40. In the alternative arrangement, p53 finger 2 spans a 4-bp subsite (3′-ACAG-5′) and finger 3 recognizes the adjacent 3′-GGT-5′ subsite. A similar spacing occurs at one point in the GLI-DNA complex (*12*).
41. We thank E. Rebar for support, encouragement, reagents, and advice that made this project possible; M. Elrod-Erickson for sharing refined coordinates of the Zif268-DNA complex and for advice on purification of zinc finger peptides; L. Nekludova for extensive discussions about modeling studies with these new zinc finger proteins and for help with computer graphics; W. El-Deiry for providing sequences before publication; and J. Pomerantz, S. Wolfe, E. Fraenkel, M. Elrod-Erickson, and E. Rebar for insightful comments on the manuscript. H.A.G. was supported by a predoctoral fellowship from the Howard Hughes Medical Institute and by a Massachusetts Institute of Technology Department of Biology fellowship from the Centocor Corporation; C.O.P. was supported by the Howard Hughes Medical Institute.

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Regulation of Neuronal Survival by the Serine-Threonine Protein Kinase Akt

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A signaling pathway was delineated by which insulin-like growth factor 1 (IGF-1) promotes the survival of cerebellar neurons. IGF-1 activation of phosphoinositide 3-kinase (PI3-K) triggered the activation of two protein kinases, the serine-threonine kinase Akt and the p70 ribosomal protein S6 kinase ($p70^{S6K}$). Experiments with pharmacological inhibitors, as well as expression of wild-type and dominant-inhibitory forms of Akt, demonstrated that Akt but not $p70^{S6K}$ mediates PI3-K–dependent survival. These findings suggest that in the developing nervous system, Akt is a critical mediator of growth factor–induced neuronal survival.

The intracellular signaling pathways by which growth factors promote survival—in particular, survival of neurons of the central nervous system—are not well characterized. The survival of certain subsets of neurons of the peripheral nervous system can be promoted by the activation of a pathway that includes the guanosine triphosphate (GTP)–binding protein Ras and a series of protein kinases leading to mitogen-activated protein kinase (MAPK) (*1, 2*). In addition, a pathway that includes the lipid kinase PI3-K is important for the survival of several cell lines (*3, 4*), although the mechanisms by which PI3-K promotes survival are unclear. We investigated the contribution of two targets of PI3-K, the serine-threonine kinase Akt (*5–7*) and $p70^{S6K}$ (*8*), to the IGF-1–mediated survival of cerebellar neurons. We found that Akt has a critical role in promoting IGF-1–dependent survival.

For these studies, we used a well-characterized culture system of cerebellar neurons (*9, 10*). Large numbers of neurons of relatively homogeneous composition (consisting primarily of granule neurons) can be obtained, thus allowing biochemical analyses (*9*). Withdrawal of survival factors leads to the rapid and synchronous apoptosis of cerebellar neurons (*9*). About 50% of the cells were apoptotic within 1 day (Fig. 1), and almost all of the cells died within 3 to 4 days. The dying neurons showed characteristic features of apoptosis, including nuclear condensation and cleavage of chromatin into oligonucleosomal fragments (Fig. 1E) (*9, 11*). The apoptosis could be inhibited by defined trophic factors (*9*), including IGF-1 (Fig. 1C); insulin at superphysiological concentrations (Fig. 1D), which is believed to act through the IGF-1 receptor (*10*); and membrane-depolarizing concentrations of KCl, which lead to increased concentrations of intracellular calcium and may therefore simulate neuronal activity (*9*). The effects of IGF-1 and high concentrations of insulin on cerebellar neuron survival may reflect an in vivo function of IGF-1, because both IGF-1 and its receptor are expressed in the cerebellum, and transgenic mice overexpressing IGF-1 show increases in cell number in the brain (*12*).

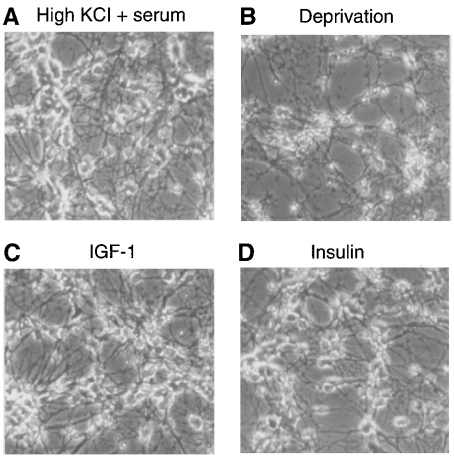

Fig. 1. Induction of apoptosis or survival of cerebellar neurons (*10*). (**A**) Phase-contrast photomicrograph of cerebellar neurons in complete medium [containing 25 mM KCl (high KCl) and 10% calf serum]. Cells were healthy, as indicated by large size and phase-brightness. (**B**) After 22 hours of deprivation (5 mM KCl, no serum), many neurons died. Death of cells was also confirmed by staining with trypan blue (*11*). (**C** and **D**) Death was inhibited by IGF-1 (25 ng/ml) (C) or insulin (10 μg/ml) (D). (**E**) Deprivation of cerebellar neurons induces chromatin cleavage. Starting at 4 to 8 hours after deprivation of trophic factors, extensive DNA laddering was present (*2*). U, untreated; positions of molecular size markers (in kilobases) are indicated on the left.

We first identified the signal transduction pathways that are activated in cerebellar neurons by IGF-1 or insulin. We examined activation of the Ras-MAPK pathway, because this pathway has been implicated in the control of nerve growth factor (NGF)–induced survival (*1, 2*). Insulin and IGF-1 failed to activate MAPK, although brain-derived neurotrophic factor (BDNF) efficiently activated MAPK (Fig. 2, A and B) (*13*). These results suggest that activation of the Ras-MAPK pathway is not critical for IGF-1–promoted or insulin-promoted survival of cerebellar neurons, and they also corroborate reports that insulin fails to activate MAPK in certain cell types, including chick forebrain neurons (*14*).

Activation of PI3-K is required for growth factor–induced survival of the PC12 neuronal cell line (*3*). By several criteria, we established that IGF-1 and insulin efficiently activate PI3-K in cerebellar neurons. First, IGF-1 and insulin induced binding of PI3-K to the receptor-associated protein IRS-1 (insulin receptor substrate 1) (Fig. 2C) (*13*), an interaction that mediates activation of PI3-K (*15*). Second, IGF-1 and insulin caused increased lipid

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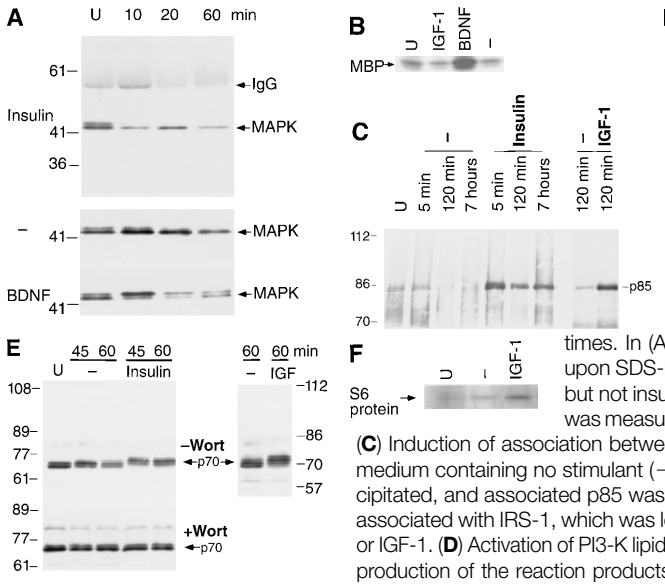


Fig. 2. Activation of signaling pathways in cerebellar neurons. (**A** and **B**) Lack of activation of MAPK by insulin (*13*). Cells were left untreated in complete medium containing 25 mM KCl and calf serum (10%) (U), or were placed in medium containing insulin, IGF-1, no stimulant (–), or BDNF for the indicated times. In (A), activation of MAPK is accompanied by phosphorylation that reduces its mobility upon SDS-PAGE (*38*). Untreated cells (U) contained a basal amount of MAPK activation; BDNF, but not insulin, caused increased activation. In (B), similar results were seen when MAPK activity was measured with an immune-complex kinase assay with MBP as substrate (10 min treatment). (**C**) Induction of association between IRS-1 and p85 by insulin and IGF-1. Cells were left untreated or were placed in medium containing no stimulant (–), insulin, or IGF-1. At the indicated times, cells were lysed, IRS-1 was immunoprecipitated, and associated p85 was detected by immunoblotting (*13*). Untreated cells contained a basal amount of p85 associated with IRS-1, which was lost when cells were starved (–) but increased when cells were stimulated with insulin or IGF-1. (**D**) Activation of PI3-K lipid kinase activity (*16*). IGF-1 [and insulin (*11*)] rapidly activated PI3-K, leading to increased production of the reaction products phosphatidylinositol-3-phosphate (PIP) and phosphatidylinositol-3,4,5-trisphosphate (PIP_3). (**E** and **F**) Induction of $p70^{S6K}$ phosphorylation and activation (*13*). Cells were left unstimulated, or were placed in media containing insulin, no stimulant (–), or IGF-1, in the absence or presence of wortmannin (Wort, 100 nM). At the indicated times, cells were lysed, and lysates were used in (E) for immunoblotting with antibody C2 to $p70^{S6K}$ (*31*) or in (F) for immune-complex kinase assays with ribosomal protein S6 as substrate. Activation of $p70^{S6K}$ is accompanied by $p70^{S6K}$ phosphorylation, which reduces its mobility upon SDS-PAGE (*8*). Insulin (and IGF-1) induced phosphorylation of $p70^{S6K}$, which was blocked by wortmannin, and also activated $p70^{S6K}$ activity. For (A), (C), and (E), the migration positions of prestained molecular size markers (in kilodaltons) are indicated.

kinase activity of PI3-K (Fig. 2D) (*16*). Third, IGF-1 and insulin activated $p70^{S6K}$ (Fig. 2, E and F) (*13*). As seen in many cell lines (*8*), phosphorylation of $p70^{S6K}$ was blocked by the PI3-K inhibitor wortmannin (Fig. 2E) (*11*), suggesting that IGF-1–induced or insulin-induced $p70^{S6K}$ activation in cerebellar neurons is dependent on PI3-K.

Consistent with a role for PI3-K in cell survival, we found that the PI3-K inhibitor LY294002 (*3, 4*) inhibited insulin-dependent survival of cerebellar neurons (Fig. 3) (*17*). LY294002 had little effect on cells grown in 25 mM KCl plus serum (Fig. 3) [or 25 mM KCl plus insulin (*17*)]. This suggests that the inhibition of insulin-dependent survival by LY294002 did not represent nonspecific toxicity, and also suggests that additional survival pathways may be activated by stimuli such as KCl.

We considered the possibility that the effects of PI3-K on cell survival might be mediated by the protein kinase Akt (also known as PKB-α or RAC-α) (*18, 19*), which is activated by a number of growth factors, including insulin, through a PI3-K–dependent mechanism (*5–7, 20*). Akt is a widely expressed cytoplasmic serine-threonine kinase, and its aberrant expression has been implicated in tumorigenesis (*18, 19, 21*). Akt contains at its NH_2-terminus a domain termed the pleckstrin homology (PH) domain, which may regulate the activation of Akt by binding D3-phosphorylated phosphoinositides that are the products of PI3-K (*6, 22*). Phosphorylation of Akt also influences its activation (*5, 20, 23*), and the PH domain may influence the activation of Akt by promoting its dimerization (*24*). The functions of Akt are mostly unknown, with the exception of the identification of glycogen synthase kinase–3 (GSK-3) as a substrate; phosphorylation of GSK-3 by Akt is believed to regulate glycogen synthesis (*20*). To determine whether Akt might mediate survival, we stimulated cerebellar neurons with IGF-1 or insulin and assayed the activity of Akt (*25*). Both insulin and IGF-1 activated Akt (Fig. 4A). Activation of Akt was blocked by wortmannin (Fig. 4A) and by LY294002 (*11*), which suggested that this activation was dependent on PI3-K. KCl was found not to activate Akt or $p70^{S6K}$ (*11*), consistent with the possibility that KCl activates other survival pathways.

To determine the importance of Akt for insulin-dependent survival, we transfected expression vectors encoding wild-type Akt (HA-Akt) or two mutant forms of Akt, a catalytically inactive mutant [HA(K179M)] and a mutant encoding the PH domain (HA-PH) (*26*), into cerebellar neurons. Transfected neurons were identified by cotransfecting an expression vector for β-galactosidase (β-Gal) and immunostaining cells for β-Gal expression (Fig. 4B). To assess the effects of Akt, we scored transfected cells in a blinded manner as healthy or apoptotic by nuclear morphology. Apoptotic cerebellar neurons showed pronounced nuclear condensation, which was visualized with the DNA dye bisbenzimide (Hoechst 33258) (*2*); these nuclei also stained positively for DNA degradation in the TUNEL assay (*11, 27*).

We first tested whether expression of the mutant forms of Akt would interfere with survival; both mutants have been found to have dominant-inhibitory activity toward wild-type Akt kinase activity (*28*). Neurons transfected with HA-Akt had a normal, noncondensed nuclear morphology (Fig. 5A, in insulin). In contrast, cells transfected with either HA-Akt(K179M) or HA-PH showed increased apoptosis, as evidenced by nuclear

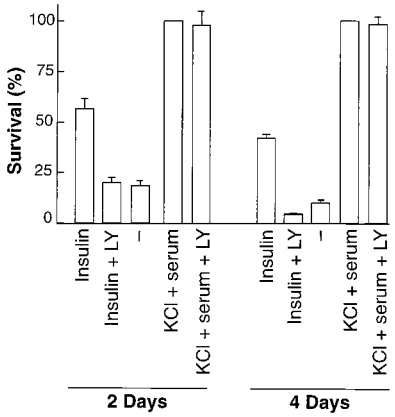
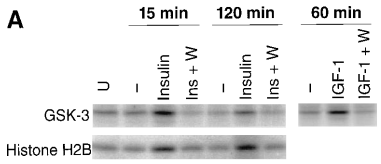


Fig. 3. Inhibition of insulin-promoted survival by the PI3-K inhibitor LY294002. Cells were placed in medium containing insulin (10 μg/ml), 25 mM KCl plus serum (10%), or no survival factor (–), in the absence or presence of LY294002 (LY, 10 μM) (*17*). After 2 or 4 days, cells were fixed, and the number of healthy cells was determined by staining with the DNA dye propidium iodide and counting nonapoptotic nuclei. Survival in the presence of both serum and 25 mM KCl (complete media) is defined as 100%. LY294002 inhibited the promotion of survival by insulin. Data are from three experiments; error bars indicate SEM.



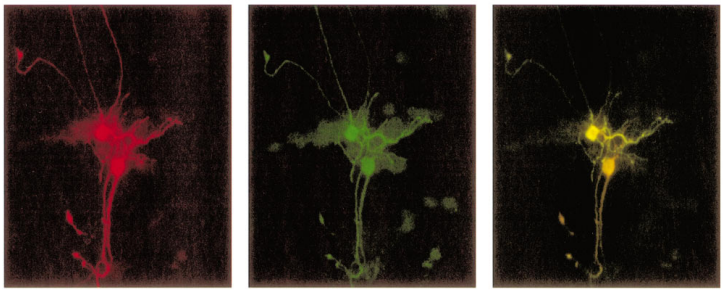


Fig. 4. Activity and expression of Akt proteins. (**A**) PI3-K–dependent activation of Akt in cerebellar neurons (*25*). Cells were left untreated (U) or were treated for the indicated times with no stimulant (–), insulin, or IGF-1, in the absence or presence of wortmannin (W). Cell lysates were immunoprecipitated with an antibody to Akt, and immune-complex kinase assays were performed with either histone H2B or recombinant GSK-3 as substrate. With H2B as substrate, activation of Akt in cells treated with insulin for 15 min ($n = 3$) was 3.3 ± 0.44 times that in unstimulated cells. (**B**) Expression of transfected Akt in cerebellar neurons (*26*). Neurons were cotransfected with HA-Akt and CMV–β-Gal, and 2 days later they were fixed and immunostained with an antibody to β-Gal (Texas Red–coupled secondary antibody, red) and 12CA5 antibody to HA (Cy2-coupled secondary antibody, green). Transfected cells efficiently expressed both β-Gal (left) and HA-tagged Akt (center); a double exposure is shown on the right.

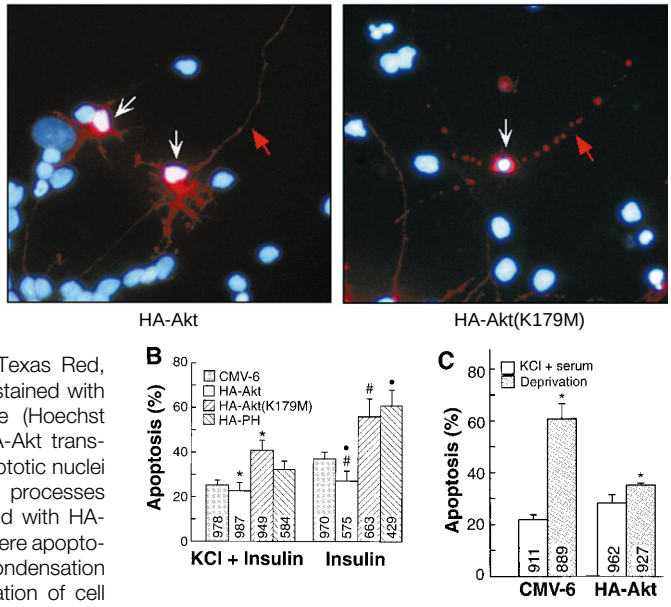


Fig. 5. Effects of expression of mutant or wild-type Akt on cerebellar neuron apoptosis. (**A**) Cells were transfected with the indicated expression vectors (along with CMV–β-Gal), and 1 day later they were placed in medium containing insulin (10 μg/ml) as the survival factor. After 16 hours, cells were fixed, immunostained, and Hoechst-stained. Transfected cells are red (Texas Red, anti–β-Gal), and nuclei are stained with the DNA dye bisbenzimide (Hoechst 33258, blue and white). HA-Akt transfectants have large, nonapoptotic nuclei (white arrows) and normal processes (red arrow). Cells transfected with HA-Akt(K179M) or HA-PH (*11*) were apoptotic, as indicated by nuclear condensation (white arrow) and disintegration of cell body and processes (red arrow). (**B**) Quantitation of induction of apoptosis by Akt mutants. Cells were transfected with the indicated expression vectors, and 1 day later they were placed in medium containing insulin alone or insulin plus 25 mM KCl. After 16 hours, cells were fixed and stained, and transfectants were scored as healthy or apoptotic. Data are from four experiments; symbols above the bars indicate significant differences between conditions with identical symbols (ANOVA with Bonferroni correction for four plasmid conditions; *, $P = 0.0034$; #, $P = 0.0047$; •, $P = 0.0017$). (**C**) Promotion of survival by Akt. Cells were transfected with either CMV-6 (control vector) or HA-Akt, and 1 day later they were placed in medium containing 25 mM KCl plus serum (10%) or in deprivation media (5 mM KCl, no serum). After 22 hours, cells were processed as in (B). Data are from three experiments; asterisks indicate significant difference (ANOVA, $P = 0.0013$). For (B) and (C), the total number of transfectants scored is shown within each bar; error bars indicate SEM. In (B) and (C), the percent apoptosis among control (CMV-6) transfectants in KCl plus insulin or KCl plus serum reflected a combination of the basal level of cell death in culture (typically 10% or less in KCl plus insulin or KCl plus serum) and transfection toxicity.

condensation and disintegration of processes and the cell body (Fig. 5, A and B). The extent of apoptosis in cells transfected with HA-Akt(K179M) or HA-PH and then grown with insulin as the sole survival factor (~60%) was as large as that in vector control transfectants in the presence of no survival factor (Fig. 5C) (*11*). Cells transfected with HA-Akt(K179M) or HA-PH showed less apoptosis when grown in the presence of both KCl and insulin [or KCl and serum (*29*)], consistent with the possibility that KCl, serum, or combinations of these factors may activate survival pathways in addition to the Akt pathway (*29*). Taken together, these results suggest that the promotion of survival by insulin requires Akt.

We next tested whether exogenously expressed Akt is sufficient to enhance survival. Cerebellar neurons were transfected with HA-Akt or with control vector and were deprived of survival factors after 1 day. Expression of HA-Akt markedly reduced the amount of apoptosis (Fig. 5C); control transfectants showed ~60% apoptosis after 1 day of deprivation, whereas HA-Akt transfectants showed only 35% apoptosis [$P = 0.0013$ by analysis of variance (ANOVA)] (*29*). The ability of HA-Akt to block apoptosis was not reduced in the presence of LY294002 (*11*), consistent with Akt acting downstream of PI3-K.

Because both PI3-K and Akt can promote activation of $p70^{S6K}$ (*5*, *23*), $p70^{S6K}$ is a potential mediator of the survival effects of IGF-1. Originally identified as a ribosomal protein S6 kinase (*30*), $p70^{S6K}$ has since been shown to regulate progression from the G_1 to the S phase of the cell cycle (*31*). We inhibited the activation of $p70^{S6K}$ with rapamycin, which blocks phosphorylation of $p70^{S6K}$ (*31*). Rapamycin had no effect on the promotion of survival by insulin or by serum plus KCl, at a range of concentrations that blocked the activation of $p70^{S6K}$ (*11*). This is consistent with the reported lack of requirement for $p70^{S6K}$ activity for PI3-K–mediated survival of PC12 cells (*4*).

Taken together, our findings reveal that a critical function of Akt is to mediate the effects of IGF-1 on neuronal survival. Akt may promote the survival of a range of cell types in response to various growth factors, particularly those that activate PI3-K. The observation that Akt promotes survival may partially explain the oncogenic potential of Akt (*18*, *21*). The promotion of survival by Akt may also be relevant to situations of pathological neuronal cell death, such as hypoxic-ischemic injury, for which IGF-1 can be protective (*32*). Because Akt is believed to be activated at least in part by lipid products of PI3-K (*6*), Akt may prove a propitious target for small-molecule therapeutics that promote cell survival.

REFERENCES AND NOTES

1. G. D. Borasio *et al.*, *Neuron* **2**, 1087 (1989); C. D. Nobes, J. B. Reppas, A. Markus, A. M. Tolkovsky, *Neuroscience* **70**, 1067 (1996).

2. Z. Xia, M. Dickens, J. Raingeaud, R. J. Davis, M. E. Greenberg, *Science* **270**, 1326 (1995).
3. R. Yao and G. M. Cooper, *ibid.* **267**, 2003 (1995).
4. _____, *Oncogene* **13**, 343 (1996).
5. B. M. Th. Burgering and P. J. Coffer, *Nature* **376**, 599 (1995).
6. T. F. Franke *et al., Cell* **81**, 727 (1995).
7. A. D. Kohn, K. S. Kovacina, R. A. Roth, *EMBO J.* **14**, 4288 (1995).
8. J. Chung, T. Grammar, K. Lemmon, A. Kazlauskas, J. Blenis, *Nature* **370**, 71 (1994).
9. S. R. D'Mello, C. Galli, T. Ciotti, P. Calissano, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10989 (1993); C. Galli *et al., J. Neurosci.* **15**, 1172 (1995); J. B. Schulz, M. Weller, T. Klockgether, *ibid.* **16**, 4696 (1996).
10. Cerebellar neurons were cultured from Long-Evans rats at postnatal day 6 to 8 (*9*) on polyornithine-coated plates (Falcon) or polyornithine plus laminin–coated glass cover slips (Bellco). Cells were grown in basal medium Eagle (BME, Sigma) with calf serum (10%, Hyclone), 25 mM KCl, 2 mM glutamine, and penicillin-streptomycin (complete media), which promotes optimal survival. On day 1 after culturing (1 DIV), the antimitotic cytosine-β-D-arabinofuranoside (10 μM) was added to prevent proliferation of non-neuronal cells. Immunostaining with the granule cell–specific antibody Q600 (*33*) indicated that these cultures were composed predominantly of granule neurons (*11*). Cells were treated at 6 to 7 DIV with the same treatment protocol for survival or biochemical assays, as follows: Cells were washed twice in medium containing no serum and 5 mM KCl (deprivation medium) and were then placed in the same medium in the absence or presence of stimulants. Media containing growth factor also included bovine serum albumin (BSA, 0.1 mg/ml) as carrier protein, which itself caused no detectable signaling responses. The following concentrations were used: insulin, 10 μg/ml; IGF-1, 50 ng/ml (except 25 ng/ml in Fig. 1); BDNF, 100 ng/ml; wortmannin, 100 nM; rapamycin, 100 pM to 20 nM; and LY294002, 10 μM. When inhibitors were used for signaling assays, cells were treated with the inhibitors for 45 min before, and for the duration of, the stimulation. In survival assays, cells that did not receive inhibitors received control vehicle (dimethyl sulfoxide for LY294002, ethanol for rapamycin). The effect of insulin on cerebellar neuron survival in this system most likely reflects cross-reactivity of insulin with the IGF-1 receptor (*34*), because lower insulin concentrations (for example, 100 ng/ml) promoted survival poorly (*11*).
11. H. Dudek, S. R. Datta, M. E. Greenberg, unpublished data.
12. F. de Pablo and E. de la Rosa, *Trends Neurosci.* **18**, 143 (1995); I. Torres-Aleman, S. Pons, M. A. Arevalo, *J. Neurosci. Res.* **39**, 117 (1994); M. J. Carson, R. R. Behringer, R. L. Brinster, F. A. McMorris, *Neuron* **10**, 729 (1993).
13. After treatment for the indicated times, cells were harvested in lysis buffer [20 mM tris (pH 7.4), 140 mM NaCl, 1% NP-40, 10 mM NaF, 1 mM Na_3VaO_4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), and 5 mM benzamidine], and MAPK was immunoprecipitated with polyclonal antibody TR2 (*35*). Immunoprecipitated proteins were resolved by electrophoresis and immunoblotted with monoclonal antibody (mAb) B3B9 to MAPK. The lack of activation of MAPK by IGF-1 or insulin was seen with multiple stimulation protocols and at a range of stimulation times, and also when analyzed by immunoblotting with mAb 4G10 to phosphotyrosine (Upstate Biotechnology) (*11*). For MAPK immune-complex kinase assays, MAPK was immunoprecipitated from cell lysates with TR2, and immunoprecipitates were washed with lysis buffer and phosphate-buffered saline and then incubated in a 40-μl reaction mixture containing 25 mM Hepes (pH 7.2), 10 mM Mg acetate, 1 mM dithiothreitol (DTT), 50 μM adenosine triphosphate (ATP), [γ-^{32}P]ATP (NEN; 10 μCi per assay), and myelin basic protein (MBP) (12.5 μg per reaction) as substrate at 30°C for 30 min. Reactions were then electrophoresed and incorporation of ^{32}P into MBP was assessed by PhosphorImager analysis. IRS-1 was immunoprecipitated with an antibody to the COOH-terminal region of IRS-1 (Upstate Biotechnology) or polyclonal antibody JD63, and p85 was detected with polyclonal antibody 137 (both provided by S. Pons and M. White). For p70^{S6K} immune-complex kinase assays, immunoprecipitates were washed once in lysis buffer, once in Buffer A [10 mM tris (pH 7.4), 1% NP-40, 0.5% Na deoxycholate, 100 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, and 2 mM DTT], once in buffer B (1 M NaCl, 0.1% NP-40, 10 mM tris, 1 mM Na_3VO_4, and 2 mM DTT), and once in ST [10 mM tris (pH 7.4) and 100 mM NaCl]. Reactions were done in 40-μl volumes in kinase buffer [10 mM Hepes (pH 7.2), 10 mM $MgCl_2$, and BSA (100 μg/ml)] supplemented with 10 μCi of [γ-^{32}P]ATP (NEN) and 0.5 A_{260} unit of 40*S* ribosomal subunits. Reactions were incubated at 30°C for 30 min, stopped with sample buffer, and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE).
14. K. A. Heidenreich and J. L. Kummer, *J. Biol. Chem.* **271**, 9891 (1996); X.-F. Ming *et al., Nature* **371**, 426 (1994).
15. M. F. White and C. R. Kahn, *J. Biol. Chem.* **269**, 1 (1994).
16. After treatment for the indicated times, cells were lysed and proteins were immunoprecipitated with phosphotyrosine mAb 4G10. Immunoprecipitates were washed twice in lysis buffer, twice in buffer containing 0.5 M LiCl and 25 mM Hepes (pH 7.2), and twice in TNE [10 mM tris (pH 7.4), 100 mM NaCl, and 1 mM EDTA]. Sonicated mixtures of phosphatidylserine (carrier lipid) and the substrates phosphatidylinositol and phosphatidylinositol-4,5-bisphosphate in a ratio of 1:2:2 (at a final concentration of 80 μg of lipid per reaction) were added to the immunoprecipitates, and the kinase reaction was started by inclusion of a reaction mixture of 20 mM Hepes (pH 7.2), 5 mM $MgCl_2$, 20 μM ATP, and 15 μCi of [γ-^{32}P]ATP. The reaction was stopped with 1 N HCl, and the lipids were extracted with a 1:1 mixture of MeOH:$CHCl_3$, spotted onto an oxalate-coated thin-layer chromatography (TLC) plate next to lipid standards, and run in 65:35 *n*-propanol:2 M acetic acid. Incorporation of ^{32}P into lipid substrates was quantitated by PhosphorImager.
17. In each of the experiments of Fig. 3, at least two cover slips per condition and at least four fields per cover slip were scored; each experiment was from a separate cerebellar culture. Relative to survival in 25 mM KCl plus serum (defined as 100%), survival in 25 mM KCl plus insulin was 102% and 103% in the absence or presence of LY294002, respectively, at 2 days, and 99% and 98% at 4 days (means of three experiments).
18. A. Bellacosa, J. R. Testa, S. P. Staal, P. N. Tsichlis, *Science* **254**, 274 (1991).
19. P. J. Coffer and J. R. Woodgett, *Eur. J. Biochem.* **201**, 475 (1991); P. F. Jones, T. Jakubowicz, F. J. Pitossi, F. Maurer, B. A. Hemmings, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4171 (1991).
20. D. A. E. Cross, D. R. Alessi, P. Cohen, M. Andjelkovic, B. A. Hemmings, *Nature* **378**, 785 (1995).
21. N. N. Ahmed *et al., Oncogene* **8**, 1957 (1993).
22. M. A. Lemmon, K. M. Ferguson, J. Schlessinger, *Cell* **85**, 621 (1996); C. L. Carpenter and L. C. Cantley, *Curr. Opin. Cell Biol.* **8**, 153 (1996); T. F. Franke, D. R. Kaplan, L. C. Cantley, A. Toker, *Science* **275**, 665 (1997).
23. A. D. Kohn, F. Takeuchi, R. A. Roth, *J. Biol. Chem.* **271**, 21920 (1996); M. Andjelkovic *et al., Proc. Natl. Acad. Sci. U.S.A.* **93**, 5699 (1996).
24. K. Datta *et al., Mol. Cell. Biol.* **15**, 2304 (1995).
25. Polyclonal antibody 31 to Akt was raised in rabbit with the Akt peptide Cys-His-Phe-Pro-Gln-Phe-Ser-Tyr-Ser-Ala-Ser-Gly-Thr-Ala coupled to keyhole limpet hemocyanin as an immunogen. This antibody detected a single prominent Akt band in 3T3 cells upon immunoblotting that was increased in intensity in cells stably overexpressing Akt. This antibody also immunoprecipitated platelet-derived growth factor–inducible wortmannin-sensitive H2B kinase activity from NIH 3T3 cells (*11*). For Akt immune-complex kinase assays, immunoprecipitates were washed twice with lysis buffer, twice with buffer containing 25 mM Hepes (pH 7.2), 1 M NaCl, 0.1% BSA, 10% glycerol, and 1% Triton X-100, and twice with kinase buffer containing 20 mM Hepes (pH 7.2), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 5 μM ATP, and 0.2 mM EGTA. Reactions were done in kinase buffer supplemented with 2 μg of PKI (Sigma), 10 μCi of [γ-^{32}P]ATP (NEN), and histone H2B (Boehringer Mannheim, 500 ng) or recombinant GSK-3 (New England Biolabs, 200 ng) per 40-μl assay reaction.
26. The cytomegalovirus (CMV) promoter–based expression vectors CMV-6, HA-Akt, and HA-Akt(K179M) have been described (*6*). HA-PH contains the coding region of HA-AH1 (*24*), including amino acids 1 through 147 of Akt, in CMV-6. The CMV–β-Gal expression vector was pON260 (*36*). All forms of Akt were tagged with a hemagglutinin (HA) epitope. Transfection of HA-Akt, but not HA(K179M), into COS cells (by DEAE-dextran) resulted in increased Akt activity relative to that in cells transfected with vector (CMV-6) (*11*). Cerebellar neurons were transfected in 24-well plates (Costar) by the calcium phosphate method on 4 or 5 DIV [or 4 or 6 DIV, in (*29*)] largely as described (*36*); cells were transfected in Dulbecco's modified Eagle's medium (DMEM) and then returned to complete medium. For some of the experiments of Fig. 5B, the glutamate receptor inhibitors kynurenate and D(-)-2-amino-5-phosphonovaleric acid (D-APV) were included in the transfection as described (*36*); however, these proved to be unnecessary and were subsequently omitted. Cells were stained as described (*2*); Cy-2 coupled antibody was from Biological Detection Systems. Scoring of transfected cells was done blinded with respect to the identity of the transfected plasmids. Transfectants were identified by immunostaining for the cotransfected β-Gal marker. This allowed comparison between empty vector and Akt transfectants. In addition, because β-Gal staining was relatively stable during apoptosis, staining for β-Gal avoids scoring bias toward healthy cells. For all neuronal transfections, a 2:1 DNA ratio of Akt expression vector to β-Gal expression vector was used, and thus all healthy cells expressing β-Gal also expressed transfected Akt (measured by anti-HA immunostaining); all Akt constructs showed similar intensity of staining.
27. Y. Gavrieli, Y. Sherman, S. A. Ben-Sasson, *J. Cell Biol.* **119**, 493 (1992).
28. T. Franke, Z. Songyang, L. Cantley, D. Kaplan, unpublished data. 3T3 fibroblasts were transfected with HA-Akt, alone or with expression vectors for the two Akt mutants. Cells were lysed, Akt was immunoprecipitated, and immune-complex kinase assays were done with histone H2B as substrate (*6*). Cotransfection of mutant Akt constructs reduced the activity of wild-type Akt.
29. Two days after transfection, the extent of apoptosis of transfected cells in medium containing 25 mM KCl plus serum was as follows (means of two experiments): CMV-6, 22%; HA-Akt, 21%; K179M, 35%; and HA-PH, 28%. The ability of the dominant negative Akt proteins to inhibit survival in KCl plus insulin or serum may indicate that they are more effective than LY294002 at blocking activation of Akt, possibly because the dominant negative Akt proteins also block PI3-K–independent activation of Akt (*37*). Transfected wild-type Akt may be effective in promoting survival because it is expressed in amounts large enough so that its basal activity is sufficient for survival. Akt is believed to be regulated by dimerization (*24*), and this may contribute to activation of transfected wild-type Akt.
30. J. Blenis, C. J. Kuo, R. L. Erikson, *J. Biol. Chem.* **262**, 14373 (1987).
31. J. Chung, C. J. Kuo, G. R. Crabtree, J. Blenis, *Cell* **69**, 1227 (1992).
32. P. Gluckman *et al., Biochem. Biophys. Res. Commun.* **182**, 593 (1992).
33. C. Gravel *et al., J. Neurosci. Methods* **21**, 145 (1987).
34. R. Schumacher *et al., J. Biol. Chem.* **268**, 1087 (1993).
35. L. B. Rosen, D. D. Ginty, M. J. Weber, M. E. Greenberg, *Neuron* **12**, 1207 (1994).
36. Z. Xia, H. Dudek, C. K. Miranti, M. E. Greenberg, *J. Neurosci.* **16**, 5425 (1996).
37. H. Konishi *et al., Proc. Natl. Acad. Sci. U.S.A.* **93**, 7639 (1996).

38. K. W. Wood, C. Sarnecki, T. M. Roberts, J. Blenis, Cell **68**, 1041 (1992).
39. We thank J. Blenis, M. Weber, S. Pons, and M. White for antibodies; M. Kozlowski and J. Avruch for 40*S* ribosomal subunits; Amgen Inc. for BDNF; K. Auger and T. Roberts for assistance with the PI3-K activity assay; S. Vasquez for assistance with cerebellar cultures; L. Lipfert for assistance generating antibodies to Akt; and members of the Greenberg lab for critical reading of the manuscript and for technical assistance. T.F.F. acknowledges L. Cantley for continuing support. Supported by American Cancer Society grant PF4059 (H.D.), NIH grants DK39519 (M.J.B.), R01 CA43855 (M.E.G.), and R01 CA18689 (G.M.C.), Mental Retardation Research Center grant NIH P30-HD18655, a K. M. Hunter fellowship in Cancer Research from the National Cancer Institute of Canada (NCIC) (T.F.F.), NCIC grant 7168 (D.R.K.), and an H. E. Johns and Canadian Cancer Society Research Scientist Award from NCIC (D.R.K.). Animal care was in accordance with institutional guidelines.

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Direct Regulation of the *Akt* Proto-Oncogene Product by Phosphatidylinositol-3,4-bisphosphate

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The regulation of the serine-threonine kinase Akt by lipid products of phosphoinositide 3-kinase (PI 3-kinase) was investigated. Akt activity was found to correlate with the amount of phosphatidylinositol-3,4-bisphosphate (PtdIns-3,4-P_2) in vivo, and synthetic PtdIns-3,4-P_2 activated Akt both in vitro and in vivo. Binding of PtdIns-3,4-P_2 occurred within the Akt pleckstrin homology (PH) domain and facilitated dimerization of Akt. Akt mutated in the PH domain was not activated by PI 3-kinase in vivo or by PtdIns-3,4-P_2 in vitro, and it was impaired in binding to PtdIns-3,4-P_2. Examination of the binding to other phosphoinositides revealed that they bound to the Akt PH domain with much lower affinity than did PtdIns-3,4-P_2 and failed to increase Akt activity. Thus, Akt is apparently regulated by the direct interaction of PtdIns-3,4-P_2 with the Akt PH domain.

Stimulation of cells by several growth factors activates PI 3-kinase (*1*). In vivo, the activation of PI 3-kinase increases the intracellular amounts of PtdIns-3,4-P_2 and phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3) (*1*). Various downstream targets of PI 3-kinase have been identified, including the serine-threonine kinase Akt (*1*–*5*). Akt (also referred to as PKBα or Racα) is encoded by the *Akt* proto-oncogene and is defined by an NH_2-terminal regulatory domain of protein-protein interaction [Akt homology (AH) domain)] that contains a PH domain (*2*). Akt participates in the activation of the p70 ribosomal protein S6 kinase ($p70^{S6K}$) (*2*) and inhibits glycogen synthase kinase-3 (*3*), and it has a role in proliferative and anti-apoptotic cell responses (*4*, *6*). Akt activation by growth factors requires PI 3-kinase activity (*2*), but there are other pathways that can also lead to Akt activation (*7*).

To examine whether PI 3-kinase is sufficient to stimulate Akt activity, we coexpressed hemagglutinin epitope–tagged Akt (HA-Akt) and activated PI 3-kinase (*8*). Activated PI 3-kinase (piSH2-p110·MT) induced the activity of HA-Akt in serum-starved COS-1 and NIH 3T3 cells (Fig. 1), and stimulation of HA-Akt by activated PI 3-kinase was blocked by the PI 3-kinase inhibitor wortmannin (*9*). Activation of HA-Akt by piSH2-p110·MT in NIH 3T3 cells was enhanced by platelet-derived growth factor (PDGF) (Fig. 1B). Mutant piSH2-p110(K227E)·MT [in which Lys (K) at position 227 is mutated to Glu (E)] that is deficient in Ras binding (*10*) stimulated the activity of HA-Akt in serum-starved cells, but it was less effective than piSH2-p110·MT at enhancing PDGF stimulation of HA-Akt (*9*). The activity of HA-Akt(R25C), which contains a point mutation of Arg (R) to Cys (C) in the Akt PH domain, was not significantly increased by PDGF treatment or coexpression of piSH2-p110·MT (Fig. 1B). The Akt PH domain was therefore important for Akt activation by PDGF and by PI 3-kinase.

We next examined the relation of Akt activity to phosphoinositide amounts in vivo. After treatment of human platelets with thrombin receptor–activating peptide (TRAP), the amounts of the PI 3-kinase products PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 increase with distinct times (*11*). We measured Akt autophosphorylation (Fig. 2A), Akt phosphorylation of histone H2B (Fig. 2B), and phosphorylation of phosphoinositides (Fig. 2B) as a function of time after the addition of TRAP (*12*). The concentration of PtdIns-3,4,5-P_3 peaked 25 s after TRAP addition, but full Akt activation did not occur until the concentration of PtdIns-3,4-P_2 had peaked

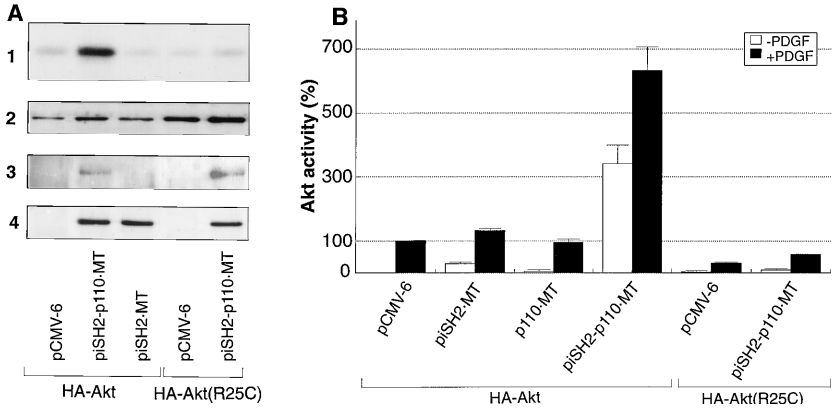

Fig. 1. Increased Akt activity in cells transfected with activated PI 3-kinase. (**A**) In vitro kinase assays of Akt immunoprecipitated from lysates of serum-starved COS-1 cells expressing the indicated constructs (*8*). Immunoprecipitations with monoclonal antibody to HA (anti-HA) (Boehringer) were followed by kinase assays with histone H2B (*3*) (panel 1). Expression of HA-Akt and HA-Akt(R25C) was determined by protein immunoblotting with anti-HA (panel 2). p110·MT (panel 3) and piSH2·MT (panel 4) were immunoprecipitated with monoclonal antibody to MT (*4*) and detected with polyclonal antibody to 9E10 (EQKLISEEDL) (*20*). (**B**) Histone H2B kinase activity in anti-HA immunoprecipitates from lysates of serum-starved or serum-starved and PDGF-stimulated [PDGF (50 ng/ml) for 5 min] NIH 3T3 cells (PDGF-stimulated HA-Akt activity equals 100% in cells transfected only with HA-Akt).

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