG-dp5 (Fig. 6A). The expression of FLAG-dp5 was measured 48 h after transfection by Western analysis using FLAG antibody. Transfection with either shdp5a or shdp5b in 293 cells led to a dramatic reduction in the level of co-expressed FLAG-dp5 protein (Fig. 6A). In contrast, the expression of the two shRNAs did not alter the expression of tubulin (Fig. 6A). These results indicate that both shRNAs knocked down the expression of dp5 effectively and specifically.

To address the role of Dp5 in apoptosis of CGNs, we co-transfected an EGFP expression plasmid with shdp5a, shdp5b, or empty vector (BS/U6). Two days after transfection, CGNs were switched from 25 K+ S to 25 K or 5 K medium. 12 h after switching, neurons were double-stained with Hoechst 33258 and PI. We found that either shdp5a or shdp5b exerted a partial but significant protection from apoptosis induced by potassium deprivation (Fig. 6B and C). Altogether, these results from endogenous knockdown indicate that Dp5 is required for apoptosis induced by potassium deprivation in CGNs.

**DISCUSSION**

To our knowledge, this is the first attempt to examine whether BH3-only Bcl2 family member dp5 is a direct c-Jun target during neuronal apoptosis. The present results show a direct binding of c-Jun to an ATF site on the dp5 promoter region after potassium deprivation can be explained by increased c-Jun expression, since c-Jun protein expression was dramatically enhanced by potassium deprivation. Furthermore, to verify the specificity of the ChIP assay and to exclude any PCR-generated artifacts, we performed PCR utilizing primers spanning a canonical ATF sequence (TRE-jun2 site) located at the c-Jun promoter. As shown in Fig. 5C, c-Jun was binding to this site, and this binding was increased when c-Jun expression was induced by potassium deprivation. Amplifications from input DNA stayed constant (Fig. 5C). These results indicate that c-Jun binds to the dp5 promoter in vivo, and following potassium deprivation, this binding is enhanced.

4 C. Ma and M. Li, unpublished data.
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A

|          | FLAG-dp5 | shdp5a | shdp5b | DP5 |
|----------|----------|--------|--------|-----|
| Tubulin  |          |        |        |      |

B

|          | 25K       | 5K      | 25K    | 5K       |
|----------|-----------|---------|--------|----------|
| EGFP     |           |         |        |          |
| Hoechst  |           |         |        |          |
| PI       |           |         |        |          |

C

FIGURE 6. Knockdown of Dp5 protects CGNs from apoptosis induced by potassium deprivation. A, the effectiveness of Dp5 shRNAs. 293 cells were co-transfected with shdp5a or shdp5b, two Dp5 shRNAs, together with a plasmid encoding FLAG-dp5. 48 h after transfection, cell lysates were harvested and subjected to Western blotting with FLAG antibody. B, Hoechst staining of EGFP-positive cells. DIV5 neurons were transfected with shdp5a or shdp5b together with pCMV-EGFP. 48 h after transfection, neurons were switched to medium containing 25 or 5 mM KCl for 12 h and then double-stained with Hoechst 33258 and PI. The upper row shows examples of EGFP-positive granule cells (green). The middle and lower rows show the Hoechst staining (blue) and PI (red) of the EGFP-positive cells shown in the same column along with a few surrounding EGFP-negative cells. Note that potassium deprivation produces an increase in the percentage of cells with pyknotic nuclei that display stronger fluorescence of Hoechst and PI, characteristic of apoptotic cells. However, the EGFP-positive cells were produced from cultures transfected with Dp5-shRNAs but not empty vector (BS/U6) and switched to 5 mM KCl. C, quantification of the results in B. Scoring of EGFP-positive cells was performed 12 h after initiating potassium deprivation. At least 600 cells were counted in each group. In cultures transfected with BS/U6, potassium deprivation greatly increases the percentage of apoptotic cells (p < 0.01). Transfection with Dp5-shRNAs reduces the increase in apoptosis elicited by potassium deprivation (5 K/shdp5a versus 5 K/BS/U6 and 5 K/shdp5b versus 5 K/BS/U6; Student’s t test; p < 0.01). The data represent the means of five independent experiments with bars showing S.E.

may promote neuronal apoptosis by activating the transcription of proapoptotic genes. It is not clear, however, what are the critical target genes for c-Jun during neuronal apoptosis.

It has been reported that the fasL promoter could be activated by the JNK/c-Jun pathway, and the fasL mRNA increased in level in CGNs deprived of survival signals (24). However, we have not observed any effects of inhibition of JNK with SP600125 or CEP11004 or inhibition of c-Jun activity with Ad-FLAG-Δ169 (Fig. 2B) on the induction of fasL mRNA in potassium-deprived CGNs, indicating that JNK/c-Jun is not involved in the up-regulation of fasL induced by potassium deprivation in CGNs. The reason for the discrepancy is not clear at present. In addition, although fasL has been reported to play a proapoptotic role during apoptosis of CGNs, studies on lpr and gld mice harboring loss-of-function mutations in Fas and FasL, respectively, showed that the lpr and gld mutations altered neither the extent nor the kinetics of potassium deprivation-induced apoptosis in CGNs (28, 29). Furthermore, activation of extrinsic pathway signaling by tumor necrosis factor α, soluble FasL, or agonistic Fas antibody Jo-2 did not induce cell death in potassium-maintained CGNs (28). Thus, although withdrawal of survival factors induces expression of both Fas and FasL in CGNs, extrinsic pathway signaling mediated by Fas/FasL may not contribute to apoptosis in CGNs.

The activation of JNK/c-Jun in neurons has also been suggested to lead to the up-regulation of one member of the proapoptotic Bcl-2 family, bim (8). The inhibition of the JNK/c-Jun signaling pathway with CEP-1347, an inhibitor of the mixed lineage kinases of JNK pathway activators, attenuated the activation of JNK/c-Jun and the induction of bim in CGNs subjected to potassium deprivation and sympathetic neurons deprived of NGF (8). Based on these, it was proposed that activation of JNK/c-Jun pathway contributed to the up-regulation of bim expression during neuronal apoptosis (8). This was to some extent corroborated by studies demonstrating that expression of a dominant negative form of c-Jun suppressed the induction of bim mRNA and protein in sympathetic neurons following NGF withdrawal (14). However, a link between activation of JNK/c-Jun and bim gene induction in CGNs was not demonstrated in our data. Several lines of our experimental approaches support the notion that bim gene activation in our experimental paradigm is independent of the JNK/c-Jun pathway. First, multiple pharmacological JNK pathway inhibitors, although effective in preventing c-Jun phosphorylation and the induction of c-jun mRNA and protein, fail to attenuate the induction of bim mRNA, protein, and promoter activity following potassium deprivation (12). Second, overexpression of a dominant negative c-Jun, which blocks the induction of c-jun mRNA (Fig. 2B) and prevents apoptosis in CGNs (12), also fails to decrease the expression of bim mRNA and its protein (Fig. 2B) (12). Together, these results demonstrate that the JNK/c-Jun pathway does not play a key role in the induction of bim expression in this particular paradigm.

In agreement with previous reports (8), our results consistently demonstrate that dp5 did increase in a manner that is clearly JNK-dependent. It is still not clear, however, whether c-Jun is involved in the induction of dp5. Several lines of our experimental approaches support the conclusion that c-Jun is involved in the induction of dp5. First, c-Jun induction and phosphorylation preceded dp5 induction, suggesting that c-Jun mediates dp5 gene transcription after potassium deprivation. Second, expression of FLAG-Δ169 greatly reduced the activation of dp5 gene promoter following potassium deprivation. Finally, when neurons were infected with the adenovirus expressing FLAG-Δ169, the increase in the level of dp5 mRNA following potassium deprivation was reduced by ~70%, indicating that the transactivity of c-Jun is required for the induction of dp5 mRNA. It is worth noting that although only 80% of
the infected neurons express detectable FLAG-Δ169 protein at the MOI used in our experiments, we cannot rule out the possibility that there are more neurons expressing the protein at lower levels not detected by immunostaining. This was highly true, because parallel detection indicated that infection of FLAG-Δ169 at the same MOI almost completely inhibited the induction of c-jun mRNA (Fig. 2B). Furthermore, JNK inhibitors, both SP600125 and CEP11004, failed to block completely the induction of dp5 mRNA following potassium deprivation. Overall, the data indicate that c-Jun cooperates with a second c-Jun-independent pathway, which is also activated by potassium deprivation, to achieve the maximum level of dp5 induction. The identification of the alternative pathways regulating dp5 expression is a matter of great interest currently under investigation.

Therefore, there is no evidence thus far suggesting that c-Jun directly regulates transcription of the dp5 gene. Here, our findings that an ATF site at positions −116 to −109 of the dp5 promoter is essential for the activation by potassium deprivation and that c-Jun binds directly to this site in vitro and in vivo are consistent with the notion that dp5 is a direct target gene of c-Jun. More importantly, mutation of this site abolished the activation of the dp5 promoter induced by potassium deprivation. Finally, we demonstrated that knockdown of Dp5 rescued CGNs from potassium deprivation-induced apoptosis. Therefore, dp5 provides the critical link between c-Jun transactivation and neuronal apoptosis and is both necessary and sufficient to mediate potassium deprivation-induced apoptosis of CGNs. In conclusion, our results suggest that dp5 is a direct c-Jun target gene and indispensable for the induction of apoptosis by potassium deprivation in CGNs.

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