THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 50, Issue of December 13, pp. 48764–48770, 2002

Printed in U.S.A.

Activation of the Rb/E2F1 Pathway by the Nonproliferative p38 MAPK during Fas (APO1/CD95)-mediated Neuronal Apoptosis*

Received for publication, June 25, 2002, and in revised form, September 11, 2002
Published, JBC Papers in Press, September 25, 2002, DOI 10.1074/jbc.M206336200

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Aberrant activation of the Rb/E2F1 pathway in cycling cells, in response to mitogenic or nonmitogenic stress signals, leads to apoptosis through hyperphosphorylation of Rb. To test whether in postmitotic neurons the Rb/E2F1 pathway can be activated by the nonmitogenic stress signaling, we examined the role of the p38 stress-activated protein kinase (SAPK) in regulating Rb phosphorylation in response to Fas (CD95/APO1)-mediated apoptosis of cultured cerebellar granule neurons (CGNs). Anti-Fas antibody induced a dramatic and early activation of p38. Activated p38 was correlated with the induction of hyperphosphorylation of both endogenous and exogenous Rb. The p38-selective inhibitor, SB203580, attenuated such an increase in pRb phosphorylation and significantly protected CGNs from Fas-induced apoptosis. The cyclin-dependent kinase-mediated Rb phosphorylation played a lesser role in this neuronal death paradigm, since cyclin-dependent kinase inhibitors, such as olomoucine, roscovitine, and flavopiridol, did not significantly prevent anti-Fas antibody-evoked neuronal apoptosis. Hyperphosphorylation of Rb by p38 SAPK resulted in the release of Rb-bound E2F1. Increased E2F1 modulated neuronal apoptosis, since E2F1−/− CGNs were significantly less susceptible to Fas-mediated apoptosis in comparison with the wild-type CGNs. Taken together, these studies demonstrate that neuronal Rb/E2F1 is modulated by the nonproliferative p38 SAPK in Fas-mediated neuronal apoptosis.

Fas (CD95/APO1) death receptor-mediated neuronal apoptosis has been implicated in the development of a variety of neurological diseases. For example, following hypoxic ischemia, the increased expression of Fas receptor and its ligand mediates neuronal apoptosis and contributes to brain and spinal cord damage (1–5). Attenuation of the expression of Fas receptor confers protection to neurons in response to cerebral ischemia (1, 6). Cross-linking of Fas by its cognate ligand or antibody results in the trimerization of the Fas receptors, which transduces death signals either via the formation of a death-inducing signaling complex, triggering the autoactivation of procaspase-8 (7, 8), or, alternatively, through an adaptor protein, Daxx, to activate the p38 SAPK[1] pathway (9–12).

The p38 SAPK, a member of the mitogen-activated protein kinase superfamily, is thought to be an important mediator for stress signal transduction from the cell surface to the nucleus. Activation of p38 SAPK in neurons causes apoptosis. Inhibition of p38 activity by SB203580, which is a pyridinyl imidazole inhibitor highly specific to p38, but not to other mitogen-activated protein kinase homologous such as c-Jun N-terminal kinase (JNK) and p42 mitogen-activated protein kinase (13, 14), confers neuroprotection (15–18), whereas an inhibitor to MEK1/2, PD98059, is not neuroprotective, such as in response to cerebral ischemia (19). The p38 SAPK is a known downstream signaling molecule of Fas-mediated apoptosis (20–22), and p38 inhibitor SB203580 blocks Fas-mediated apoptosis (23). Once activated, the p38 SAPK can phosphorylate transcription factors, such as ATF-2, Elk-1, myelin basic protein (23–25), and Rb (22). However, in postmitotic neurons, the downstream target of the p38 SAPK in Fas-mediated neuronal apoptosis is not yet clear.

One of the possible candidates is the Rb/E2F1 pathway. Works reported from many other laboratories, including our own, showed that the transcription factor E2F1 is an important mediator of neuronal apoptosis (26–31). E2F1-mediated apoptosis is tightly regulated by the Rb protein, which efficiently blocks E2F1-mediated apoptosis (32, 33). Hyperphosphorylation of Rb by the CDK4/6-cyclin D complex leads to the dissociation of pRb from its binding partner E2F1 (34). Inhibitors of the CDK-cyclin complex, such as olomoucine, roscovitine, and flavopiridol, reduce the phosphorylation of Rb, increase Rb binding to E2F1, and protect both cycling cells and neurons from E2F1-mediated apoptosis (35–39). Because the Rb/E2F1 pathway plays a critical role in determining the fate of a cell, these proteins are the logical targets of multiple signaling pathways. In addition to the mitogenic signals such as the CDK-cyclin complex, other nonproliferative stress signals such as p38 SAPK may also target Rb to modulate E2F1-mediated apoptosis through phosphorylation of Rb, as has been demonstrated in Jurkat cells by Wang *et al.* (22). However, little is known of whether the p38 SAPK modulates the Rb/E2F1 pathway to induce apoptosis of postmitotic neurons under physiological settings.

In the present study, we used Fas-mediated apoptosis of cultured CGNs as a physiological model to examine the activation of p38 and to investigate whether p38 targets the Rb/E2F1 pathway to induce neuronal apoptosis. We show that Rb is targeted by the p38 SAPK via hyperphosphorylation, which leads to increased E2F1-mediated neuronal apoptosis.

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‖ Supported by the Canadian Institutes of Health Research.

[1] The abbreviations used are: SAPK, stress-activated kinase; JNK, c-Jun N-terminal kinase; CDK, cyclin-dependent kinase; CGN, cerebellar granule neuron; IP, immunoprecipitation; GST, glutathione S-transferase; ERK, extracellular signal-regulated kinase.

MATERIALS AND METHODS

Primary Cerebellar Granule Neuron Cultures—CGNs were prepared from 6–9-day postnatal mice (E2F1+/+ and E2F1−/− mice were obtained from Jackson Laboratories, bred, and genotyped locally as described previously (40)). The cerebellum was removed and cultured exactly as described (26). Cells were cultured for at least 7 days before experimentation.

Neuronal Viability Assay—CGNs cultured on a 24-well plate were treated with 2 μg/ml anti-Fas antibody (catalog no. sc-7886; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2, 4, 6, 12, and 18 h or at 0.4, 0.8, 1.2, 2, and 2.4 μg/ml for 12 h. Neuronal viability was measured by adding the nonfluorescent hydrophobic dye 5(6)-carboxyfluorescein diacetate (Molecular Probes, Inc., Eugene, OR) to the culture medium at a final concentration of 5 μg/ml as described before (26). Cultured CGNs were also pretreated with SB203580 dissolved in Me_2SO for 15 min and then stimulated with the anti-Fas antibody. The cells were subsequently fixed with 10% formalin for 20 min and stained with Hoechst 33528 for 5 min. Healthy and dead cells were counted under a fluorescent microscope. At least 500 nuclei were randomly selected and counted.

Double Immunofluorescent Staining—The procedures for fluorescent immunocytochemistry were exactly as previously described (26). The primary antibody against Fas receptor was purchased from Santa Cruz Biotechnology. Neuron-specific antibody to NeuN was a gift from Dr. R. J. Mullen (School of Medicine, University of Utah). A specific mouse monoclonal antibody to E2F1 (KH129) was a gift from C. Ngwu (Laboratory of Molecular Oncology, Harvard Medical School). Antibodies to p38, phospho-p38, and phospho-pRb were purchased from Cell Signaling Technology (Beverly, MA).

Western Blotting—The procedures for Western blotting were exactly as described previously (30). The intensities of the bands were quantitated using Amersham Biosciences ImageQuant Software.

Caspase-3-like Activity Assay—The assay was performed as described previously (29). The results were presented as absolute fluorescent units/μg of protein/h. The -fold induction was normalized with the untreated controls.

Immunoprecipitation (IP), E2F1 DNA Binding Assay, and Kinase Assay—IP was performed using a streptavidin-conjugated Dynabeads method (Dynal Inc., Lake Success, NY) exactly as described (41). Briefly, 250 μg of total protein was incubated overnight at 4 °C with a monoclonal antibody to Rb (BD Biosciences, Missisauga, Canada) or phospho-p38 (Cell Signaling Technology). These primary antibodies had already been captured by the Dynal beads-secondary antibody complex. After washing away unbound proteins, the immunoprecipitated Rb/E2F1-beads protein complex was subjected to either Western blotting for E2F1 (see above) or E2F1 DNA binding assay; *i.e.* IP complex (Rb/E2F1-beads) was first washed with the DNA binding buffer three times and then resuspended in the binding buffer. The binding buffer contained 10 mM HEPES, pH 7.5, 80 mM KCl, 1 mM EDTA, 1 mM EGTA, 6% glycerol, 50 μg of denatured herring sperm DNA and 50 μg/ml poly(dI-dC). The Rb/E2F1-beads complex was then co-incubated with 0.2 ng of [α-^{32}P]dCTP-labeled E2F1 specific double-stranded DNA (upper strand, 5′-GCTCTTTCGCGGCAAAAAGG-3′; lower strand, 5′-CCTTTTGCCGCAAAGAGC-3′) for 30 min at room temperature. The mixture was then washed three times with the binding buffer to eliminate the unbound radioactive oligonucleotides. The reaction was stopped by boiling for 10 min in the loading buffer. Magnetic beads were recaptured, and the supernatant at 10 μl was spotted onto a nitrocellulose membrane, dried, and analyzed using autoradiography by exposure to Eastman Kodak Co. X-Omat films. In order to show the specificity of E2F1 binding, excess amounts (100-fold) of unlabeled E2F1 probe or nonrelated, sequence-scrambled DNA probe were added to the mixture to either compete out or reduce the binding of the specific E2F1 band, respectively.

To measure p38 SAPK activity, a nonradioactive IP kinase assay kit (Cell Signaling Technology) was used exactly as described by the manufacturer. Briefly, p38 was immunoprecipitated from 250 μg of Fas-treated CGNs using an antibody to p38. The IP product was mixed with 2 μg of GST-ATF-2 or 2 μg of bacterially produced Rb protein (QED Biosciences) in the presence of 200 μM ATP and incubated at 30 °C for 30 min. The reaction was stopped by adding 1× gel loading buffer. The mixture was resolved on a 12% SDS-PAGE gel. Phosphorylated ATF-2 and Rb were detected using phospho-specific antibodies.

All of the above described experiments were repeated at least three times, and the statistical significance was calculated using the method indicated in the figure legend.

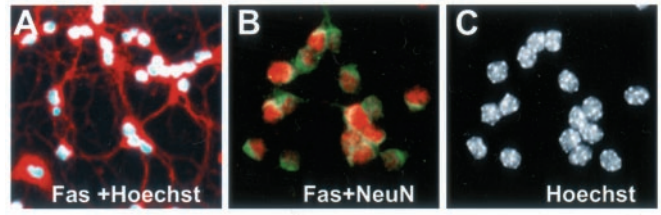

FIG. 1. **Fas is localized on neurons.** Cultured CGNs were fixed with formalin and double-stained with Hoechst 32558 and Fas antibody (*A*) or antibodies to Fas and NeuN (*B*). *A*, superimposition of Hoechst (*white*) with Fas antibody staining (*red*). *B*, superimposition of Fas (*green*) with NeuN (*red*) immunostaining. *C*, Hoechst staining of *B*.

RESULTS

Fas Functions as a Death Receptor in CGNs—To understand the mechanisms of Fas-mediated neuronal death, we used cultured mouse CGNs as an *in vitro* model. The following three experiments showed that Fas indeed functioned as a death receptor in CGNs. (i) Fas expressed on the surface of postmitotic neurons (Fig. 1) as shown by double immunolabeling with an antibody to Fas and a neuron-specific antibody to NeuN (Fig 1*B*). (ii) Treating CGNs with the Fas antibody (sc-7886) induced an increased expression of Fas (not shown) and a decreased neuronal viability in a time- and dose-dependent manner (Fig. 2, *A* and *B*). The viability of the Fas antibody-treated cultures was examined by the 5(6)-carboxyfluorescein diacetate assay, which monitored the activities of esterase in cells. After an 18-h treatment with 2 μg/ml of the Fas antibody, the intensity of the 5(6)-carboxyfluorescein diacetate assay decreased to about 40% of that of the untreated control cultures. The antibody interaction with Fas is specific, since the denatured Fas antibody (boiled for 10 min) and a nonspecific rabbit preimmune serum failed to induce neuronal death (not shown). (iii) Fas overexpression killed cultured CGNs (Fig. 2*C*) as has been shown in other cell types (42). A replication-defective adenovirus containing the Fas cDNA was added to the cultured CGNs at a concentration of 100 units of multiplicity of infection. After a 72-h infection with the virus, significant neuronal death occurred in comparison with the cultures treated with the control virus containing only green fluorescent protein. Neuronal death induced by the Fas antibody had condensed and punctate nuclear morphology, indicative of apoptosis (see Fig. 4*B*).

Activation of p38 SAPK during Fas-induced Neuronal Apoptosis—To see whether Fas-mediated neuronal apoptosis activates p38, antibodies to phospho-p38 and total p38 were used for Western blotting. As shown in Fig. 3*A*, p38 was expressed ubiquitously in CGNs, whereas the phosphorylated form of p38 was expressed at a very low level almost beyond detection by the Western blotting (Fig. 3*A*). After 2-h treatment with the Fas receptor antibody, the expression of phosphorylated p38 increased dramatically, and the level of phospho-p38 stayed elevated after 4 and 6 h of treatment. Such a fast kinetics of induction of the phosphorylated p38 suggested that the p38 kinase was involved in the Fas receptor-mediated death signal transduction in neurons. Indeed, anti-Fas antibody induced a sharp increase in p38 kinase activity as measured by the increased phosphorylation of p38 substrate ATF-2 (Fig. 3*B*, *lane 3*, *top panel*), whereas the p38 kinase-specific inhibitor SB203580 dramatically reduced p38 phosphorylation of ATF-2 (Fig. 3*B*, *lane 4*). An equal amount of p38 was immunoprecipitated as shown by Western blotting in the *lower panel* of Fig. 3*B*. Bacterially expressed GST-Rb (*lane 5*) and ATF-2 (*lane 6*) were loaded on the same gel to serve as negative controls. Such a reduction in p38 kinase activity was associated with neuroprotection (Fig. 4, *A–C* and *G*). As indicated in Fig 4*G*, after

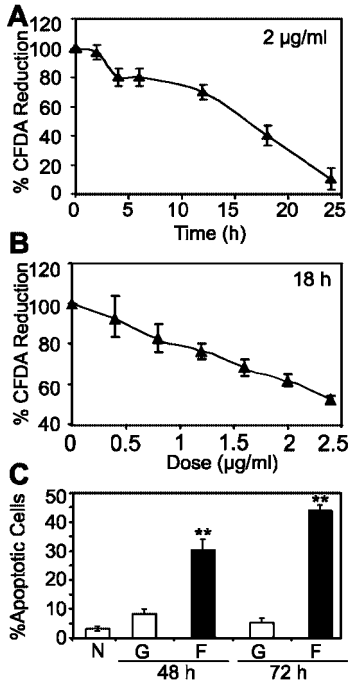

FIG. 2. **Fas-mediated reduction in neuronal viability.** Cultured CGNs were treated with 2 μg/ml anti-Fas antibody for 0–24 h (*A*) or with a range of concentrations of anti-Fas antibody (*B*). The cellular viability was measured with the 5(6)-carboxyfluorescein diacetate (*CFDA*) assay. A replication-defective adenovirus expressing Fas was added to the CGN culture at a multiplicity of infection of 100 for 48 or 72 h. Infected cultures were fixed with 10% formalin and stained with Hoechst. Normal and apoptotic nuclei were counted and plotted. Overexpression of Fas (labeled as *F* in *C*) clearly induced significant neuronal death in comparison with the control virus (expressing only green fluorescence protein, labeled as *G* in *C*) and the nontreated control (*N* in *C*). **, statistical significance by Student's *t* test ($p < 0.001$).

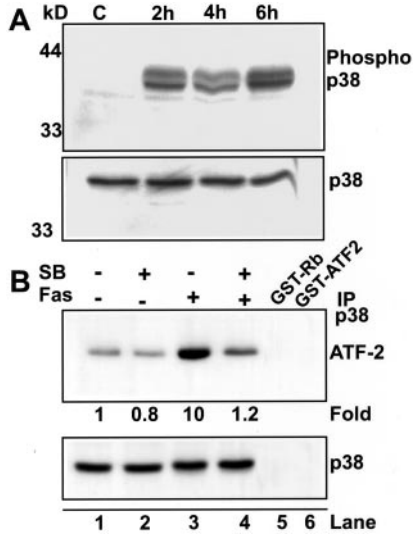

FIG. 3. **Activation of p38 in response to Fas-mediated neuronal death.** CGNs were treated with 2 μg/ml of anti-Fas antibody and harvested for Western blotting with antibody specific to p38 or phospho-p38. p38 was expressed ubiquitously in CGNs (*A*, *lower panel*), whereas phospho-p38 was dramatically induced after a 2-h treatment with anti-Fas antibody (*A*, *upper panel*). Antibody to p38 was then used to immunoprecipitate total p38. The immunoprecipitated p38 was mixed with 2 μg of GST-ATF-2 for *in vitro* kinase assay (*B*, *top panel*). *Lanes 5* and *6* were loaded with bacterially expressed Rb and GST-ATF-2, respectively, to serve as negative controls. An equal amount of immunoprecipitated p38 is shown in the *bottom panel* of *B*.

24-h treatment with 2 μg/ml anti-Fas antibody, 90% of the CGNs were apoptotic in comparison with about 45% of apoptotic cells in SB203580-treated cultures.

p38 Kinase Mediates Fas-induced Hyperphosphorylation of Rb—One of the potential targets of p38 SAPK is the retinoblastoma gene product Rb. Rb is expressed in CGNs as a 110-kDa band on Western blotting (Fig. 5*A*). In response to anti-Fas antibody treatment, Rb phosphorylation increased. Hyperphosphorylation of Rb created a band shift of the 110-kDa Rb band to a molecular mass close to 116 kDa (Fig. 5*A*). A phospho-specific Rb antibody was also used to specifically detect increased hyperphosphorylation of Rb in response to the anti-Fas antibody (Fig. 5*B*). The induction of Rb phosphorylation correlated temporally with the increased activation of p38 (*i.e.* 2 h after treatment with the anti-Fas antibody). The level of pRb remained high after 6 h of treatment, albeit at a relatively lower level in comparison with that of the 2-h treated CGNs (Fig. 5, *A* and *B*).

Such an induction of pRb phosphorylation is mediated by the p38 kinase but not the CDKs. The p38 inhibitor, SB203580, reduced the hyperphosphorylation of Rb in response to anti-Fas antibody (Fig. 5, *A*, *lanes 3* and *6*, and *B*, *lanes 3*, *5*, and *7*) and protected CGNs from anti-Fas antibody-induced neuronal death (Fig. 4, *A–C* and *G*), whereas potent CDK inhibitors such as olomoucine (200 μM) and roscovitine (50 μM) and flavopirodal (2 μM) did not reduce the hyperphosphorylation state of the pRb in response to anti-Fas antibody treatment (not shown) and also failed to provide any neuroprotection (Fig. 4, *D–G*), indicating that Fas-mediated induction in hyperphosphorylation of Rb is unlikely to be mediated by the CDKs.

To see whether the endogenous neuronal p38 can phosphorylate Rb *in vitro*, cell extracts were first immunoprecipitated with antibody to phospho-p38 and then mixed with bacterially expressed Rb as substrate. Indeed, Fas-activated p38 induced an increase in phosphorylation of exogenous Rb (Fig. 5*C*, *lane 2*), whereas SB203580 inhibited such an induction in Rb phosphorylation (Fig. 5*C*, *lane 3*). Nonphosphorylated Rb (*lane 4*) and ATF-2 (*lane 5*) were loaded on the same gel, serving as negative controls, and they were not recognized by the phospho-Rb-specific antibody.

p38-mediated Rb Hyperphosphorylation Correlates with the Activation of E2F1—The consequence of increased phosphorylation of Rb is the release of Rb-bound E2F1, which, when overexpressed, is known to induce neuronal apoptosis (26, 28, 36). To see whether the total cellular E2F1 level changes following anti-Fas antibody treatment, we first used Western blotting to detect neuronal E2F1. No dramatic changes in the level of total E2F1 occurred (Fig. 6*A*). An electrophoretic mobility shift assay was performed to see changes in the level of "free" E2F1 capable of binding to DNA (Fig. 6*B*). Anti-Fas antibody elicited an induction in the "free" E2F1 band (*arrow* in *lane 3* compared with that in *lane 2* in *B*) and also the level of complexed E2F1, most likely bound with Rb. An excess amount of cold probe (100-fold) almost completely competed out these bands (*lane 4*). An adenovirus expressing E2F1 was used to overexpress E2F1 to serve as a positive control for the E2F1 band (*lane 5*). These experiments showed that anti-Fas antibody did not alter the expression level of E2F1; however, it increased the amount of E2F1 capable of binding to DNA.

We next examined whether the interaction of Rb with E2F1 had changed. To do this, Rb-bound E2F1 was pulled out by immunoprecipitation with an antibody to Rb. The level of Rb-bound E2F1 was detected either by Western blotting or E2F1 DNA binding assay. First, Rb/E2F1-beads complex was dissociated from its bound magnetic beads by treatment with an acid elution buffer at pH 2.5. The eluted protein was renatured by adjusting the pH back to 7.4 using 1 M Tris buffer. The

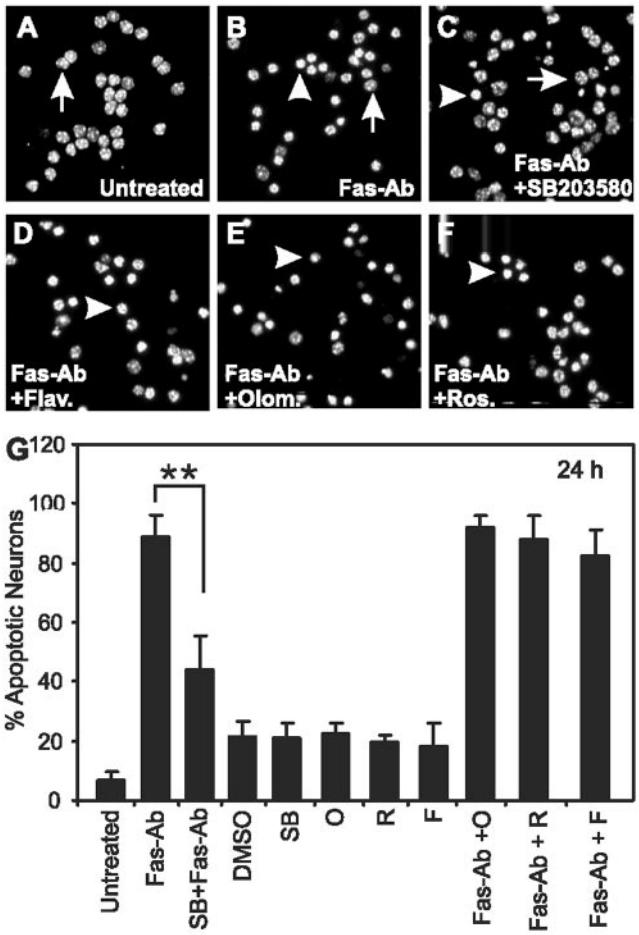

FIG. 4. **Inhibition of p38 SAPK confers neuroprotection.** The working concentrations of p38 inhibitor SB203580 (10 μM, labeled as *SB* in *G*) and CDK inhibitors, olomoucine (200 μM, labeled as *O* in *G*), roscovitine (50 μM, labeled as *R* in *G*), and flavopiridol (2 μM, labeled as *F* in *G*), were determined empirically. The inhibitors at the above stated concentrations were added 15 min prior to treatment with 2 μg/ml of the anti-Fas antibody. The treated CGNs were then fixed with 10% formalin and stained with Hoechst. The number of apoptotic cells (*arrowheads*) were counted (*A–F*) and plotted (*G*) against healthy nuclei (*arrows*). SB203580 provided significant protection to the treated CGNs (** in *G*), whereas other inhibitors did not. **, statistical significance by Student's *t* test ($p < 0.001$). *DMSO*, Me_2SO.

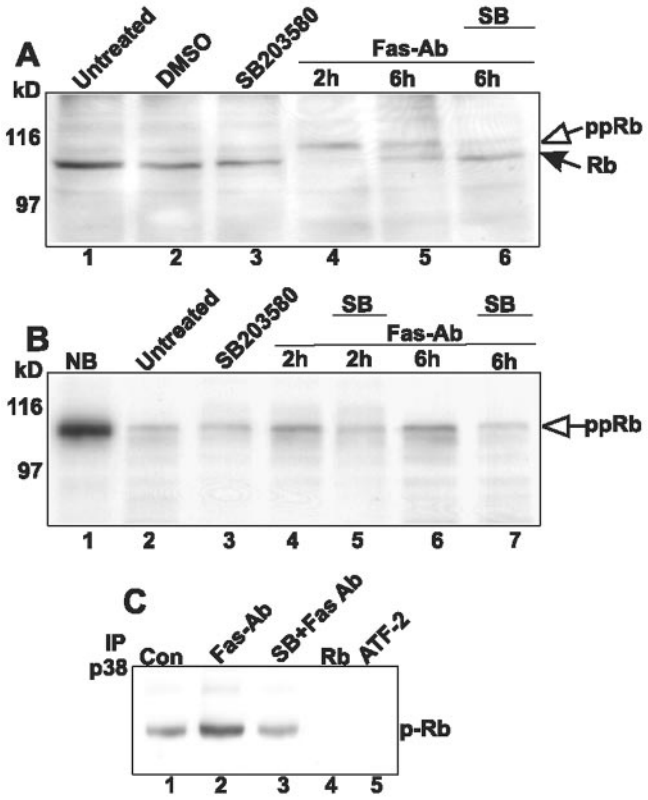

FIG. 5. **p38-mediated hyperphosphorylation of Rb.** Cultured CGNs was treated with 2 μg/ml anti-Fas antibody either with or without SB203580. Me_2SO (*DMSO*) was used as a vehicle control. Cell extracts were subjected to Western blotting using antibodies to Rb (*A*) or phospho-Rb (*B*). After 2 h of anti-Fas antibody treatment, Rb phosphorylation increased dramatically, creating a band shift (*A*, *lane 4*). Using an antibody specific to phospho-Rb confirmed that phosphorylation of Rb increased in response to anti-Fas antibody (*B*, *lanes 4* and *6*), whereas treating CGNs with SB203580 reduced Rb phosphorylation (*B*, *lanes 5* and *7*). Neuroblastoma cell (*NB*) extract served as a positive control for hyperphosphorylated Rb (*B*, *lane 1*). *C*, CGNs treated with anti-Fas antibody was immunoprecipitated with antibody to phospho-p38 and then incubated with 2 μg of bacterially expressed Rb as substrate to show that the activated endogenous neuronal p38 can phosphorylate Rb *in vitro*. *Lanes 4* and *5* were only loaded with the substrate Rb and GST-ATF-2, respectively, to serve as negative controls.

mixture was then boiled in loading buffer and subjected to Western blotting to detect E2F1 (Fig. 6*C*). Control CGNs showed a visible amount of E2F1 (Fig. 6*C*, *lane 1*), indicating the existence of Rb/E2F1-beads complexes in untreated neurons. CGNs treated with SB203580 alone had a slight increase in the level of E2F1 (*lane 2*) compared with the control, suggesting that inhibition of endogenous p38 activity reduced phosphorylation of Rb and increased the amount of unphosphorylated Rb that bound to E2F1. After a 2-h treatment with anti-Fas antibody, CGNs showed a much reduced level of Rb-bound E2F1 (*lane 3*) to a level barely visible on the Western blot when compared with the control, indicating increased release of Rb-bound E2F1. In the sample treated with both SB203580 and anti-Fas antibody, the E2F1 level was high (*lane 4*), again suggesting that inhibition of p38 activity with SB203580 reduced Rb phosphorylation and increased Rb binding with E2F1.

The second method we used to detect Rb-bound E2F1 was by an E2F1 DNA binding assay. The Rb/E2F1-beads complex was captured on magnetic beads as described above. However, this made it impossible to elute the bound proteins under nondenaturing conditions for a gel mobility shift assay. Therefore, an alternative E2F1 DNA binding assay was used to examine the amount of Rb-bound E2F1. The immunoprecipitation product was washed three times under nondenaturing conditions using DNA binding buffer (see "Materials and Methods") and then mixed with ^{32}P-labeled dsE2F1 oligonucleotides. After a 30-min incubation, the mixture was washed three times to eliminate the unbound probes. The mixture was boiled for 5 min and then loaded onto a nitrocellulose membrane. The membrane was then exposed to an x-ray film. The dot intensity reflected the amount of Rb-bound E2F1 that was captured by immunoprecipitation. As shown in Fig. 6*D*, CGNs treated with the anti-Fas antibody had reduced dot intensity in comparison with the control, indicating less Rb-bound E2F1, whereas CGNs treated with both SB203580 and anti-Fas antibody had stronger dot intensity than that from the sample treated with anti-Fas antibody alone, indicating the presence of more Rb-bound E2F1. Taken together, these experiments showed that p38 phosphorylation of Rb affected E2F1.

E2F1−/− CGNs Are Less Susceptible to Fas-induced Death—To investigate whether E2F1 is indeed implicated in the pathway of Fas-mediated neuronal death, we tested the susceptibility to Fas-mediated apoptosis in CGNs derived from E2F1 knockout (E2F1−/−) mice. E2F1−/− CGNs were first

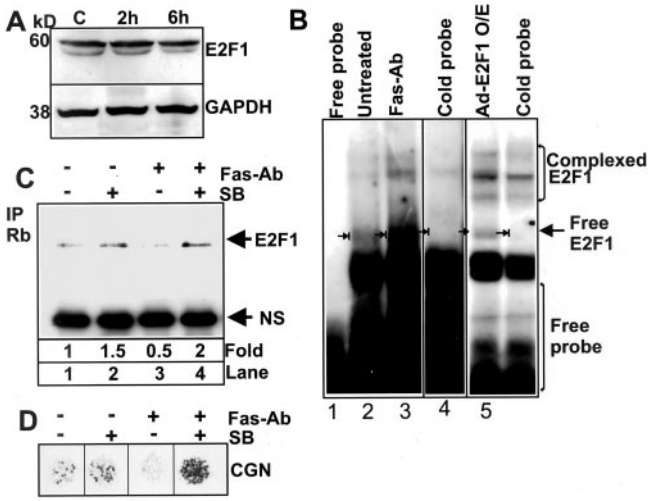

FIG. 6. **Increased release of Rb-bound E2F1 in response to anti-Fas antibody.** CGNs were treated with 2 μg/ml of anti-Fas antibody for 0, 2, and 6 h. Total cell extracts (250 μg) were subjected to Western blotting to detect E2F1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*A*). Cell extracts (20 μg) were also incubated with [α-^{32}P]dCTP-labeled E2F1-specific double-stranded DNA probes and then subjected to electrophoretic mobility shift assay (*B*). *B*, *lane 1* was loaded only with radioactive free probes. *Lanes 2* and *3* show untreated and anti-Fas antibody-treated CGNs, respectively. CGN extracts were also mixed with a 100-fold excess amount of cold probe for competition with the hot probes (*lane 4*). CGN extracts overexpressing E2F1 were subjected to electrophoretic mobility shift assay (*lane 5*) or cold probe competition (*lane 6*) to identify the E2F1 band (*arrows* in *B*). *C* and *D*, cell extracts (250 μg) were subjected to IP with antibody to Rb as described under "Materials and Methods." *C*, Rb/E2F1-beads complex was eluted using an acid elution buffer. E2F1 dissociated from the IP complexes was then detected by Western blotting (*C*). -Fold change in the E2F1 band was calculated from densitometry measurements. *NS*, a nonspecific band indicating equal protein loading. In *D*, Rb/E2F1-beads complexes, which were still attached to the magnetic beads, were incubated with [α-^{32}P]dCTP-labeled E2F1 probe at 37 °C for 30 min and then washed three times with the binding buffer. After boiling for 5 min, 10 μl of each sample was spotted onto a nitrocellulose membrane and exposed to x-ray film. The intensity of the dot reflected the amount of E2F1-bound radioactive oligonucleotides.

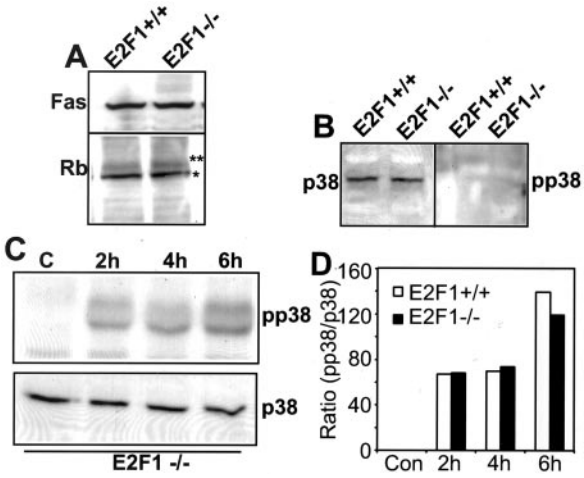

FIG. 7. **E2F1+/+ and E2F1−/− CGNs express similar levels of Fas, Rb, and p38.** CGN cultures (50 μg of protein/lane) derived from E2F1+/+ and E2F1−/− mice were subjected to Western blotting using antibodies to Fas (*A*, *top panel*), Rb (*A*, *bottom panel*), total p38, and phospho-p38 (*B*, *left* and *right panel*, respectively). E2F1−/− CGNs treated with anti-Fas antibody also elicited a robust and early induction of phospho-p38 (*C*) to the same level compared with the wild-type CGNs (*D*). *D*, a bar graph comparing changes in phosho-p38 between E2F1+/+ (data from Fig. 3*A*) and E2F1−/− (data from *C*) CGNs. The band intensities of pp38 and p38 were measured by densitometry. The ratio of pp38 to p38 was calculated and plotted as in *D*.

compared with the wild-type (+/+) CGNs in their expression of Fas, p38, and Rb using immunostaining or Western blotting. Both the localization (not shown) and the level of Fas expression were indistinguishable between the E2F1+/+ and E2F1−/− CGNs (Fig. 7*A*). Similarly, there were no differences in the expression levels of p38, Rb, and their phosphorylated forms (Fig. 7, *A* and *B*). In response to the same dose of anti-Fas antibody, E2F1−/− CGNs also showed a similar level of induction of phospho-p38 as the wild-type CGNs (Fig. 7, *C* and *D*). Despite all of these similarities, E2F1−/− CGNs were significantly less susceptible to the anti-Fas antibody-induced apoptosis (Fig. 8) and showed less caspase-3-like activity (Fig. 8*E*). Interestingly, when treated with the same dose of SB203580 and the anti-Fas antibody, E2F1−/− CGNs consistently showed only a marginally better protection than the similarly treated wild-type CGNs, indicating a small additive effect of inhibition of p38 function with the absence of functional E2F1 (Fig. 8*D*).

DISCUSSION

Information regarding how the Rb/E2F1 pathway is regulated during apoptosis of postmitotic neurons is scarce. To the best of our knowledge, this is the first demonstration that the nonproliferative stress kinase, p38 SAPK, modulates Rb/E2F1 by phosphorylating Rb in a similar manner as described in cycling Jurkat cells by Wang *et al.* (22). Furthermore, CGNs derived from E2F1-deficient mice were significantly less susceptible to Fas-induced apoptosis, indicating that such neuronal death is E2F1-dependent. A schematic diagram depicting Fas-mediated neuronal death is proposed in Fig. 9 in that activated p38 SAPK increases phosphorylation of Rb, leading to the release of otherwise tightly bound E2F1. Elevated E2F1 may cause neuronal apoptosis either via activation of death genes or repression of survival genes (29, 43, 44).

Recently, there has been a surge in the literature implicating Fas in mediating neuronal death in a number of neurodegenerative diseases and traumatic brain injury (1–4, 21, 45, 46). Using cultured CGNs as a model system, we demonstrated that Fas can indeed function as death receptors in neurons, since cross-linking of Fas using a Fas antibody or overexpressing Fas caused apoptosis of CGNs. Understanding the mechanism of Fas signaling in neurons and targeting Fas-mediated death may contribute to neuroprotection.

Major members of the SAPK family include extracellular signal-regulated kinase (ERK), JNK, and p38. However, the role of ERK and JNK in neuronal death and survival are confusing and sometimes paradoxical (*i.e.* they modulate both neuronal death and survival). Activation of ERK or JNK is generally associated with neuronal survival (47–51). ERK protects cortical neurons from hypoxia via increased phosphorylation and inactivation of BAD (52). JNK activities provide survival signals following stress through increased phosphorylation of Bcl2. This may enhance the binding of Bcl2 with the proapoptotic Bax (53). The p38 SAPK, however, is almost exclusively associated with mediating apoptosis (54, 55). Activation of p38 SAPK occurs in response to a variety of neuronal stresses such as trophic factor withdrawal (56–58), glutamate toxicity (59), ceramide toxicity (60), cerebral ischemia (15, 19, 61, 62), and Fas antibody (20). Blocking p38 activity with selective inhibitor SB203580 had no effect on JNK, ERK, or several other protein kinases (13). Although inhibition of p38 by SB203580 may induce ERK phosphorylation, which may contribute to protection, Willaime *et al.* (60) showed that ERK inhibitor PD98059 did not change the protective effect of SB203580, suggesting that SB203580 protection is due to inhibition of p38 rather than ERK activation. SB203580 has been

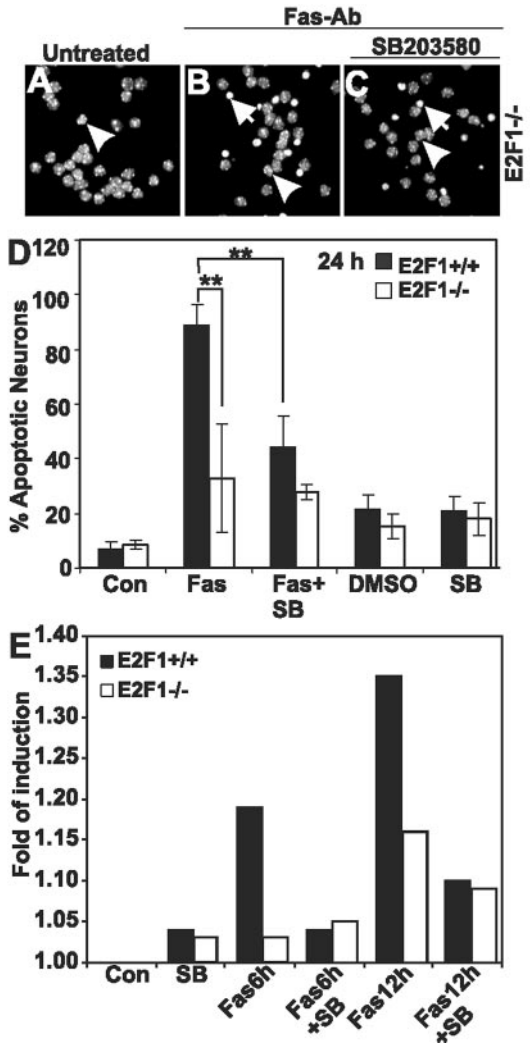

FIG. 8. **E2F1−/− CGNs are less susceptible to anti-Fas antibody-induced death.** E2F1+/+ and E2F1−/− CGNs were cultured under the same conditions for 7 days and then subjected to treatment with 2 μg/ml anti-Fas antibody in the presence/absence of SB203580. The number of dead cells was counted and plotted as shown in *D*. Cell extracts were mixed with Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl-7-amide) as described under "Materials and Methods" to show caspase-3-like activity (*E*). The -fold induction of caspase-3-like activity was calculated by normalizing to the untreated controls. The *arrowheads* and *arrows* in *A–C* indicate live and dead cells, respectively. *D*, ** indicates statistical significance by Student's *t* test ($p < 0.001$).

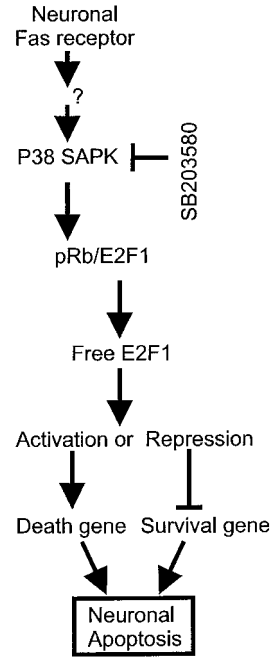

FIG. 9. **A schematic diagram depicts the pathway for Fas-mediated neuronal apoptosis.** Fas activates p38, leading to hyperphosphorylation of Rb and the release of E2F1, thereby causing neuronal apoptosis either through activation or repression of target genes.

shown to protect delayed neuronal death following transient global ischemia, but PD98059 is not neuroprotective (19). In the present study, the association of induction of p38 activity in response to anti-Fas antibody with neuronal death and the protective effects of SB203580 confirmed the above reports. Furthermore, the fast kinetics of p38 induction (2 h after treatment) could also trigger the expression of other death mediators such as Fas and Fas ligand, which, in turn, would further exacerbate neuronal apoptosis. Indeed, Western blotting showed relatively delayed induction of Fas (*i.e.* after 6 h of treatment with the Fas antibody (not shown)) in comparison with the early induction of phspho-p38 and phospho-Rb.

Altered cell cycle control mechanisms have detrimental consequences to the survival of neurons. Unbalanced expression of cell cycle genes is associated with many neurodegenerative diseases (38, 63). It is suggested that, in response to insults, postmitotic neurons may attempt to reenter the cell cycle and that failure to complete the cell cycle leads to the demise of neurons. Although much of the theory remains to be verified, examples of elevation of cell cycle genes in damaged neurons have been widely reported (26, 27, 29, 30, 37, 38, 40, 64). The Rb/E2F1 pathway is a vital regulator for the G_1-S phase transition. In addition to being targeted by the mitogenic signals, there is also ample evidence from studies of cycling cells that Rb/E2F1 acts as a converging point for multiple signaling events such as in response to hypoxia and DNA damage (34, 65). In Jurkat cells, Rb/E2F1 are modulated by the p38 SAPK in response to Fas activation (22). In the present report, we demonstrate that indeed neuronal Rb/E2F1 is also targeted by Fas-activated p38 SAPK. CDKs play a lesser role in Fas-mediated activation of Rb/E2F1 pathway in CGNs, since inhibitors to a broad range of CDKs failed to provide neuroprotection. In addition, other studies showing that Fas may actually repress CDK activity (35) further support the notion that p38 plays a key role in inactivation of Rb. These studies confirmed that Rb/E2F1 signals may serve as a common pathway in different paradigms of cell death and that Rb/E2F1 can be modulated by multiple upstream signals including CDKs and p38.

The result of increased Rb phosphorylation is the release of the transcription factor E2F1. Although E2F1 was not induced in response to Fas-mediated neuronal death, we detected a reduced level of Rb-bound E2F1 in the Rb immunoprecipitation complex, indicating that there are more active E2F1 proteins following anti-Fas antibody treatment. Those E2F1 proteins released from Rb may be responsible for modulating neuronal death, since, in the absence of E2F1, E2F1−/− CGNs were less susceptible to Fas-mediated death, albeit expressing similar levels of Fas, Rb, and p38. The mechanism of E2F1-induced neuronal apoptosis is not currently clear, but there are several possibilities that may be either dependent on p53 or independent of p53. In the latter case, E2F1 may either block the antiapoptotic signal transduction pathway, such as NF-κB (29, 44), or by derepressing of death genes such as transcription factor B and c-Myb (43).

To conclude, we have provided evidence to show that Rb/E2F1 pathway can be activated in postmitotic neurons at least

in part by the p38 SAPK. Future studies aimed at identifying neuronal E2F1 downstream target genes may provide opportunities to block Fas-mediated neuronal death and aid in the design of therapeutics for neurodegenerative diseases.

Acknowledgments—We thank the Institute for Biological Sciences animal facility for timely supply of animals and Drs. John P. MacManus and Robert A. Smith for critical reading of the manuscript.

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Activation of the Rb/E2F1 Pathway by the Nonproliferative p38 MAPK during Fas (APO1/CD95)-mediated Neuronal Apoptosis

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J. Biol. Chem. 2002, 277:48764-48770.
doi: 10.1074/jbc.M206336200 originally published online September 25, 2002
