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 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 p70S6K (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, A and B), 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 supraphysiological 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).
kinase activity of PI3-K (Fig. 2D) (16). Third, IGF-1 and insulin activated p70S6K (Fig. 2, E and F) (13). As seen in many cell lines (8), phosphorylation of p70S6K was blocked by the PI3-K inhibitor wortmannin (Fig. 2E) (11), suggesting that IGF-1–induced or insulin-induced p70S6K 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 NH₂-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 p70S6K (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 cotransferring 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 bisbenzimid (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 control). In contrast, cells transfected with either HA-Akt(K179M) or HA-PH showed increased apoptosis, as evidenced by nuclear
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 transfecants 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 transfected 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 p70S6K (5, 23), p70S6K is a potential mediator of the survival effects of IGF-1. Originally identified as a ribosomal protein S6 kinase (30), p70S6K has since been shown to regulate progression from the G1 to the S phase of the cell cycle (31). We inhibited the activation of p70S6K with rapamycin, which blocks phosphorylation of p70S6K (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 p70S6K (11). This is consistent with the reported lack of requirement for p70S6K 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

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 coverslips (Becton). 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 promote optimal survival. On day 1 after culturing (DIV), the antimitotic cytokine β-2-arabino-nucleoside (10 μM) was added to prevent proliferation of non-neuronal cells. Immunostaining with the granule cell–specific antibody Q600 (33) indicated that these cultures contained more than 90% of the constitutively grown neurons (17). 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 in the absence of serum and then incubated in serum-containing medium (24 h) and then placed in the same medium in the absence or presence of stimulants. Media containing growth factor also included bovine serum albumin (100 μg/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 μM; 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-reaction of activity with the IGF-1 receptor (34), because lower insulin concentrations (for example, 10 ng/ml) promoted survival poorly (17).

After treatment for the indicated times, cells were lysed and proteins were immunoprecipitated with phosphospecific mAb 4G10. Immunoprecipitates were washed twice in lysis buffer, twice in buffer containing 10 μM tris (pH 7.2), and twice in TNE (10 μM tris (pH 7.4), 100 mM NaCl, and 1 mM EDTA). Sonicated mixtures of phosphatidylinerine (cancer lipid) and the substrates phosphatidylinositol 3-phosphate (P3P) or phosphatidylinositol 3,4-diphosphate (P3,4P2) were added to the immunoprecipitates, and the kinase reaction was started by addition of a reaction mixture of 20 mM Hepes (pH 7.2), 5 mM MgCl2, 20 μM ATP, and 15 μCl of [γ-32P]ATP (NEN) (10 μl). The reaction was stopped with 1 N HCl, and the lipids were extracted with a 1:1 mixture of MeOH/CH3OH solutin onto an octadeylated thin-layer chromatography (TLC) plate next to lipid standards, and run in 6:3:5 tris-propanoic acid. Incorporation of radioactivity into substrates was quantitated by PhosphorImager analysis. IRS-1 was immunoprecipitated with an antibody to the COOH-terminal region of IRS-1 (Upstate Biotechnology) or polyclonal antibody D638, and p88 was detected with polyclonal antibody 137 (both provided by S. Pons and M. Greenberg). Unless otherwise stated, as in most cases, immunoprecipitates were washed once in lysis buffer, once in buffer A (10 mM tris (pH 7.4), 1% NP-40, 10 mM NaCl, 1 mM NaOAc, 1 mM EDTA, 1 mM Na3VO4, and 2 mM DTT), once in buffer B (1 M NaCl, 0.1% NP-40, 10 mM tris, 1 mM NaCl, 1 mM NaOAc, and 2 mM DTT), and once in TNE at 30°C (7.4) and 100 mM NaCl. Reactions were done in 40-μl volumes in kinase buffer (10 μM tris (pH 7.2), 7 mM MgCl2, and BSA (100 μg/ml) supplemented with 10 μCl of [γ-32P]ATP (NEN) and 0.5 μg/ml of SH3600 (Ribbit). Immunoprecipitates were washed twice with lysis buffer, twice with buffer containing 0.5 M LiCl and 25 mM Hepes (pH 7.2), were stained with Coomassie blue, and then visualized by autoradiography after electrophoresis (PAGE).

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In each of the experiments of Fig. 3, at least two coverslip per condition and at least four fields per coverslip were scored; each experiment was from a separate cerebella culture. Relative to survival in 25 mM KCi plus serum (defined as 100%), survival in 25 mM KCi 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).


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-P2) in vivo, and synthetic PtdIns-3,4-P2 activated Akt both in vitro and in vivo. Binding of PtdIns-3,4-P2 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-P2 in vitro, and it was impaired in binding to PtdIns-3,4-P2. 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-P2, and failed to increase Akt activity. Thus, Akt is apparently regulated by the direct interaction of PtdIns-3,4-P2 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-P2 and phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P3) (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 Raca) is encoded by the AKT proto-oncogene and is defined by an NH2-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 (p70S6K) (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 (pSH2-p110MT) 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 pSH2-p110MT in NIH 3T3 cells was enhanced by platelet-derived growth factor (PDGF) (Fig. 1B). Mutant pSH2-p110K227E (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 pSH2-p110MT 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 pSH2-p110MT (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-P2 and PtdIns-3,4,5-P3 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-P3 peaked 25 s after TRAP addition, but full Akt activation did not occur until the concentration of PtdIns-3,4,5-P3 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 (9) (panel 1). Expression of HA-Akt and HA-Akt(R25C) was determined by protein immunoblotting with anti-HA (panel 2), p110MT (panel 3) and pSH2/MIT (panel 4) were immunoprecipitated with monoclonal antibody to MT (4) and detected with polyclonal antibody to 9E10 (EQQKLISEEDL) (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).