Growing evidence suggests that certain cell cycle regulators also mediate neuronal death. Of relevance, cyclin D1-associated kinase activity is increased and the retinoblastoma protein (Rb), a substrate of the cyclin D1-Cdk4/6 complex, is phosphorylated during K⁺ deprivation-evoked death of cerebellar granule neurons (CGNs). Cyclin-dependent kinase (CDK) inhibitors block this death, suggesting a requirement for the cyclin D1/Cdk4/6-Rb pathway. However, the downstream target(s) of this pathway are not well defined. The transcription factor E2F-1 is regulated by Rb and is reported to evoke death in proliferating cells when overexpressed. Accordingly, we examined whether E2F-1 was sufficient to evoke death of CGNs and whether it was required for death evoked by low K⁺. We show that adenovirus-mediated expression of E2F-1 in CGNs results in apoptotic death, which is independent of p53, dependent upon Bax, and associated with caspase 3-like activity. In addition, we demonstrate that levels of E2F-1 mRNA and protein increase during K⁺ deprivation-evoked death. The increase in E2F-1 protein is blocked by the CDK inhibitor flavopiridol. Finally, E2F-1-deficient neurons are modestly resistant to death induced by low K⁺. These results indicate that E2F-1 expression is sufficient to promote neuronal apoptosis and that endogenous E2F-1 modulates the death of CGNs evoked by low K⁺.

Increasing evidence suggests that apoptosis may be involved in a number of neurological disorders, including Alzheimer’s disease (1–3) and stroke (4–7). A greater understanding of the cellular signaling pathways that regulate neuronal apoptosis may lead to novel therapeutic targets for these and other neuropathologies. However, the pathways that regulate neuronal death are not fully delineated.

The cell cycle is regulated through a complex series of events controlled through the actions of cyclin-dependent kinases (CDKs) and their obligate binding partners, the cyclins (8). Interestingly, a growing body of work has implicated these CDKs in some instances of neuronal death. This evidence includes both in vivo and in vitro up-regulation of the activity and/or the expression of cyclin-CDK complexes during neuronal death. For example, levels of cyclin D and B and activity of Cdk2 and Cdk4 increase during PC12 cell death evoked by trophic factor deprivation (9, 10). Increased levels of these cell cycle proteins have also been found in Alzheimer’s (11–13) and ischemic (14, 15) brain tissue. In addition, cyclin D1-associated kinase activity increases during DNA damage (16) and low K⁺ induced neuronal death (17). Finally, pharmacological CDK inhibitors and/or expression of dominant negative Cdk4/6 inhibit death of neurons by DNA damage (16, 18), β-amyloid (19), and K⁺ deprivation (17).

Although the above evidence implicates cyclin D1/Cdk4/6 in neuronal death, the downstream effector(s) of the activated complex are unclear. It has been demonstrated that the tumor suppressor Rb, a substrate of Cdk4/6, is phosphorylated in response to β-amyloid (19), K⁺ deprivation (17), and cisplatin-induced death (20). Rb is also capable of protecting neurons from DNA damage-induced death when overexpressed (21). While this evidence suggests Rb involvement, the manner by which Rb controls neuronal death is not fully delineated.

The function of Rb within the nervous system is complex, and it probably plays a role in both cell cycle control and differentiation (22, 23). Although Rb interacts with a large number of proteins, its regulation of E2F members is best characterized (24). E2F-1 is a transcription factor that, along with its heterodimeric partner DP1/2, plays a pivotal role in the G1/S transition of the cell cycle (25, 26). Binding of Rb to the E2F-1-DP complex inhibits transcriptional activity either through sequestration of active E2F-1 complexes or formation of active transcriptional repressor complexes. Phosphorylation of Rb by CDKs results in loss of Rb-E2F-1 binding, loss of repression, and promotion of transcriptional activity (24). Interestingly, several groups have reported that E2F-1 can induce apoptosis in proliferating cells when overexpressed (27–29), and this death can be inhibited by Rb (30).

Previous evidence demonstrated that dominant negative DP-1 can block neuronal death evoked by DNA damage (21) and β-amyloid (19). These results have led us to hypothesize that E2F-1 may also be capable of signaling apoptosis in neurons. In order to further test this model and extend our observations of E2F-1 involvement in neuronal death, we determined whether E2F-1 expression alone was sufficient to evoke death in primary cultures of cerebellar granule neurons (CGNs). We presently show that expression of E2F-1 evokes
death of CGNs in a manner dependent upon Bax and independent of p53. We also examined whether E2F-1 was required for death of CGNs evoked by K+/Ca2+ withdrawal, a model of death known to activate the Cdk4/6-Rb pathway (17). We demonstrated increased E2F-1 mRNA and protein levels following K+/Ca2+ withdrawal and a delay of death in E2F-1-deficient CGNs.

MATERIALS AND METHODS

Cell Culture/K+/Ca2+ Deprivation—Primary CGN cultures were prepared from 7−9-day postnatal mice as described previously (31). Neurons were maintained in complete medium (Eagle’s minimum essential medium (Sigma) containing 2 mM glutamine, 25 mM glucose, 0.02 mg/ml gentamicin (Sigma), 10% dialyzed FBS) supplemented with K+ to a final concentration of 25 mM. An equal number of neurons (in any given experiment) were plated in 24-well dishes coated with poly (I)-lysine (Sigma) at a density of 500,000 cells/well. The antimitic cytosome-arabinoside (10 μM final concentration; Sigma), which kills dividing cells, was added 18−24 h after plating to reduce the amount of glia cells in the culture. Cytosome-arabinoside administered at these doses had no toxic effects on CGNs. Recombinant adenovirus expressing human E2F-1 or LacZ (multiplicity of infection (m.o.i.) of 6 or 10 as indicated) was added to cell suspensions immediately before plating. K+ deprivation was performed 7 days after plating by washing each well twice with 750 μl of complete medium (final K+ concentration of 5 mM) and then incubating in 1 ml of complete medium (5 mM K+ final). Control cells were washed similarly with 5 mM K+ medium and then maintained in 1 ml of complete medium containing 25 mM K+. Cells exposed to flavopiridol were washed as above with complete medium containing 5 mM K+ and 1 μM flavopiridol and then maintained in 1 ml of this same medium for the indicated times.

Knockout Mice—CD-1 mice were obtained from Charles River Laboratories. Bax- and p53-deficient neurons were obtained from pups derived from heterozygous parents maintained on a C57BL6 genetic background. E2F-1-deficient neurons were obtained from pups produced by mating heterozygous or heterozygous and homozygous mice from a B6/129 background. For each experiment, neurons from heterozygote or wild type littermates were used as control. Each animal was genotyped prior to dissection. Genotyping of Bax mice was performed as described previously (32). E2F-1 was genotyped using GGATATGATTCTTGAGGATGTTCCG (E2F-1−5′), CTAATCTGAGCACCAACAGCG (E2F-1+3′), and CAAGTGCCAGCGGGCTGCTAAAG (PGKB) primers. The primers were used in one PCR to amplify an untargeted 170-bp fragment and a targeted 230-bp fragment. PCR conditions are as follows: 94 °C, 1 min (1 cycle); 94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min (30 cycles); 72 °C, 5 min, p53 animals were genotyped using GTATCTGG- GAAGACGGCCAGAC (P-o53–1) and GTATCTGAGTAGGATGGG (P-o53–2) primers to detect the wild type allele (450 bp) and PATACT- CAGAGCCGCCCT (P-o53-X7) and TTTCTCCTGGCTTACTCGGTATC (P-o neo-2) primers to detect the targeted allele (333 bp). PCR conditions were as follows: 94 °C, 5 min (1 cycle); 94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min; 72 °C, 5 min (30 cycles).

Cell Survival—At the indicated time points, medium was aspirated and cells were lysed in 200 μl of lysis buffer (0.1X PBS, pH 7.4, 0.4 mM Na2HPO4, 0.15 mM KH2PO4, 13.5 mM NaCl, 0.25 mM KCl, 0.5% Triton X-100, 2 mM MgCl2, and 0.5 g/100 ml cetyldimethylethyleneammonium bromide). This solution disrupts cell membranes while nuclei remain intact and distinguishable under light microscopy (17, 33, 34). 10 μl of loaded onto a hemacytometer, and the number of healthy intact nuclei were counted, while those displaying blebbing, disruption of nuclear membrane, or phase bright bodies were excluded. The percentage of surviving neurons is expressed relative to the number of neurons in uninfected or non-K+/Ca²⁺-deprived wells plated at the same time as the experimental wells. Each data point is the mean ± S.E. from three samples. Alternatively, neurons were fixed in 0.2% glutaraldehyde and incubated with Hoescht 33258 (0.25 μg/ml) for 30 min at room temperature. After washing with 1X PBS, the percentage of shrunken and condensed nuclei was assessed. Each data point is the mean ± S.E. from three samples.

Caspase Activity Assay—At the indicated time points, cells were washed three times in cold 1X PBS and then processed in a lysis buffer as described previously (35). Lysates were incubated on ice for 20 min and sonicated for 3 s. The samples were then centrifuged for 15 min at 12,000 rpm on an Eppendorf tabletop centrifuge. Supernatant was collected, and protein concentration was assayed using the Bradford reagent (Bio-Rad). Equivalent protein (3 μg) for each sample was incubated with DEVD-AFC (BIOMOL) in a buffer as described (35), and the increase in fluorescence at 505 nm was measured using an excitation wavelength of 400 nm. Measurements were performed on Perkin-Elmer LS 50B spectrometer with a slit width setting of 10 and integration time of 3 s. DEVD-AFC cleavage activity was determined by plotting the change in fluorescence over time. The slope of the curve was then determined and set as DEVD-AFC cleavage activity (in arbitrary units).

Western Blot—At the indicated time points following either infection or K+/Ca²⁺ deprivation, neurons were washed in 1X PBS and then collected in solubilization buffer (0.0625 M Tris, 2.5 mM EDTA, 2.5 mM EGTA, 10% glycerol, 2% SDS, 0.001% bromphenol blue, and 5% β-mercaptoethanol). Protein was loaded onto SDS-polyacrylamide gels and transferred to nitrocellulose membranes as described previously. Membranes were then probed with anti-E2F-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-β-actin (Sigma) antibody.

Immunofluorescence and β-Galactosidase Staining—Neuronal cultures were fixed in 4% paraformaldehyde for 30 min at 4 °C. After washing three times for 10 min in 1X PBS, polyclonal E2F-1 antibody (1:500 in 1X PBS; Santa Cruz Biotechnology) was added to cells for 1 h at room temperature. After washing with 1X PBS, Cy3-conjugated secondary antibody (1:200; Amersham Pharmacia Biotech) was added to cells for 1 h and then washed with 1X PBS. Cells were then washed three times in 1X PBS and visualized by fluorescent microscopy. β-Galactosidase staining was performed as described (31).

Reverse Transcriptase-PCR—Total RNA was extracted from rat brains using TRIzol reagent (Life Technologies, Inc.). First strand cDNAs were reverse transcribed from 4 μg of total RNA. The same amount of cDNAs was subsequently used for PCR amplification for a total of 25 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min of each cycle. These optimal amplification conditions and cycle numbers were determined experimentally to ensure specific and linear signal generation. Expression of β-actin mRNA was used as a standard to quantify the relative amount of expression of E2F-1 as described previously (36). The mouse-specific E2F-1 PCR primers (CAGTGTGACTT- TGGGACC and TGTTCACCTTCATTCCC) and actin primers (AACAC- CCAAGCATGTACGTAG and GTGTTGGCATAGAGGTCTTTACGG) were used to generate an E2F-1 product of 434 bp and a β-actin fragment of 509 bp. The PCR products were fractionated on a 1% agarose gel, photographed, and quantified by scanning laser densitometry. The authenticity of the PCR products was confirmed by cloning into a pCR2.1 vector (Invitrogen) and sequencing using an automated ABI373A DNA sequencer.

RESULTS

Overexpression of E2F-1 Evoke Death of CGNs—We first examined whether expression of E2F-1 was sufficient to induce death of CGNs. CGNs cultured from neonates (CD-1) were infected with recombinant adenoviral constructs expressing
E2F-1 or LacZ as a control. As shown in Fig. 1, 4 days following infection, expression of E2F-1 resulted in 70% reduction in survival compared with control noninfected neurons, while infection with LacZ resulted in only 9% reduction in survival. Western blot analyses confirmed increased expression of E2F-1 in E2F-1-infected cultures (Fig. 2H). With higher exposure of the identical blot, a low level of endogenous E2F-1 was observed in control cultures (data not shown). E2F-1 expression was also confirmed by immunocytochemistry (Fig. 2, A–D). We found that 62% of the neurons in E2F-1 infected cultures present at day 4 stained for E2F-1 (Fig. 2G). The lower number of E2F-1-positive neurons compared with dying neurons, as assessed by nuclear counts, is probably a result of some E2F-1-expressing cells having died and lifted off the plate prior to fixation. Staining for β-galactosidase verified 75% expression in LacZ-infected cultures (Fig. 2, E–G). CGNs infected with E2F-1-expressing adenovirus died with nuclear signs of apoptosis (Fig. 3, A and B). Quantification of Hoescht staining shows that 40% of the neurons in E2F-1-infected cultures and 5% of LacZ-infected cultures displayed condensed and/or fragmented nuclei (Fig. 3C).

**E2F-1 Induces Caspase-3-like Activity and Neuronal Death in a Bax-dependent Manner**—Neuronal death evoked by numerous death stimuli is dependent upon Bax. Of relevance, several reports have indicated that CGN death evoked by either low K+ concentration or p53 overexpression requires Bax activity (31, 37, 38). Accordingly, we examined whether Bax was required for E2F-1-mediated neuronal death by comparing the effects of E2F-1 expression in neuronal cultures obtained from Bax-deficient mice and littermate controls. As shown in Fig. 4, Bax-deficient neurons expressing E2F-1 were significantly more resistant to death as compared with Bax wild type littermates (88% survival for Bax (−/−) versus 36% for Bax (+/+) 4 days following infection). Bax-deficient neurons infected with E2F-1 displayed healthy soma and intact processes typical of healthy neurons, while neurons from Bax wild type littermates infected with E2F-1 show degenerating processes and apoptotic bodies (Fig. 4, B–D).

The resistance of Bax-deficient neurons to E2F-1-induced death indicates that Bax acts downstream of the E2F-1 death signal. One model by which Bax mediates death involves its ability to facilitate the release of cytochrome c from the mitochondria, which leads to formation of the apoptosome complex and subsequent caspase activation (39, 40). To test whether Bax-dependent E2F-1-induced death was associated with caspase activation, we measured caspase-3-like activity in Bax null and wild type littermates infected with E2F-1 or LacZ adenovirus. Caspase-3-like activity was measured by DEVD-AFC cleavage activity. CGNs from Bax (+/+ and (+/−) mice showed a 7–8-fold increase in DEVD-AFC cleavage activity 4 days after infection with E2F-1 (Fig. 5). DEVD-AFC cleavage activity, however, is not detected in Bax-deficient CGNs expressing E2F-1. These results indicate that death of CGNs induced by E2F-1 expression is associated with increased caspase-3-like activity and that this activity is dependent on Bax.

**Death of CGNs Induced by Expression of E2F-1 Is Not Dependent on p53**—Multiple reports in proliferating cells have indicated that E2F-1-mediated death can be a p53-dependent (29, 41, 42) or -independent (30, 43) phenomenon. In addition, E2F-1-dependent neuronal death in vivo due to Rb deficiency can be either p53-dependent or p53-independent according to neuronal type (44–46). In order to establish the relevance of p53 in E2F-1-induced neuronal death, we examined whether p53 (−/−) neurons were resistant to E2F-1 expression. We found that neurons from p53-deficient and heterozygous mice displayed no significant differences in survival in response to E2F-1 expression (Fig. 6). This indicates that p53 is not required for death of CGNs induced by E2F-1 expression and that...
the activation of Bax-mediated death signals occurs independently of p53.

**E2F1 Involvement in Death of Neurons Evoked by K⁺ Deprivation**—The above studies indicate that E2F-1 expression is sufficient to cause neuronal death. We next asked whether E2F-1 was involved in a CGN model of death in which E2F-1 itself is not the death stimulus. To address this question, we examined the requirement for E2F-1 in K⁺ deprivation-induced death of CGNs. In this death paradigm, CGNs die apoptotically when switched from medium containing a high K⁺ concentration (25 mM) to medium with a low K⁺ concentration (5 mM) (47–49). Moreover, similar to our results with E2F-1 overexpression, K⁺ deprivation-induced death is dependent upon Bax and induces caspase-3-like activity (38, 50).

We first determined whether E2F-1 was required for death of CGNs evoked by K⁺ deprivation. CGNs from E2F-1-deficient animals displayed a small but significant delay of death compared with neurons from heterozygous E2F-1 littermates. The effect was most significant 12 h following deprivation, with 68% of E2F-1 (+/−) neurons surviving versus 39% survival for E2F-1 (−/−) (Fig. 7). Consistent with this observed protection, E2F-1 transcripts increase during CGN death induced by K⁺ withdrawal, as determined by semiquantitative reverse transcr.
scriptase-PCR. We detected increases in E2F-1 message as early as 1 h following the induction of K^+ deprivation (Fig. 8A). As an internal control, β-actin message was co-amplified with E2F-1. The densitometric ratio of E2F-1 to β-actin increases approximately 3-fold at 1 h and plateaus 12 h following K^+ deprivation (Fig. 8B). Combined data from three independent experiments shows that E2F-1 transcript levels increase 4-fold over base line within 6 h of K^+ deprivation (Fig. 8C). Because the E2F-1 gene is itself regulated by E2F activity (51), our data suggests a deregulation of E2F-1 transcriptional complexes.

To determine if the increase in E2F-1 message is associated with an increase in E2F-1 protein levels, we performed Western blot analysis of protein isolated from CGNs deprived of K^+. Prior to K^+ deprivation, E2F-1 protein levels are low but detectable. However, as early as 6 h following the lowering of K^+ concentration, we detected an increase in total E2F-1 protein (Fig. 9). To investigate the involvement of CDKs in the increase in E2F-1 expression, we treated K^+-deprived CGNs with flavopiridol. Flavopiridol inhibits Cdk4/6 activity and Rb phosphorylation and prevents death of CGNs evoked by low K^+ (17). Treatment with flavopiridol suppressed the increase in E2F-1 protein, suggesting that the Cdk4/6-Rb pathway is required for increased E2F-1 expression during K^+ deprivation (Fig. 9B). An E2F-1-reactive band was not detected in E2F-1-deficient neurons deprived of K^+ (data not shown).

Taken together, our results indicate that E2F-1 levels increase and that this increase plays a modulatory role in K^+ deprivation-induced death of CGNs. However, our data also indicate that factors other than E2F-1 must contribute to the death signal.

**DISCUSSION**

Previous results suggested that death of CGNs evoked by low K^+ is regulated by activation of cyclin-dependent kinases (17). Accordingly, we examined the involvement of E2F-1, a transcription factor regulated by Rb and a potential effector of the CDK/Rb pathway. We investigated whether expression of E2F-1 was sufficient to evoke neuronal death and whether E2F-1 was required to promote CGN death evoked by low K^+.

**E2F-1 Expression Evokes Caspase Activation and Death in a Bax-dependent and p53-independent Manner**—The ability of E2F-1 to induce apoptosis was previously reported in proliferating cell systems and was thought to be a protection mechanism against uncontrolled cell division (28, 52). In support of this, E2F-1 knockout mice show increased tumorigenesis (53). However, its ability to evoke neuronal death and the mechanism by which it may cause this death are not clear. We presently show that E2F-1 expression evokes CGN death in a manner independent of p53 but dependent on Bax.

In dividing cells, E2F-1-mediated death is reported to proceed through both p53-dependent (29, 41, 42) and -independent (30, 43) processes. In the case of the former, E2F-1 is thought to signal via the tumor suppressor p19ARF. E2F-1 is reported to
directly activate transcription of p19Arf, which binds to MDM2, a negative regulator of p53, with consequent stabilization of p53 levels (54). Our results suggest that E2F-1-evoked death of CGNs is independent of p53. This is significant in light of previous evidence demonstrating that apoptosis in the developing embryo due to Rb loss is dependent on both E2F-1 and p53 in the central nervous system (44, 46). This indicates that neuronal death due to Rb loss in the central nervous system proceeds through a different signaling mechanism from that of E2F-1-mediated overexpression alone.

Very little is known regarding the factors that mediate E2F-1-induced apoptosis in the absence of p53. Experiments using mutant forms of E2F-1 have shown that the transactivation domain of E2F-1 is dispensable for induction of p53-independent apoptosis in Saos-2 cells, whereas the DNA binding domain is required (30, 43). In addition, Phillips et al. have suggested that p53-independent E2F-1-mediated apoptosis involves the inhibition of antiapoptotic signals and may require signaling via death receptors (56). Whether this is true in neurons remains to be determined. The recent identification of p63 and p73 (57) raise the possibility that E2F-2 and E2F-3 may signal apoptosis in different cell types through multiple p53 family members.

E2F-1 expression results in caspase activation and death, which is dependent upon the presence of Bax. We found that caspase activity, as indicated byDEVDD-AFC cleavage, increases in response to E2F-1 expression in wild type CGNs but not Bax-deficient CGNs. Most importantly, these Bax-deficient CGNs are also resistant to the death induced by E2F-1. These results suggest an E2F-1-mediated death signaling pathway proceeding through Bax and subsequent caspase activity. Bax has been reported as an upstream activator of caspase activity in a number of cell death systems (31, 38, 58). Bax activation of caspases is thought to occur via translocation to the mitochondrial membrane, consequent cytochrome-c release into the cytoplasm, and activation of the Apaf1/caspase 9-containing apoptosome (39). Bax activity has also been shown to be required for other neuronal death paradigms, including K+ deprivation of CGNs (37, 38). Accordingly, our results are consistent with the importance of Bax in apoptotic death of CGNs.

Role of E2F-1 in Death of CGNs Evoked by Low K+—We have shown that E2F-1 levels increase during death evoked by low K+ and that this increase appears to be regulated by CDKs. Because E2F-1 is known to regulate its own transcription, this result is consistent with the notion of deregulated activation of E2F-1 complexes during death induced by low K+. A concept suggested by previous reports demonstrating both Rb phosphorylation and loss in this same death paradigm (17). Our findings of significant neuroprotection as a result of E2F-1 deficiency are also consistent with the in vivo observations of others showing that E2F-1 deficiency is partially protective against death induced by Rb deficiency (46) and ischemia (59). In addition, we have also recently shown that E2F-1 deficiency is protective against β-amyloid toxicity (60).

Our present findings extend these observations of E2F-1 involvement in neuronal death. However, our results indicating incomplete protection with E2F-1 deficiency suggest that other Rb-interacting members may also be involved in the CDK-mediated death signal. In support of this, recent studies have raised the possibility that expression of E2F-2 and E2F-3 can also evoke death in proliferating cell systems (61, 62). Accordingly, it is possible that functional redundancy among E2F members may control death of CGNs by low K+. Other studies, however, have shown that E2F-1 is the only E2F member capable of evoking apoptosis in proliferating cells (63). In addition, although E2F-1 deficiency prolongs the life of Rb-deficient mice, it does not protect from all forms of apoptosis, suggesting that Rb functions through proteins other than E2F-1 (46).

The involvement of numerous signaling pathways other than those directly related to the cell cycle have also been well established in K+ deprivation-induced death of CGNs. For example, activation of the c-Jun N-terminal kinase (64) and the Fas/FasL (40) pathway have been demonstrated in CGNs exposed to low K+ conditions. Other signals mediated through phosphatidylinositol 3-kinase (65) and mitogen-activated protein kinase (66) have been demonstrated to be neuroprotective in these cells. This involvement of multiple factors suggests that neurons simultaneously activate several biochemically distinct but functionally cooperative pathways that regulate the death response to low K+. It will be important to relate these signaling events to obtain a fuller understanding of how neuronal death is controlled.

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