11. S. Patel and M. Latterich, *Trends Cell Biol.* **8**, 65 (1998).
12. P. I. Hanson *et al.*, *Cell* **90**, 523 (1997).
13. K. G. Fleming *et al.*, *J. Biol. Chem.* **273**, 15,675 (1998).
14. J. J. Hartman and R. D. Vale, data not shown.
15. Katanin p60 was cloned into the Bam HI site of pFastBac HT B (Life Technologies). The resulting baculovirus construct expresses p60 (amino acids 1 to 516) with the following additional NH_2-terminal amino acids: MSYY-HHHHHHDYDIPTTENLYFQGS. The GFP mut2 [B. P. Cormack *et al.*, *Gene* **173**, 33 (1996)] coding sequences were amplified with primers containing Kas I sites at both ends and inserted into the Kas I site in pFastBac HT B. The resulting baculovirus constructs express GFP fused to the NH_2-terminus of p60. We prepared recombinant p60 proteins as described in (*7*), except that we did not perform Mono Q chromatography, and the proteins were stored at −80°C in Ni-NTA elution buffer [20 mM tris (pH 8.0), 100 mM NaCl, 100 mM imidazole, 2 mM $MgCl_2$, 0.02% Triton X-100, 10 mM 2-mercaptoethanol, 10% glycerol, 250 μM ATP]. Storage in ATP was necessary to preserve enzyme activity. Glycerol gradient centrifugation was modified from other methods (*12*). Gradients were prepared by layering equal steps of 10%, 16.3%, 22.5%, 28.8%, and 35% glycerol in gradient buffer [20 mM potassium Hepes (pH 8.0), 75 mM potassium glutamate, 2 mM $MgCl_2$, 0.02% Triton X-100, 1 mM dithiothreitol], with the indicated nucleotide (2 mM) and equimolar $MgCl_2$; gradients were allowed to form overnight at 4°C. Proteins in Ni-NTA elution buffer were diluted ~1:1 with gradient buffer (final concentration, ~5 μM) and incubated with the appropriate nucleotide (2 mM) and 2 mM $MgCl_2$ for 15 min on ice before centrifugation. The NSF D2 domain was expressed and purified as described in (*12*) and handled as described above. Bovine serum albumin (BSA) (4.1*S*), catalase (11.2*S*), and thyroglobulin (19.4*S*) were used as standards. Gradients were centrifuged for 12 hours at 285,000*g* in an SW60 Ti rotor (Beckman Instruments) at 4°C. GFP-tagged p60 was quantitated by fluorescence (λ_{ex} 470, λ_{em} 508) using either an SLM 8100 in photon counting mode (Spectronics) or a Perkin-Elmer LS-5B (Perkin-Elmer). Standards and NSF D2 were quantitated by the Bradford assay.
16. A. Miyawaki *et al.*, *Nature* **388**, 882 (1997).
17. ECFP (Clonetech) and EYFP (Clonetech) coding sequences were inserted into the pFastBac-p60 expression plasmid as in (*15*).
18. R. Tsien, personal communication.
19. Amino acid residues are abbreviated as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.
20. The E334Q mutant was prepared by overlap extension amplification [S. N. Ho *et al.*, *Gene* **77**, 51 (1989)] of the wild-type p60 cDNA, followed by cloning of the mutant sequence into pFastBac HT B as in (*15*) and sequencing to check for amplification errors.
21. S. W. Whiteheart *et al.*, *J. Cell Biol.* **126**, 945 (1994).
22. M. Babst, B. Wendland, E. J. Estepa, S. D. Emr, *EMBO J.* **17**, 2982 (1998).
23. Separately purified CFP-p60 and YFP-p60 were preincubated together in the indicated ratios at a 10× assay concentration for 30 to 60 min on ice. The CFP-YFP–p60 mixture was then diluted into reaction mixtures containing 2 mM nucleotide and equimolar $MgCl_2$ in ATPase buffer [20 mM potassium Hepes (pH 7.5), 25 mM potassium glutamate, 2 mM $MgCl_2$, 0.02% Triton X-100, 10% glycerol, 20 μM paclitaxel]. Reaction mixtures contained BSA at 1 mg/ml to prevent surface adsorption; 20 mM glucose and hexokinase at 0.1 mg/ml, which converted residual ATP to ADP, were used to measure FRET in the ADP state. For FRET measurements with microtubules, we preincubated p60 with the nucleotide mixture for 10 min at 22°C before we added microtubules. Microtubules were prepared in ATPase buffer containing 20 μM paclitaxel, as described in (*7*). After a 10- to 15-min incubation with microtubules, we quantitated energy transfer by exciting the sample at 433 nm and measuring the fluorescence at 480 and 535 nm. We used the ratio of emission at 535 nm/480 nm as the measure of energy transfer (*16*). For emission scans, we excited the sample at 433 nm and measured the fluorescence emission between 450 and 600 nm with excitation and emission slit sizes of 1 nm.
24. We performed gel filtration chromatography on about 15 μg of $p60^{E334Q}$ with a 0.78 × 30 cm TSK-4000SWXL column (TosoHaas) equilibrated with 20 mM potassium Hepes (pH 7.8), 75 mM potassium glutamate, 2 mM $MgCl_2$, 0.02% Triton X-100, 5% glycerol, and 1 mM ATP. Standard proteins were BSA (Stokes radius 35 Å), catalase (52 Å), and thyroglobulin (86 Å). We estimated molecular mass by using the measured Stokes radius and the *S* value [L. M. Siegel and K. J. Monty, *Biochim. Biophys. Acta* **112**, 346 (1966)].
25. GFP-p60 (0.4 μM final concentration) was mixed with paclitaxel-stabilized microtubules prepared as in (*7*) and BSA (1 mg/ml) in ATPase buffer and incubated at 22°C for 20 min. We used 5 mM glucose and hexokinase (0.1 mg/ml), which converted residual ATP to ADP, to measure binding in ADP. Samples (50 μl) were incubated at 22°C for 20 min before loading onto a 50-μl cushion of 66% glycerol in ATPase buffer, followed by centrifugation for 5 min at 436,000*g* (20°C). The supernatant and cushion were removed and diluted 1:1 with water to reduce sample viscosity. The microtubule pellets were depolymerized by incubation on ice for 30 min in ATPase buffer containing 5 mM $CaCl_2$, followed by 1:1 dilution with water. Bound and free GFP-p60 were determined by fluorescence (λ_{ex} 470, λ_{em} 508).
26. The p60ΔAAA construct was prepared by amplifying the region coding for amino acids 1 to 210 of p60 and inserting this into pFastBac HT B at the Bam HI and Xho I sites. We amplified the GFP coding sequence and inserted it at Kpn I and Hind III sites, which resulted in a baculovirus that expresses p60 (amino acids 1 to 210) with the same additional NH_2-terminal amino acids as wild-type p60 fused to the NH_2-terminus of GFP.
27. E. E. Nagiec, A. Bernstein, S. W. Whiteheart, *J. Biol. Chem.* **270**, 29182 (1995).
28. S. P. Gilbert and K. A. Johnson, *Biochemistry* **32**, 4677 (1993).
29. We performed ATPase assays by using the malachite green method as described in (*7*). We included DEAE-purified ATP at 1 mM.
30. We also examined FRET when the katanin concentration was varied with a constant microtubule concentration in the presence of ATP-γ-S. The signal did not increase in a hyperbolic manner. Instead, an increase in the FRET signal was observed only when the katanin reached a critical concentration, which again suggests a cooperative phenomenon.
31. We measured the microtubule severing rate with a fluorescence-based assay for 4′,6-diamidino-2-phenylindole binding as described in (*7*). To calculate the amount of tubulin dimers released, we divided the rate of change of fluorescence ($\Delta F/s$) by {[($F_{unsevered}$) − ($F_{Ca^{2+}\ depolymerized}$)]/microtubule concentration, μM}.
32. P. B. Sigler *et al.*, *Annu. Rev. Biochem* **67**, 581 (1998).
33. Z. Xu, A. L. Horwich, P. B. Sigler, *Nature* **388**, 741 (1997).
34. M. Shtilerman, G. H. Lorimer, S. W. Englander, *Science* **284**, 822 (1999).
35. A. Desai, S. Verma, T. J. Mitchison, C. E. Walczak, *Cell* **96**, 69 (1999).
36. S. M. Sweitzer and J. E. Hinshaw, *ibid.* **93**, 1021 (1998).

37. We thank F. J. McNally, R. D. Mullins, K. S. Thorn, and J. E. Wilhelm for critical reading of the manuscript.

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Neuronal Activity-Dependent Cell Survival Mediated by Transcription Factor MEF2

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During mammalian development, electrical activity promotes the calcium-dependent survival of neurons that have made appropriate synaptic connections. However, the mechanisms by which calcium mediates neuronal survival during development are not well characterized. A transcription-dependent mechanism was identified by which calcium influx into neurons promoted cell survival. The transcription factor MEF2 was selectively expressed in newly generated postmitotic neurons and was required for the survival of these neurons. Calcium influx into cerebellar granule neurons led to activation of p38 mitogen-activated protein kinase-dependent phosphorylation and activation of MEF2. Once activated, MEF2 regulated neuronal survival by stimulating MEF2-dependent gene transcription. These findings demonstrate that MEF2 is a calcium-regulated transcription factor and define a function for MEF2 during nervous system development that is distinct from previously well-characterized functions of MEF2 during muscle differentiation.

The MEF2 proteins constitute a family of transcription factors that play a critical role in the process of cell differentiation during the development of multicellular organisms (*1–7*). MEF2 proteins cooperate with members of the MyoD family in specifying the differentiation of skeletal muscle (*8, 9*). During neurogenesis, MEF2 mRNAs are robustly transcribed in the developing mammalian central nervous system (CNS) (*10–12*), although the functions of MEF2s during nervous system development have not been known.

To investigate the role of the MEF2 proteins during mammalian CNS development, we first characterized the expression of

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MEF2 proteins in the rat brain by immunohistochemistry with antibodies that recognize specific MEF2 isoforms (*13*). Among the four MEF2 proteins, MEF2C was the predominant isoform expressed in the developing rat cerebral cortex. MEF2C expression was detectable at embryonic day 17 (E17) and peaked around E21 (Fig. 1A) (*14*). MEF2C is expressed in the cortical plate but is not detectable in the ventricular zone (Fig. 1A), which indicates that MEF2C is primarily expressed in differentiating neurons but not in actively dividing neuronal precursor cells that populate the ventricular zone. Cells expressing the neuron-specific marker β-tubulin type III (TuJ1) express MEF2C (Fig. 1B), whereas cells expressing the glial marker glial fibrillary acidic protein (GFAP) do not (*14*). This suggests that MEF2C expression is restricted to neurons and that MEF2C may have a role in neuronal differentiation.

To determine whether MEF2C expression is specifically induced in postmitotic neurons, we prepared primary cultures of cortical neurons in which proliferating precursor cells and postmitotic differentiating neurons could be distinguished. In these cultures, proliferating cerebral cortical precursor cells incorporate bromodeoxyuridine (BrdU) into DNA as they undergo DNA synthesis and express nestin, an intermediate filament protein that is a marker for actively dividing ventricular zone cells. Cultured cortical precursors do not express TuJ1, an isoform of tubulin that is specifically expressed in postmitotic neurons (*15, 16*). When cortical cultures that contained both precursors and postmitotic neurons were stained with an antibody to MEF2C, MEF2C was found to be expressed in large amounts in postmitotic neurons and was not detectable in BrdU-positive proliferating precursor cells (Fig. 1C). Thus, once cortical precursor cells withdraw from the cell cycle and differentiate into neurons, they begin to express MEF2C. These findings raised the possibility that during neurogenesis MEF2s might control the maturation or function of newly differentiated neurons.

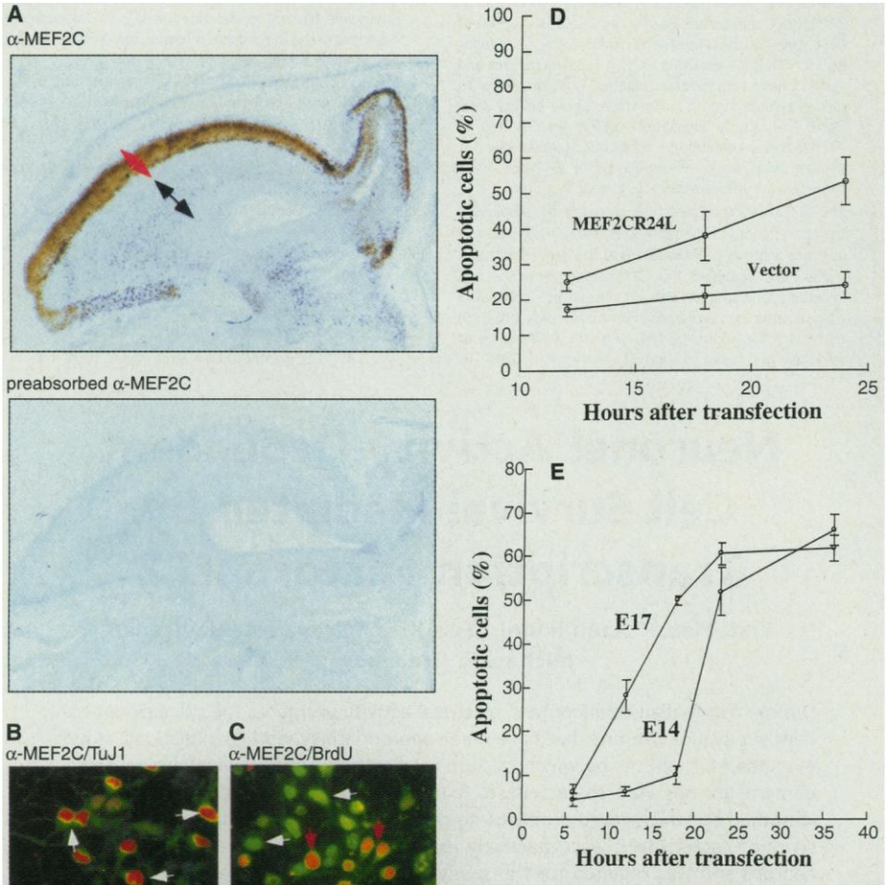


Fig. 1. Expression of MEF2C protein and its role in the developing rat cerebral cortex. (**A**) Immunohistochemistry of E21 rat brain. (**Top**) Sections of rat brain were fixed and immunostained with MEF2C-specific antibodies. Red arrow indicates neocortex–cortical plate; black arrow indicates the subventricular zone–neuroepithelium. (**Bottom**) Sections of rat brain were fixed and immunostained with MEF2C antibodies preabsorbed with MEF2C protein. Immunostaining with preimmune serum showed no signal. (**B**) Expression of MEF2C protein in cultured neurons from E17 rat cerebral cortex. E17 cortical neurons were cultured in vitro for 2 days. Cells were fixed and immunostained with a MEF2C-specific antibody and an antibody to TuJ1, a marker for differentiating neurons. White arrows indicate cells positive for MEF2C and TuJ1. (**C**) Expression of MEF2C protein in postmitotic neurons. E14 cortical neurons were cultured in vitro for 2 days. BrdU was then added to the culture medium at a final concentration of 1 μM for 8 hours and cells were fixed and immunostained with a polyclonal antibody to MEF2C and a monoclonal antibody to BrdU. White and red arrows indicate BrdU- and MEF2C-positive cells, respectively. (**D**) Kinetics of cell apoptosis induced by a dominant-interfering form of MEF2 (MEF2CR24L). Cultured E17 cortical neurons were transfected with either a control vector or MEF2CR24L together with CMV-β-Gal. Transfected cells were scored by two independent investigators in a blinded fashion as healthy or apoptotic based on the extent of nuclear condensation and fragmentation and process integrity. Apoptotic cells showed a pyknotic or fragmented nucleus and disintegrated cell body or neurites (*29–31*). Results are presented as mean ± SEM ($n = 3$). (**E**) Effects of blocking MEF2 on E14 and E17 cerebral cortical neurons. After 2 days in vitro, neurons from E14 and E17 cerebral cortical cultures were transfected with MEF2CR24L or a control vector along with CMV-β-Gal. Cultures were analyzed as in (D) ($n = 3$).

To investigate the role of MEF2 proteins during neurogenesis, we examined the effect of blocking the function of endogenous MEF2 in neurons. For these experiments, we transfected cortical neurons with a plasmid containing a gene encoding a dominant-interfering form of MEF2 (MEF2CR24L) and a plasmid that directs the expression of β-galactosidase (β-Gal) so that transfected cells can be easily identified by staining with an antibody to β-Gal (anti–β-Gal). MEF2CR24L contains a point mutation in its DNA binding domain that prevents its binding to the specific MEF2 DNA recognition site. However, MEF2CR24L still dimerizes with wild-type MEF2s and therefore can inhibit the function of endogenous MEF2s (*17*). After transfection, we analyzed cultures by immunofluorescence microscopy with anti–β-Gal to visualize transfected cells and with antibodies to TuJ1 to confirm the neuronal identity of the transfected cells. We also stained the cultures with the DNA dye Hoechst 33258 to visualize the nuclear morphology of transfected cells. We found that inhibition of MEF2 function by MEF2CR24L increased the number of apoptotic cells (Fig. 1D).

Inhibition of MEF2 function induced apoptosis with a time course that depended on the stage of neurogenesis. In cerebral cortical neurons cultured from E17 rat embryos, in which endogenous MEF2C is highly expressed at the time of transfection, inhibition of MEF2 function by transfected MEF2 CR24L induced apoptosis within 12 hours (Fig. 1E). However, in E14 cultures, in which most of the cells are dividing neuronal precursors and endogenous MEF2C is not detectable until about 24 hours after transfec-

tion, inhibition of MEF2C had little effect until 18 hours after transfection. Around 18 hours, just as MEF2C was beginning to be expressed in the cultures, the expression of the dominant-interfering form of MEF2 triggered apoptosis in a large percentage of the transfected neurons (Fig. 1E). We cannot rule out the possibility that there is a difference in sensitivity of cells to the inhibition of MEF2 function due to a difference in embryonic age. However, the simplest explanation for these findings is that MEF2 specifically promotes the survival of postmitotic neurons. Consistent with this hypothesis, when MEF2 CR24L was expressed in cortical precursor cells or glial cells, this dominant-interfering form of MEF2 had little effect on cell survival (*14*). These results indicate that MEF2 proteins are required for the survival of neurons as they differentiate and raise the possibility that a primary function of MEF2 might be to promote the survival of newly differentiated neurons.

We next examined the role of MEF2 in promoting the survival of a relatively homogeneous population of CNS neurons, the granule neurons of the postnatal cerebellum.

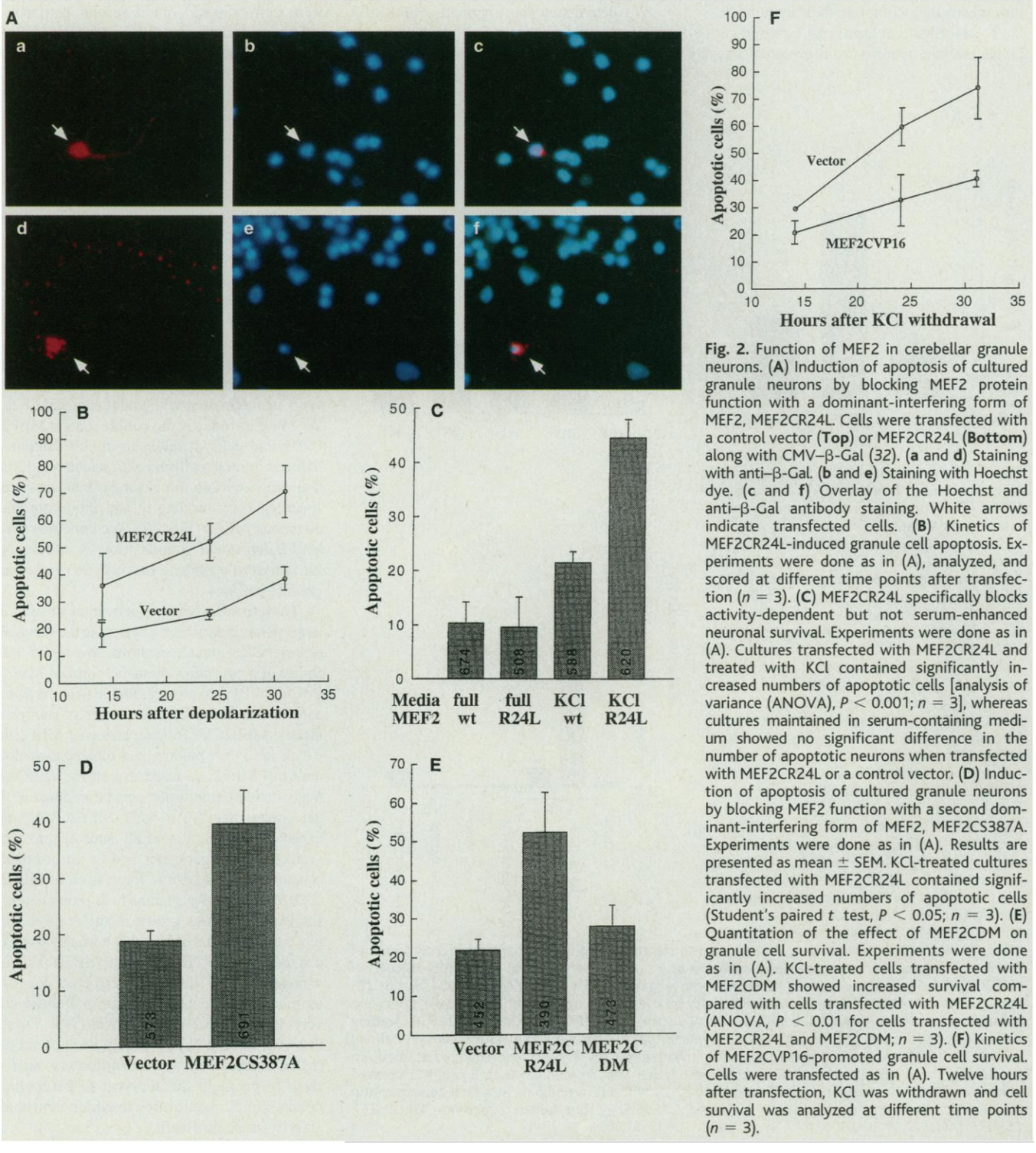


Fig. 2. Function of MEF2 in cerebellar granule neurons. (**A**) Induction of apoptosis of cultured granule neurons by blocking MEF2 protein function with a dominant-interfering form of MEF2, MEF2CR24L. Cells were transfected with a control vector (**Top**) or MEF2CR24L (**Bottom**) along with CMV–β-Gal (*32*). (**a** and **d**) Staining with anti–β-Gal. (**b** and **e**) Staining with Hoechst dye. (**c** and **f**) Overlay of the Hoechst and anti–β-Gal antibody staining. White arrows indicate transfected cells. (**B**) Kinetics of MEF2CR24L-induced granule cell apoptosis. Experiments were done as in (A), analyzed, and scored at different time points after transfection ($n = 3$). (**C**) MEF2CR24L specifically blocks activity-dependent but not serum-enhanced neuronal survival. Experiments were done as in (A). Cultures transfected with MEF2CR24L and treated with KCl contained significantly increased numbers of apoptotic cells [analysis of variance (ANOVA), $P < 0.001$; $n = 3$], whereas cultures maintained in serum-containing medium showed no significant difference in the number of apoptotic neurons when transfected with MEF2CR24L or a control vector. (**D**) Induction of apoptosis of cultured granule neurons by blocking MEF2 function with a second dominant-interfering form of MEF2, MEF2CS387A. Experiments were done as in (A). Results are presented as mean ± SEM. KCl-treated cultures transfected with MEF2CR24L contained significantly increased numbers of apoptotic cells (Student's paired t test, $P < 0.05$; $n = 3$). (**E**) Quantitation of the effect of MEF2CDM on granule cell survival. Experiments were done as in (A). KCl-treated cells transfected with MEF2CDM showed increased survival compared with cells transfected with MEF2CR24L (ANOVA, $P < 0.01$ for cells transfected with MEF2CR24L and MEF2CDM; $n = 3$). (**F**) Kinetics of MEF2CVP16-promoted granule cell survival. Cells were transfected as in (A). Twelve hours after transfection, KCl was withdrawn and cell survival was analyzed at different time points ($n = 3$).

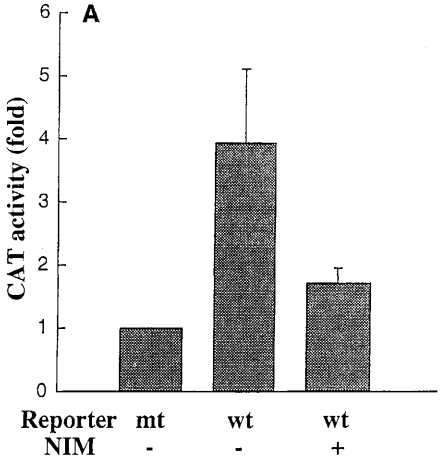
MEF2A is highly expressed in developing rat cerebellum and is expressed predominantly in the internal granule layer and the Purkinje cell layer but not in the proliferating zone of the external granular layer (*14, 18*). The pattern of MEF2A expression is consistent with a possible role for the MEF2 proteins in promoting the survival of newly generated postmitotic cerebellar granule neurons after their migration to the sites within the cerebellum where they complete their maturation.

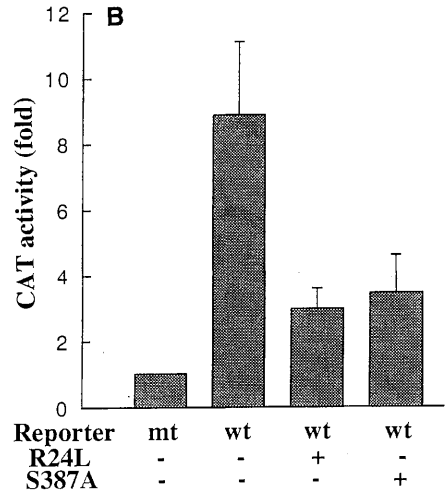
To test this hypothesis, we cultured cerebellar granule neurons from postnatal day 6 (P6) rat cerebella in the presence of depolarizing concentrations of KCl, concentrations that mimic the effects of neuronal activity on neuronal survival. Increased concentrations of KCl that promote calcium influx through L-type voltage-sensitive calcium channels (VSCCs) enhance the survival of cerebellar granule neurons. When the concentration of extracellular KCl is lowered, these neurons undergo cell death by apoptosis (*14, 19–21*).

We determined the importance of MEF2 activity for the survival of cerebellar granule cells by blocking MEF2 function and examining the effect on the survival of these cells under various conditions. Expression of the dominant-interfering form of MEF2, MEF2 CR24L, reduced the survival of membrane-depolarized granule neurons (Fig. 2, A and B) but did not affect the survival of cerebellar neurons grown in the presence of serum-containing medium (Fig. 2C), which suggests that MEF2s were specifically required for activity-mediated neuronal survival. These findings were corroborated with a second dominant-interfering form of MEF2, MEF2CS387A, which functions by a different mechanism than MEF2CR24L (*22*). MEF2CS387A dimerizes and competes with endogenous MEF2s for binding to MEF2 DNA regulatory sites. However, because of a mutation in its transactivation domain MEF2CS387A cannot be activated by extracellular stimuli and therefore inhibits the function of endogenous MEF2s. When expressed in large amounts in cerebellar granule neurons, MEF2CS387A inhibited calcium-dependent survival of these cells (Fig. 2D).

To rule out the possibility that the dominant-interfering forms of MEF2 might be blocking cell survival nonspecifically by sequestering cellular factors other than MEF2, we engineered a second mutation within the dimerizing domain of MEF2CR24L that prevents its interaction with endogenous MEF2s. When we introduced the double mutant MEF2 CDM into cells, it failed to inhibit calcium-induced granule cell survival, which suggests that MEF2CR24L blocks neuronal survival specifically by binding to and inhibiting endogenous MEF2 (Fig. 2E). We conclude that MEF2-dependent transcription is necessary for activity-dependent survival of cerebellar granule neurons.

To determine whether activation of MEF2 target genes is sufficient to promote the survival of cerebellar granule neurons, we tested the ability of a constitutively active form of MEF2 (MEF2CVP16) to protect granule neurons from apoptosis induced by withdrawal of survival factors. MEF2CVP16 is a version of MEF2 in which the DNA binding and dimerization domains of MEF2 are fused to a strong constitutively active transcription activation domain of the viral protein VP16 (*22*). MEF2CVP16 is expected to bind to MEF2 sites within the promoters of target genes and to lead to their constitutive transcription. Expression of MEF2 CVP16 in cerebellar granule cells promoted the survival of neurons grown in the presence of low concentrations of KCl that normally trigger cell death (Fig. 2F). Overall, MEF2CVP16-expressing cells displayed a robust cellular morphology, bore intact neurites, and displayed very little nuclear condensation (*14*). These data suggest that MEF2 recognition element (MRE)–dependent transcription is sufficient to promote the survival of cerebellar neurons under conditions in which neuronal activity is suppressed.





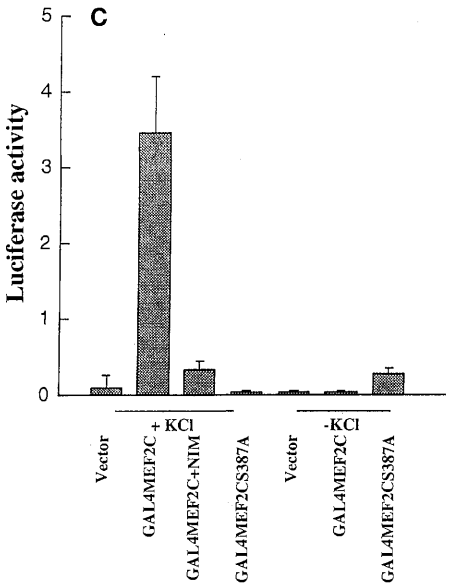


Fig. 3. Mechanism by which neuronal activity regulates MEF2 function. (**A**) Activation of MRE-CAT reporter gene expression by membrane depolarization. Granule cells were cultured as in Fig. 2A and transfected with Rous sarcoma virus–β-Gal (RSV–β-Gal) and a MRE-CAT reporter gene containing either wild-type MREs (indicated as wt) or a mutant MRE (indicated as mt). Two hours after transfection, cells were placed in medium containing 25 mM KCl without serum. After 10 hours, nimodipine was added to the medium to a final concentration of 20 μM (indicated as NIM). Twelve hours later, cell lysates were prepared and analyzed for both CAT and β-Gal activity ($n = 4$) (*33*). Membrane depolarization significantly induced MRE-CAT reporter gene activity, which required the influx of extracellular calcium through VSCCs and an intact MRE (ANOVA, $P < 0.01$ for wt and mt, $P < 0.05$ for wt and NIM; $n = 3$). (**B**) Inhibition of membrane depolarization-induced MRE-CAT reporter gene activity by dominant-interfering forms of MEF2. Granule neurons were cultured and transfected as in (A). Cells were kept in full medium for 8 hours after transfection and then placed in medium containing 25 mM KCl. Twelve hours later, before overt morphological changes occurred in cells transfected with the dominant-interfering forms of MEF2, cell lysates were prepared and analyzed as in (A). Transfection of either MEF2CR24L or MEF2CS387A significantly reduced MRE-CAT reporter gene activity (ANOVA, $P < 0.001$; $n = 3$). (**C**) Induction of GAL4MEF2C transactivation activity by membrane depolarization. Granule cells were cultured as in (A) and transfected with a control vector, GAL4MEF2C, or GAL4MEF2CS387A along with a GAL4-luciferase (*22*) and RSV–β-Gal reporter gene. Cells were kept in medium containing 25 mM KCl without serum immediately after transfection for 2 hours and changed to medium containing 5 mM KCl. Two hours later, cells were either kept in medium with 5 mM KCl or were placed for 5 hours in medium with 25 mM KCl with or without nimodipine at a final concentration of 20 μM. Membrane depolarization activated GAL4MEF2C but failed to activate GAL4MEF2 CS387A (ANOVA, $P < 0.001$; $n = 3$).

We next investigated whether membrane depolarization increased MEF2 activity in cerebellar neurons. Membrane depolarization enhanced the expression of a chloramphenicol acetyltransferase (CAT) reporter gene that contained two MEF2 DNA binding elements (MREs) within its regulatory region (Fig. 3A). The enhancement in CAT expression was MRE dependent because a mutation in the MRE that blocks MEF2 binding reduced CAT activity to the low amount detected in the absence of calcium influx. MRE-driven reporter gene expression was blocked by the VSCC blocker nimodipine, indicating that induction of the reporter gene by membrane depolarization required an influx of calcium through VSCCs (Fig. 3A). Cotransfection of the dominant-inhibitory forms of MEF2 blocked MRE-dependent transcription (Fig. 3B), confirming that calcium induction of MRE-dependent gene transcription was mediated by MEF2.

To characterize further the mechanism by which calcium regulates MEF2 activity, we used an assay that allows the activity of exogenous MEF2 in neurons to be distinguished from that of endogenous MEF2. For this assay, we used GAL4MEF2C, a fusion of the transcription activation domain of MEF2 C and the DNA binding domain of the yeast transcription factor GAL4. We transfected GAL4MEF2C into neurons with a reporter construct in which five copies of the GAL4 binding site were cloned within the 5' regulatory region of the luciferase gene (*22*). When the transfected neurons were exposed to increasing concentrations of KCl, transcription of the GAL4MEF2C-dependent construct was markedly enhanced (Fig. 3C). The enhancement in luciferase expression was blocked by the VSCC antagonist nimodipine, indicating that the increased luciferase expression required an influx of calcium through VSCCs. These findings indicate that, when bound to the regulatory region of a reporter gene via a GAL4 DNA binding domain, MEF2 on its own can mediate a calcium-dependent transcriptional response. These findings further suggest that MEF2 may undergo a calcium-dependent modification that enhances the ability of MEF2 to activate transcription.

Phosphorylation of MEF2C at Ser^{387} within its transactivation domain stimulates MEF2C-dependent transcription in macrophages (*22*). To determine whether calcium influx stimulates MEF2-dependent transcription by inducing the phosphorylation of MEF2C at Ser^{387}, we tested whether mutation of Ser^{387} to Ala affected the ability of GAL4MEF2C to activate luciferase reporter gene transcription. A version of GAL4MEF2 C in which Ser^{387} was mutated to Ala was unresponsive to membrane depolarization (Fig. 3C), indicating that phosphorylation of MEF2C at Ser^{387} is critical for calcium stimulation of MEF2-dependent transcription.

We next characterized the signaling pathway(s) that triggers MEF2 phosphorylation and activation in membrane-depolarized neurons. Extracellular stimuli that trigger activation of the p38 mitogen-activated protein kinase (p38 MAP kinase) induce phosphorylation of MEF2C at Ser^{387}. The phosphorylation of MEF2C at Ser^{387} appears to be catalyzed directly by the p38 mitogen-activated protein kinase (MAP kinase) (*22*). Therefore, we tested whether calcium influx into neurons leads to activation of p38 MAP kinase and if, once activated, the p38 MAP kinase catalyzes MEF2 phosphorylation and activation. Protein immunoblot analysis revealed that calcium influx through VSCCs induced phosphorylation of the p38 MAP kinase at sites that lead to p38 MAPK activation (Fig. 4A). Inhibition of the p38 MAP kinase signaling cascade with a dominant-interfering form of the p38 MAP kinase [p38(M)] reduced MEF2-dependent transcription in membrane-depolarized neurons (Fig. 4B). Because inhibition of the p38 MAPK signaling pathway blocked MEF2 function, we asked if blocking this signaling pathway might also promote apoptosis of newly generated neurons that require MEF2 for survival. Consistent with this possibility, cerebellar neurons transfected with the dominant-interfering forms of p38 MAP kinase or the p38 MAP kinase activator MAP kinase kinase 6 (MKK6) readily underwent apoptosis, even when exposed to increased concentrations of KCl (Fig. 4C) (*14*). The effect of the dominant-interfering p38 MAP kinase on cell survival appears to be due to inhibition of MEF2 activity because apoptosis induced by the dominant-negative protein was abolished when the dominant-interfering p38 MAP kinase mutant was expressed in cerebellar neurons together with a constitutive active form of MEF2 (MEF2CVP16) (Fig. 4C).

These observations, taken together with the previous finding that the p38 MAP kinase phosphorylates MEF2C at Ser^{387} in vitro (*22*), suggest that calcium influx into cerebellar neurons triggers the activation of the MKK6–p38 MAP kinase cascade and that the p38 MAP kinase then phosphorylates and activates MEF2s. Once activated by this calcium-dependent p38 MAP kinase signaling

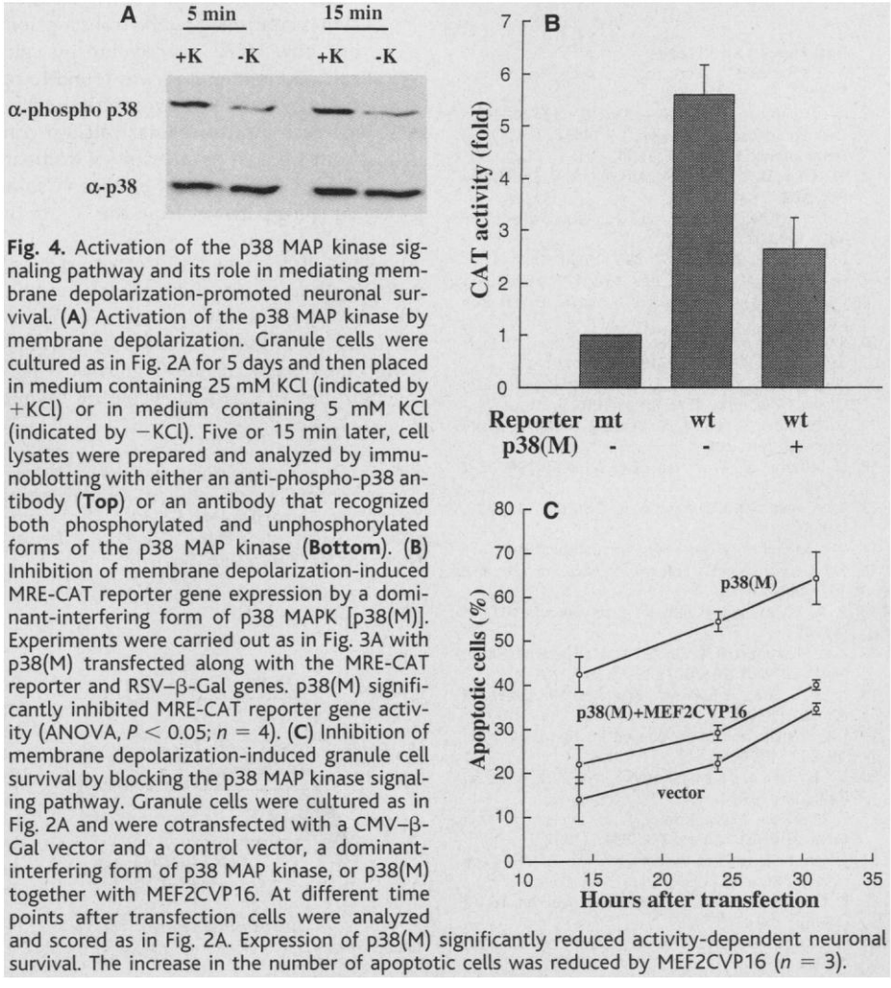


Fig. 4. Activation of the p38 MAP kinase signaling pathway and its role in mediating membrane depolarization-promoted neuronal survival. (**A**) Activation of the p38 MAP kinase by membrane depolarization. Granule cells were cultured as in Fig. 2A for 5 days and then placed in medium containing 25 mM KCl (indicated by +KCl) or in medium containing 5 mM KCl (indicated by −KCl). Five or 15 min later, cell lysates were prepared and analyzed by immunoblotting with either an anti-phospho-p38 antibody (**Top**) or an antibody that recognized both phosphorylated and unphosphorylated forms of the p38 MAP kinase (**Bottom**). (**B**) Inhibition of membrane depolarization-induced MRE-CAT reporter gene expression by a dominant-interfering form of p38 MAPK [p38(M)]. Experiments were carried out as in Fig. 3A with p38(M) transfected along with the MRE-CAT reporter and RSV–β-Gal genes. p38(M) significantly inhibited MRE-CAT reporter gene activity (ANOVA, $P < 0.05$; $n = 4$). (**C**) Inhibition of membrane depolarization-induced granule cell survival by blocking the p38 MAP kinase signaling pathway. Granule cells were cultured as in Fig. 2A and were cotransfected with a CMV–β-Gal vector and a control vector, a dominant-interfering form of p38 MAP kinase, or p38(M) together with MEF2CVP16. At different time points after transfection cells were analyzed and scored as in Fig. 2A. Expression of p38(M) significantly reduced activity-dependent neuronal survival. The increase in the number of apoptotic cells was reduced by MEF2CVP16 ($n = 3$).

pathway, MEF2 may regulate the expression of genes that are critical for survival of newly differentiated neurons. Consistent with this possibility is our finding that mutations of MEF2 that cause a loss of MEF2-dependent transcription also lead to a loss of calcium-dependent neuronal survival.

These findings extend the scope of the cellular responses that are controlled by MEF2 proteins. Although neuronal survival has been thought to occur largely through direct posttranslational modifications of components of the cell death machinery, our results suggest that transcription-dependent events regulated by MEF2 proteins also have a critical role in mediating the survival of neurons. The observation that MEF2 activity is regulated by calcium raises the possibility that MEF2s may mediate other calcium-dependent signaling events in addition to calcium-dependent neuronal survival. It is possible that, like the cyclic adenosine monophosphate response element binding protein (CREB), a well-characterized mediator of calcium-dependent transcriptional responses (*23–28*), MEF2s may regulate calcium-dependent changes in transcription that affect synaptic function and thereby mediate adaptive neuronal responses.

References and Notes

1. A. E. Chambers, S. Kotecha, N. Towers, T. J. Mohun, *EMBO J.* **11**, 4981 (1992).
2. A. E. Chambers *et al.*, *Genes Dev.* **8**, 1324 (1994).
3. D. G. Edmondson, G. E. Lyons, J. F. Martin, E. N. Olson, *Development* **120**, 1251 (1994).
4. M. H. Lin, B. A. Bour, S. M. Abmayr, R. V. Storti, *Dev. Biol.* **182**, 240 (1997).
5. Q. Lin, J. Schwarz, C. Bucana, E. N. Olson, *Science* **276**, 1404 (1997).
6. B. Lilly *et al.*, *Science* **267**, 688 (1995).
7. G. Ranganayakulu *et al.*, *Dev. Biol.* **171**, 169 (1995).
8. J. D. Molkentin, B. L. Black, J. F. Martin, E. N. Olson, *Cell* **83**, 1125 (1995).
9. S. Kaushal, J. W. Schneider, B. Nadal-Ginard, V. Mahdavi, *Science* **266**, 1236 (1994).
10. G. E. Lyons, B. K. Micales, J. Schwarz, J. F. Martin, E. N. Olson, *J. Neurosci.* **15**, 5727 (1995).
11. H. Ikeshima, S. Imai, K. Shimoda, J. Hata, T. Takano, *Neurosci. Lett.* **200**, 117 (1995).
12. D. Leifer *et al.*, *Proc Natl Acad Sci U.S.A.* **90**, 1546 (1993).
13. T. H. Han and R. Prywes, *Mol. Cell. Biol.* **15**, 2907 (1995).
14. Z. Mao and M. E. Greenberg, unpublished data.
15. E. E. Geisert Jr. and A. Frankfurter, *Neurosci. Lett.* **102**, 137 (1989).
16. S. A. Bayer and J. Altman, *Exp. Neurol.* **107**, 48 (1990).
17. J. D. Molkentin, B. L. Black, J. F. Martin, E. N. Olson, *Mol. Cell. Biol.* **16**, 2627 (1996).
18. X. Lin, S. Shah, R. F. Bulleit, *Brain Res. Mol. Brain Res.* **42**, 307 (1996).
19. J. L. Franklin and E. M. Johnson Jr., *Trends Neurosci.* **15**, 501 (1992).
20. J. L. Franklin and E. M. Johnson Jr., *Ann. N.Y. Acad. Sci.* **747**, 195 (1994).
21. T. M. Miller, M. G. Tansey, E. M. Johnson Jr., D. J. Creedon, *J. Biol. Chem.* **272**, 9847 (1997).
22. J. Han, Y. Jiang, Z. Li, V. V. Kravchenko, R. J. Ulevitch, *Nature* **386**, 296 (1997).
23. K. Deisseroth, H. Bito, R. W. Tsien, *Neuron* **16**, 89 (1996).
24. H. Bito, K. Deisseroth, R. W. Tsien, *Cell* **87**, 1203 (1996).
25. H. Bito, K. Deisseroth, R. W. Tsien, *Curr. Opin. Neurobiol.* **7**, 419 (1997).
26. M. Sheng, M. A. Thompson, M. E. Greenberg, *Science* **252**, 1427 (1991).
27. L. Santella and E. Carafoli, *FASEB J.* **11**, 1091 (1997).
28. M. R. Montminy *et al.*, *J. Neurosci.* **6**, 1171 (1986).
29. F. Saudou, S. Finkbeiner, D. Devys, M. E. Greenberg, *Cell* **95**, 55 (1998).
30. H. Dudek *et al.*, *Science* **275**, 661 (1997).
31. Z. Xia, M. Dickens, J. Raingeaud, R. J. Davis, M. E. Greenberg, *Science* **270**, 1326 (1995).
32. Granule neurons from P6 rat cerebellum were cultured in vitro for 5 days in full medium (serum + KCl) and transfected with the indicated constructs along with cytomegalovirus (CMV)–β-Gal. Sixteen hours after transfection, cells were washed and placed in medium containing 25 mM KCl but no serum (Fig. 2, A to E), 5 mM KCl (Fig. 2F), or serum-containing medium (Fig. 2C). After 12 hours, cells were fixed and stained with an antibody to β-Gal and Hoechst dye. For kinetics studies, cells were fixed at various time points after transfection. Cells were scored in a blinded fashion as apoptotic or healthy based on their nucleic and cell body–neurite morphology.
33. Y. T. Yu *et al.*, *Genes Dev.* **6**, 1783 (1992).

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Apoptosis of T Cells Mediated by Ca^{2+}-Induced Release of the Transcription Factor MEF2

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T cell receptor (TCR)–induced apoptosis of thymocytes is mediated by calcium-dependent expression of the steroid receptors Nur77 and Nor1. Nur77 expression is controlled by the transcription factor myocyte enhancer factor 2 (MEF2), but how MEF2 is activated by calcium signaling is still obscure. Cabin1, a calcineurin inhibitor, was found to regulate MEF2. MEF2 was normally sequestered by Cabin1 in a transcriptionally inactive state. TCR engagement led to an increase in intracellular calcium concentration and the dissociation of MEF2 from Cabin1, as a result of competitive binding of activated calmodulin to Cabin1. The interplay between Cabin1, MEF2, and calmodulin defines a distinct signaling pathway from the TCR to the Nur77 promoter during T cell apoptosis.

Apoptosis of T lymphocytes can be induced by multiple signaling pathways. Whereas the Fas and tumor necrosis factor–α pathways are involved in the elimination of activated peripheral T cells, a distinct pathway emanating from the TCR is responsible for thymic negative selection (*1, 2*). Orphan steroid receptors including Nur77 and Nor1 have been identified as crucial mediators of TCR-induced apoptosis (*3–5*). TCR-mediated Nur77 expression requires an increase in intracellular calcium concentration (*6*). Two Ca^{2+}-regulated DNA elements in the Nur77 promoter were identified as consensus binding sites for MEF2 (*6*). These observations implicated MEF2, originally discovered as a transcription factor for muscle-specific gene expression (*7–12*), as a Ca^{2+}-dependent transcription factor for Nur77 expression.

The protein phosphatase calcineurin has

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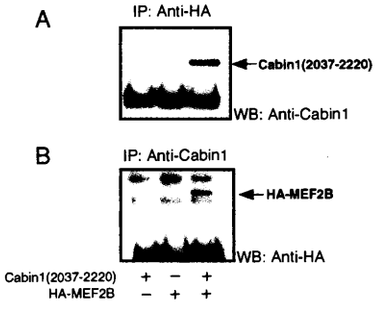

Fig. 1. The COOH-terminal fragment of Cabin1 [Cabin1(2037–2220)] interacts with MEF2B in a Ca^{2+}-sensitive manner. (**A** and **B**) Coimmunoprecipitation of Cabin1(2037–2220) and MEF2B. Jurkat T cells transfected with pSGCabin1(2037–2220) and pSGHA-MEF2B were incubated for 24 hours. The cells were lysed, followed by immunoprecipitation using either anti-Cabin1 or HA mAb. The precipitated protein was subjected to protein immunoblot with appropriate antibodies.

References and Notes

[5] **Control of Mouse Cardiac Morphogenesis and Myogenesis by Transcription Factor MEF2C**
Qing Lin; John Schwarz; Corazon Bucana; Eric N. Olson
Science, New Series, Vol. 276, No. 5317. (May 30, 1997), pp. 1404-1407.
Stable URL:
http://links.jstor.org/sici?sici=0036-8075%2819970530%293%3A276%3A5317%3C1404%3ACOMCMA%3E2.0.CO%3B2-O

[6] **Requirement of MADS Domain Transcription Factor D-MEF2 for Muscle Formation in Drosophila**
Brenda Lilly; Bin Zhao; Gogineni Ranganayakulu; Bruce M. Paterson; Robert A. Schulz; Eric N. Olson
Science, New Series, Vol. 267, No. 5198. (Feb. 3, 1995), pp. 688-693.
Stable URL:
http://links.jstor.org/sici?sici=0036-8075%2819950203%293%3A267%3A5198%3C688%3AROMDTF%3E2.0.CO%3B2-6

[9] **Activation of the Myogenic Lineage by MEF2A, a Factor That Induces and Cooperates with MyoD**
Sunjay Kaushal; Jay W. Schneider; Bernardo Nadal-Ginard; Vijak Mahdavi
Science, New Series, Vol. 266, No. 5188. (Nov. 18, 1994), pp. 1236-1240.
Stable URL:
http://links.jstor.org/sici?sici=0036-8075%2819941118%293%3A266%3A5188%3C1236%3AAOTMLB%3E2.0.CO%3B2-G

LINKED CITATIONS

[12] **MEF2C, a MADS/MEF2-Family Transcription Factor Expressed in a Laminar Distribution in Cerebral Cortex**

Dana Leifer; Dimitri Krainc; Yie-Teh Yu; John McDermott; Roger E. Breitbart; John Heng; Rachael L. Neve; Barry Kosofsky; Bernardo Nadal-Ginard; Stuart A. Lipton

Proceedings of the National Academy of Sciences of the United States of America, Vol. 90, No. 4. (Feb. 15, 1993), pp. 1546-1550.

Stable URL:

http://links.jstor.org/sici?sici=0027-8424%2819930215%2990%3A4%3C1546%3AMAMTFE%3E2.0.CO%3B2-X

[26] **CREB: A Ca ²⁺ -Regulated Transcription Factor Phosphorylated by Calmodulin-Dependent Kinases**

Morgan Sheng; Margaret A. Thompson; Michael E. Greenberg

Science, New Series, Vol. 252, No. 5011. (Jun. 7, 1991), pp. 1427-1430.

Stable URL:

http://links.jstor.org/sici?sici=0036-8075%2819910607%293%3A252%3A5011%3C1427%3ACACTFP%3E2.0.CO%3B2-M

[30] **Regulation of Neuronal Survival by the Serine-Threonine Protein Kinase Akt**

Henryk Dudek; Sandeep Robert Datta; Thomas F. Franke; Morris J. Birnbaum; Ryoji Yao; Geoffrey M. Cooper; Rosalind A. Segal; David R. Kaplan; Michael E. Greenberg

Science, New Series, Vol. 275, No. 5300. (Jan. 31, 1997), pp. 661-665.

Stable URL:

http://links.jstor.org/sici?sici=0036-8075%2819970131%293%3A275%3A5300%3C661%3ARONSBT%3E2.0.CO%3B2-O

[31] **Opposing Effects of ERK and JNK-p38 MAP Kinases on Apoptosis**

Zhengui Xia; Martin Dickens; Joël Raingeaud; Roger J. Davis; Michael E. Greenberg

Science, New Series, Vol. 270, No. 5240. (Nov. 24, 1995), pp. 1326-1331.

Stable URL:

http://links.jstor.org/sici?sici=0036-8075%2819951124%293%3A270%3A5240%3C1326%3AOEOEAJ%3E2.0.CO%3B2-4