Activated Phosphatidylinositol 3-Kinase and Akt Kinase Promote Survival of Superior Cervical Neurons

Karen L. Philpott,* Mary Jane McCarthy,* Anke Klippel,‡ and Lee L. Rubin*

*Eisai London Research Laboratories Ltd., Bernard Katz Building, University College London, London WC1E 6BT, United Kingdom; and ‡Chiron Corporation, Emeryville, California 94608

Abstract. The signaling pathways that mediate the ability of NGF to support survival of dependent neurons are not yet completely clear. However previous work has shown that the c-Jun pathway is activated after NGF withdrawal, and blocking this pathway blocks neuronal cell death. In this paper we show that overexpression in sympathetic neurons of phosphatidylinositol (PI) 3-kinase or its downstream effector Akt kinase blocks cell death after NGF withdrawal, in spite of the fact that the c-Jun pathway is activated. Yet, neither the PI 3-kinase inhibitor LY294002 nor a dominant negative PI 3-kinase cause sympathetic neurons to die if they are maintained in NGF. Thus, although NGF may regulate multiple pathways involved in neuronal survival, stimulation of the PI 3-kinase pathway is sufficient to allow cells to survive in the absence of this factor.

Neurons are critically dependent upon growth factors during development, and up to 50% of neurons born die by apoptosis during this period. This process is thought to match the number of neurons to their targets and allows for plasticity during development (Oppenheim, 1991). The role of apoptosis in neuronal survival is not limited to development, however. There is now increasing evidence that it also plays a role in many neuropathological conditions such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and stroke (for review see Thompson, 1995; Choi, 1996; Nicholson, 1996). Understanding the control of neuronal cell death could therefore lead to more effective therapies for these disorders.

Growth factors are thought to prevent apoptosis by signaling to effector molecules within the cell, and considerable effort has been placed in dissecting these signaling pathways. In the case of neurons, NGF has been investigated in some depth (see Fantl et al., 1993; Segal and Greenberg, 1996). The binding of NGF to its high-affinity receptor, TrkA, leads to its dimerization and activation of an intrinsic kinase domain that autophosphorylates tyrosine residues within the cytoplasmic domain of the receptor (Jing et al., 1992). The phosphorylated residues lie within specific amino acid motifs and act as docking sites for a number of proteins generally containing an SH2 domain (Koch et al., 1991; Kaplan and Stephens, 1994). These include proteins that regulate the activity of the $p21^{ras}$/MAPK pathway, such as SHC, rasGAP, and Grb2, and proteins that modulate phosphatidylinositol metabolism, including phospholipase C-γ (PLCγ) and the p85 subunit of phosphatidylinositol (PI)[1] 3-kinase (Soltoff et al., 1992; Obermeier et al., 1993, 1994; Stephens et al., 1994; Carter and Downes, 1995).

The role of an individual pathway in neurons may be somewhat different to that observed in other cell types. For example, signaling through ras in neurons leads to differentiation and neurite outgrowth, while in fibroblasts it can lead to proliferation (for review see Wood and Roberts, 1993). Activation of these pathways has been studied in neurons in a number of ways. Trk mutants in the rat PC12 cell line have demonstrated that the SHC pathway is important for neurite outgrowth (Obermeier et al., 1994; Stephens et al., 1994). In addition, scrape loading of ras into certain types of neurons promotes their survival after growth factor withdrawal (Borasio et al., 1989; Downward, 1994; Nobes et al., 1996; Weng et al., 1996), and blocking antibodies directed against ras inhibit survival in freshly dissociated rat SCG neurons (Nobes and Tolkovsky, 1995). However, ras does not promote survival in all neuronal cell types (Borasio et al., 1993), and downstream inhibition of this pathway using PD98059, a MAPKK inhibitor, does not result in the death of rat SCG neurons (Virdee and Tolkovsky, 1996). The ras/MAPK pathway may signal to multiple pathways that are important in dif-

Address all correspondence to Karen Philpott, Eisai London Research Laboratories Ltd., Bernard Katz Building, University College London, London WC1E 6BT, United Kingdom. Tel.: (44) 171-388-4746. Fax: (44) 171-413-1121. E-mail: kphilpott@elrl.co.uk

1. *Abbreviations used in this paper*: CGN, cerebellar granule neurons; GSK, glycogen synthase kinase; IGF, insulin-like growth factor; PI, phosphatidylinositol; SCG, superior cervical ganglion.

ferentiation and neurite outgrowth or survival, depending on the type of neuron.

Evidence that PI 3-kinase mediates neurite outgrowth is somewhat contradictory. Studies using receptor mutants suggested it is not crucial (Obermeier et al., 1994; Stephens et al., 1994), while those directly analyzing the effect of PI 3-kinase inhibitors on PC12 cells suggested that it does indeed play a role (Kimura et al., 1994; Jackson et al., 1996). More recently, there has been the suggestion that PI 3-kinase may be critical in the survival of several types of cells, including neurons (Scheid et al., 1995; Ernhardt and Cooper, 1996; Minshall et al., 1996; Vemuri and McMorris, 1996). Yao and Cooper (1995) showed that wortmannin, an irreversible inhibitor of PI 3-kinase (Yano et al., 1993; Okada et al., 1994), caused PC12 cells to die in the presence of NGF. This is supported by the observation that PC12 cells transfected with receptor chimeras containing the extracellular domain of the PDGF receptor and mutated cytoplasmic domains were able to survive in the presence of PDGF, but only when the PI 3-kinase binding site was intact.

Downstream effectors of PI 3-kinase have recently been described. Akt (also called RAC a or PKB kinase) is a protein kinase with homology to protein kinases A and C within the catalytic domain and is the cellular homologue of vAkt (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991). Akt has been shown to be activated by the phosphatidylinositol products of PI 3-kinase, and its activation can be prevented by inhibition of PI 3-kinase (Burgering and Coffer, 1995; Franke et al., 1995; Park et al., 1996). Recently Akt kinase has been shown to promote survival in cerebellar granule neurons under certain circumstances and to protect against c-myc–induced fibroblast cell death (Dudek et al., 1997; Kauffmann-Zeh et al., 1997). It is also known that $p70^{S6}$ kinase, which lies downstream of PI 3-kinase, plays a significant role during mitogenesis (Downward 1994; Chou and Blenis, 1995; Weng et al., 1995) but does not appear to be involved in survival mechanisms (Yao and Cooper, 1996).

We wished to examine the effects of PI 3-kinase and of downstream effectors on the survival of NGF-dependent superior cervical ganglion (SCG) neurons. We have found that although inhibition of PI 3-kinase does not induce apoptosis of neurons maintained in NGF, both activated PI 3-kinase and Akt can prevent the death of these cells after NGF withdrawal. In addition we have examined the effect of PI 3-kinase on the transcription factor c-Jun, which is activated in the absence of NGF and which we and others have previously postulated to play a role in apoptosis (Estus et al., 1994; Ham et al., 1995). We show that PI 3-kinase does not interfere with the changes in expression of c-Jun seen upon induction of apoptosis in SCG neurons; therefore, PI 3-kinase must be either acting downstream or independently of c-Jun. We conclude that NGF activates multiple signaling pathways and that stimulation of PI 3-kinase is sufficient, but not obligatory, for the survival of SCG neurons.

Materials and Methods

Cell Culture

SCG neurons were removed from newborn rat pups and dissociated in 0.025% trypsin for 30 min after which an equal volume of 0.4% collagenase (Worthington, Reading, UK) was added for a further 30 min. The cells were triturated with a 19-gauge needle until a single cell suspension was obtained and preplated for 2 h to allow non-neuronal cells to attach (Deckwerth and Johnson, 1993). The neurons were gently rinsed off, centrifuged, and resuspended in culture medium. Neurons were plated on poly-L-lysine/laminin–coated 13-mm glass coverslips at 8×10^3 cells/coverslip. The cell culture medium was DME (GIBCO BRL Laboratories, Paisley, UK) containing 10% fetal calf serum (Globepharm Limited, Surrey, UK), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 100 ng/ml NGF (Promega, Southampton, UK). To limit the growth of any contaminating non-neuronal cells, 20 μM fluorodeoxyuridine, and 20 μM uridine (Sigma Chemical Co., Dorset, UK) were added. Cells were cultured for 5 to 8 d before use in a humidified 10% CO_2 incubator at 37°C.

Microinjection

Microinjection was carried out using a microscope (Axiovert 135M; Zeiss, Inc., Thornwood, NY) with an Eppendorf transjector (model 517; Campden Instruments, Leicester, UK) and micromanipulator (model 5246; Campden Instruments). Microinjection needles were pulled from glass capillaries using a horizontal electrode puller (model 773; Campden Instruments). DNA was injected into the nucleus in 0.5× PBS with either 5 μg/ml of purified guinea pig IgG (Sigma Chemical Co.) for cell staining experiments or 5 μg/ml of neutral 70 kD Texas red dextran (Molecular Probes, Eugene, OR) for cell survival experiments. We found some protective effect (~20%) due to injection of plasmid DNA and Texas red dextran and therefore used equivalent DNA concentrations in all microinjection experiments.

Survival Assays

After microinjection, cells were left 4–24 h to allow the protein to be expressed, and the number of Texas red dextran-positive cells was then counted (100% value). The cells were washed twice with DME and fresh medium lacking NGF but containing 100 ng/ml of anti-NGF antibody (Boehringer Mannheim, East-Sussex, UK). After 72 h the cells were incubated for 30 min with 1 μM calcein AM, which is converted to a green fluorescent derivative in live cells (Live/Dead kit; Molecular Probes). Cells that were positive for both Texas red dextran and calcein were counted as viable. In experiments that did not involve microinjection, calcein-positive cells in 10 random fields around the perimeter of the coverslip were counted at a magnification of 200. All experiments were done in a blinded manner.

In experiments in which LY294002 (Affiniti Research Products Ltd., Nottingham, UK) was used, 0.6% methyl cellulose was added to the medium to prevent cells from mechanically detaching (Hawrot and Patterson, 1979). Rapamycin was purchased from Calbiochem-Novochem (Nottingham, UK).

Immunofluorescence

When analyzing the protein expression of plasmid-injected neurons, purified guinea pig IgG was co-injected. The cells were injected and stained 24 h later unless stated otherwise. Cells were generally fixed in 3% paraformaldehyde for 20 min, permeabilized for 5 min in 0.5% Triton X-100, and blocked in 50% goat serum/50% antibody diluting buffer (1% BSA in PBS) for 30 min. Cells were incubated with primary antibodies for 1 h at room temperature, rinsed, and incubated in FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) plus rhodamine-conjugated donkey anti–guinea pig IgG for a further 30 min. Cells were costained with 1 μg/ml of Hoechst 33342 for 5 min before mounting in Citifluor (Citifluor Ltd., Canterbury, UK). Primary antibodies included an anti–c-Jun antibody, raised against a gst–c-Jun fusion protein, an anti–phospho-c-Jun monoclonal antibody raised against the phosphopeptide amino acids 57–68 (phospho-serine 63) from the mouse c-Jun sequence, both provided by Dominique Lallemand (Pasteur Institute, Paris, France), an anti-p85 antibody, a gift from Ivor Gout (Ludwig Institute, London, UK), and an anti-myc antibody (9E10; Boehringer Mannheim). In the case of the anti–c-myc antibody, cells were fixed in 50% methanol/50% acetone and subsequently processed as above. Photographs of cells were taken on a fluorescent microscope (Microphot-FXA; Nikon) using Kodak 400 ASA film.

Constructs

The PI 3-kinase plasmids used in this paper were p110* PI 3-K (constitutively active kinase) and p110*Δ PI 3-kinase (kinase dead construct) and have been previously described (Hu et al., 1995). The wild-type, K179M, and viral Akt constructs were supplied by Dr. Philip Tschillis (Fox Chase Cancer Center, Philadelphia, PA); the p85 construct was obtained from Dr. Len Stephens (Babraham Institute, Cambridge, UK) with the permission of Professor Kasuga (Kobe University School of Medicine, Kobe, Japan), and the Δ169 c-Jun and pCDbcl-2 plasmids were obtained from Dr. John Ham (Eisai Laboratories, London, UK).

Results

Inhibition of PI 3-Kinase Does Not Kill SCGs in the Presence of NGF

To determine the effects of inhibiting PI 3-kinase in primary neurons, we incubated cells in 50 μM LY294002 for 3 d in the presence of NGF. We had found that this concentration of LY294002 inhibited neurite outgrowth in newly plated cells (data not shown). However we could detect no decrease in the survival of differentiated cells, using the Live/Dead assay after 72 h incubation in LY294002 (Fig. 1 *A*). We did not use wortmannin in these experiments since it has a half-life of ~3 h. The gross morphology of cells grown in LY294002 appeared normal (Fig. 1 *B*), but they appeared slightly smaller, perhaps due to their decreased substrate attachment, which is consistent with a postulated role of PI 3-kinase in substrate adhesion (Serve et al., 1995).

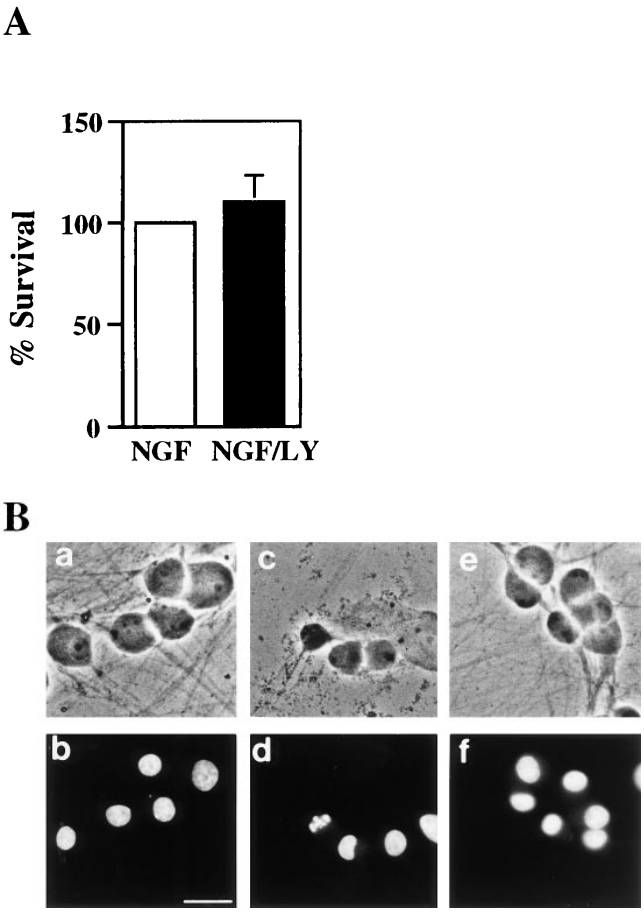

Figure 1. LY294002 does not kill SCG neurons maintained in NGF. Cells were treated with or without 50 μM LY294002 in the presence of NGF for 3 d. (*A*) Results are the mean ± SEM from three experiments. (*B*) Cells were maintained in NGF (*a* and *b*), NGF deprived (*c* and *d*), or maintained in NGF in the presence of 50 μM LY294002 (*e* and *f*) for 3 d. Phase micrographs are shown in *a*, *c*, and *e*, and Hoechst-stained cells are shown in *b*, *d*, and *f*. Bar, 30 μm.

As an alternative method for inhibiting the action of PI 3-kinase, we microinjected a dominant negative p85 construct. The canonical PI 3-kinase consists of a p110 catalytic subunit and a p85 regulatory unit which is important for receptor binding and interacting with other cellular proteins (Carpenter and Cantley, 1996). The dominant negative p85 lacks a 35-amino acid stretch within the inter-SH2 domain necessary for p110 binding and is able to bind to the TrkA receptor, but not to the PI 3-kinase p110 subunit (Hara et al., 1994). We found this modified p85 to be expressed throughout the cytoplasm (Fig. 2 *A*), but it did not induce apoptosis in SCG neurons in the presence of NGF (Fig. 2 *B*), even though it has been reported to block neurite outgrowth in NGF-stimulated PC12 cells (Jackson et al., 1996). These data indicate that the activity of PI 3-kinase is not essential for survival when cells are maintained in NGF.

Constitutively Active PI 3-Kinase Protects SCG Neurons from NGF Withdrawal-Induced Death

A constitutively active myc-tagged version of PI 3-kinase (p110*), containing the p110 kinase subunit linked to the inter-SH2 domain of the p85 subunit (Hu et al., 1995), was used in these experiments. Expression of this protein was verified by staining with an anti-myc–tagged antibody (9E10), as shown in Fig. 3 *A*. For survival experiments, DNA was microinjected into 5- to 8-d-old neurons and the neurons withdrawn from NGF 16–24 h later, having allowed sufficient time for the protein to be expressed. After an additional 72 h, survival was quantified. Three control plasmids were used: pCD*bcl-2*, known to enhance SCG survival after NGF withdrawal (Garcia et al., 1992; Ham et al., 1995); the backbone vector pCG; and a kinase-dead version of PI 3-kinase, p110*Δ, in which amino acids 917–950 of wild-type p110 had been removed. PI 3-kinase displayed a striking ability to support survival of NGF-deprived neurons, being approximately as effective as bcl-2. On the other hand, the kinase-dead variant was completely ineffective (Fig. 3 *B*). If we were indeed promoting survival by introducing activated PI 3-kinase, this effect should be completely reversed by the addition of the PI 3-kinase inhibitor, LY294002. As expected, when added at the time of NGF removal, LY294002 did block the protective effects of PI 3-kinase (Fig. 4). Hence our results indicate that, in the absence of NGF, expression of PI 3-kinase activity is sufficient to prevent apoptosis.

The Kinase Akt Promotes Survival of SCG Neurons

One of the kinases known to lie downstream of PI 3-kinase is Akt, which can be directly activated by products of PI 3-kinase (Franke et al., 1995, 1997). We injected wild-type (wt), viral (v) Akt, or a kinase-dead version, Akt K179M, into 5- to 8-d-old SCG neurons and confirmed that wtAkt and K179M Akt were expressed using an anti-HA antibody to the 3′ tag (data not shown). We found that wtAkt

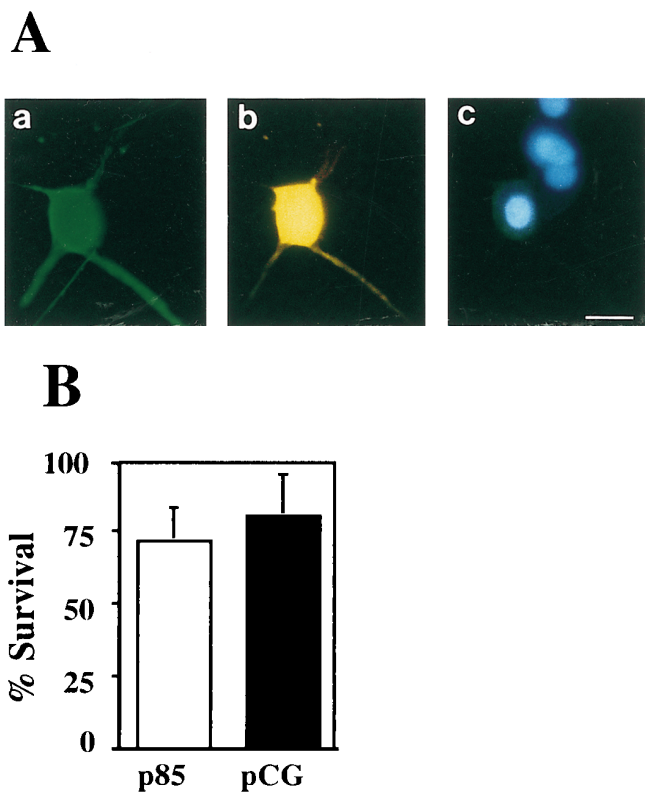

Figure 2. A dominant negative PI 3-kinase construct, Δp85, does not induce SCG neuron death. (*A*) Cells were microinjected and stained 24 h later for p85 (*a*), for guinea pig IgG (*b*), and Hoechst (*c*). (*B*) 200 cells/coverslip were injected with 100 ng/ml of either Δp85 or pCG. Cells were scored after 3 d for survival in the presence of NGF. Results are the mean of three independent experiments ± SEM. Bar, 20 μm.

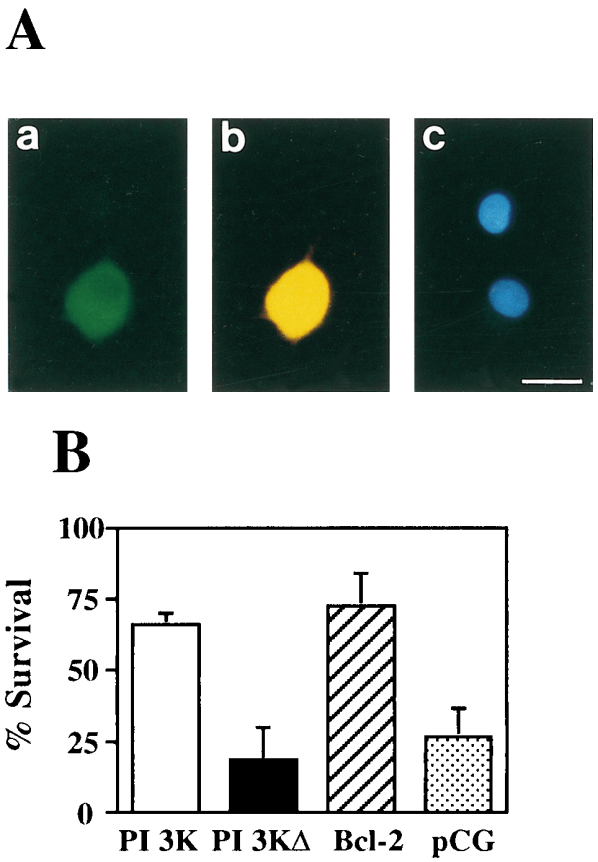

Figure 3. Activated PI 3-kinase protects SCG neurons from NGF withdrawal-induced death. (*A*) Cells were injected with 100 ng/ml of activated PI 3-kinase vector and stained 24 h later for the myc-tag epitope present on the PI 3-kinase construct (*a*), guinea pig IgG (*b*), and Hoechst (*c*). (*B*) 200 cells were injected per coverslip with 100 ng/ml of plasmid DNA and scored for survival 3 d later. Results are the mean of six experiments (five for Bcl-2) ± SEM. Bar, 20 μm.

or vAkt maintained survival of SCG neurons withdrawn from NGF but that K179M Akt did not (Fig. 4). To ascertain whether the protection by Akt was specific to the PI 3-kinase pathway, we treated injected cells with LY294002. LY294002 prevented protection by injected PI 3-kinase (Fig. 4) but did not prevent survival promoted by the Akt constructs. $p70^{S6}$ kinase is known to lie downstream of PI 3-kinase and can be inhibited by the macrolide antibiotic rapamycin (Chung et al., 1992; Price et al., 1992; Soltoff et al., 1992). Rapamycin did not induce neuronal apoptosis when cells were maintained in NGF (data not shown) and did not inhibit the survival-promoting effect of Akt (Fig. 4).

PI 3-Kinase Does Not Inhibit Increased c-Jun Expression or Phosphorylation after NGF Withdrawal

We have previously shown that after the withdrawal of NGF from neurons there is a marked increase in the expression and phosphorylation of the transcription factor c-Jun (Ham et al., 1995; A. Watson, personal communication). This increase in expression and phosphorylation can be inhibited by a dominant negative form of c-Jun, Δ169 c-Jun, in which the transactivation domain has been deleted. This mutant can also protect the cells from apoptosis. We have therefore postulated that c-Jun plays an important role in the death process in neurons by initiating the transcription of genes essential for their death. To determine, therefore, whether the survival-promoting activity of PI 3-kinase could act by inhibiting the c-Jun pathway, we injected neurons with either activated PI 3-kinase, a vector control, or Δ169 c-Jun. NGF was withdrawn 16–24 h later and the cells stained for either c-Jun or phospho–c-Jun expression after an additional 24 h. The phospho–c-Jun antibody used was raised against a peptide containing phosphorylated serine 63 in the c-Jun sequence, a site known to be phosphorylated in activated c-Jun (Smeal et al., 1991). Activated PI 3-kinase was unable to inhibit either the increase in nuclear staining of c-Jun or of phosphorylated c-Jun that follows NGF withdrawal. However, Δ169 c-Jun prevented both changes as expected (Fig. 5, *A–C*). This suggests that PI 3-kinase is acting downstream of c-Jun or independently of it, and that apoptosis can be prevented even when c-Jun has been activated.

Discussion

Recently there have been a number of reports examining the effects of PI 3-kinase inhibition on cell survival. These have included studies on cells of hemopoeitic origin that were maintained with different survival factors. It was

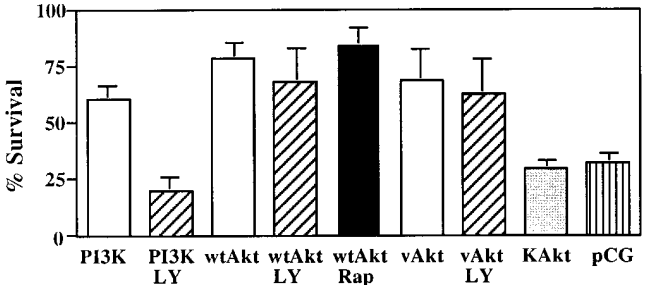

Figure 4. Akt Kinase protects SCG neurons from NGF withdrawal-induced death. 200 cells/coverslip were microinjected with plasmid DNA at 100 ng/ml, withdrawn from NGF 16–24 h later, and treated with no compound or with 10 μM LY294002 (*LY*) or 2 nM rapamycin (*Rap*) as indicated. Results are the mean of three experiments ± SEM.

noted that PI 3-kinase was important for survival of MC-9 cells when they were grown in IL-3 or -4, but not when they were grown in GMCSF or IL-5 (Scheid et al., 1995). However, other types of hemopoeitic cells were dependent on PI 3-kinase for survival when they were maintained in IGF-1, but not in IL-3 (Minshall et al., 1996). Further, the survival of other types of cells, such as oligodendrocytes and fibroblasts, seems to be mediated by PI 3-kinase under all growth conditions (Vemuri and McMorris, 1996; Yao and Cooper, 1996). PI 3-kinase activity has also been shown to be important in the survival of PC12 cells, a model system for sympathetic neurons (Rukenstein et al., 1991; Yao and Cooper, 1995), and most recently in cerebellar granule neurons grown in IGF-1 (Dudek et al., 1997). Thus it seems that multiple survival pathways accessed via different receptors are often present within cells and that their dependence upon PI 3-kinase cannot necessarily be predicted by the use of a particular survival agent.

To investigate the role of PI 3-kinase in a more physiologically relevant system than PC12 cells, we chose to study NGF-dependent SCG neurons, primary sympathetic neurons. PI 3-kinase activity can be inhibited in a number of ways, and we used two different methods. The first was the PI 3-kinase inhibitor LY294002, a reversible inhibitor that competes with ATP for binding to the enzyme (Vlahos et al., 1994). Addition of LY294002 to neurons did not induce apoptosis (Fig. 1), although this compound effectively inhibited PI 3-kinase, since it blocked the effects of overexpressed enzymatically active kinase (Fig. 4, and below). We also employed an alternative way of inhibiting PI 3-kinase by microinjecting a dominant negative form of p85. This construct lacks the region necessary for binding to the p110 subunit while retaining its ability to interact with the receptor or other docking molecules (Klippel et al., 1993; Hara et al., 1994). It is thus able to block endogenous PI 3-kinase activity. It has been shown to effectively inhibit PI 3-kinase in a number of cell types including CHO cells stimulated with insulin or EGF (Hara et al., 1994; Kido et al., 1995) and it inhibits NGF-induced neurite outgrowth in PC12 cells (Jackson et al., 1996). Dominant negative p85 also did not induce apoptosis in SCG neurons grown in NGF (Fig. 2 *B*) which, together with the inhibitor data, indicates that in the presence of NGF, PI 3-kinase activity is not necessary for survival of SCG neurons. This is in contrast

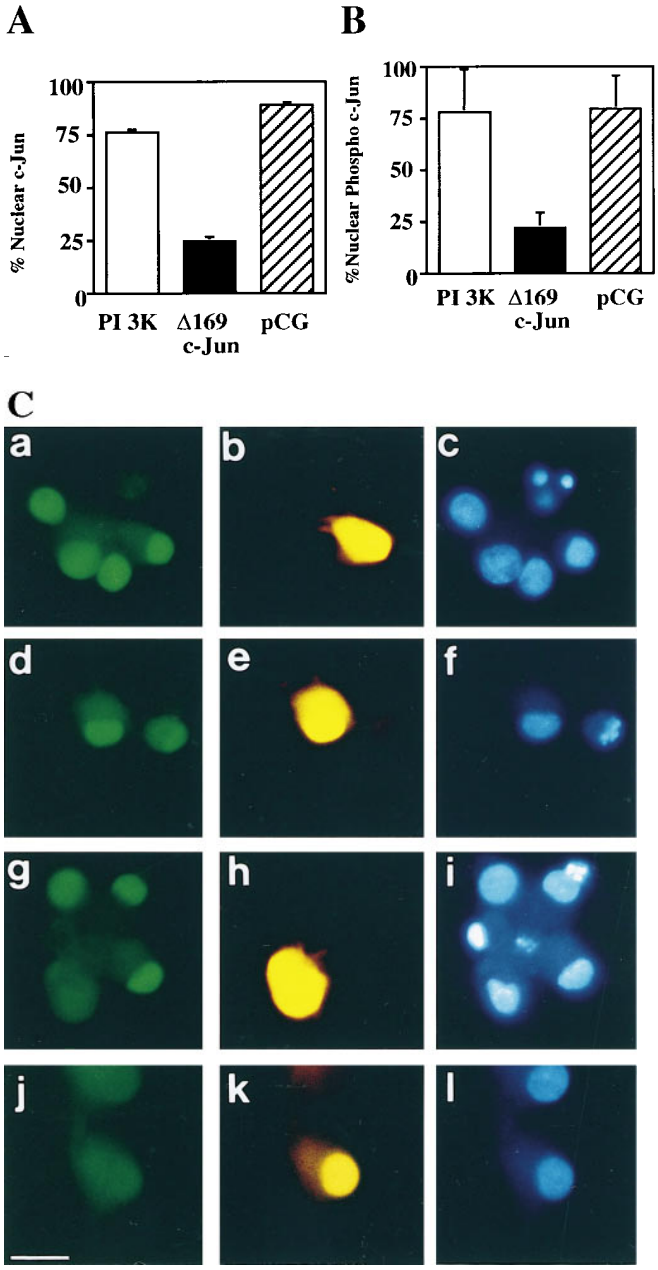

Figure 5. Activated PI 3-kinase does not affect the expression of c-Jun after NGF withdrawal. Cells were injected with activated PI 3-kinase, pCG, or Δ169 c-Jun. After 16–24 h they were withdrawn from NGF, fixed 24 h later, and stained for c-Jun (*A*) or phospho-c-Jun (*B* and *C*). The graphs in *A* and *B* represent data from three independent experiments each, in which 200 cells were injected per coverslip and scored positive for c-Jun or phospho-c-Jun if there was increased staining in the nucleus. (*C*) Injected cells were withdrawn from NGF (*a–i*) or maintained in NGF (*j–l*). Cells were injected with PI 3-kinase (*a–c*), pCG (*d–f*, and *j–l*), or Δ169 c-Jun (*g–i*). Cells were stained for phospho-c-Jun (*a*, *d*, *g*, and *j*), for guinea pig IgG (*b*, *e*, *h*, and *k*), or with Hoechst (*c*, *f*, *i*, and *l*). Bar, 20 μm.

to the results obtained with PC12 cells (Yao and Cooper, 1995), where PI 3-kinase inhibitors induced death, and highlights the importance of using primary cells.

We also studied the effects of over-expression of activated PI 3-kinase on the survival of NGF-deprived neu-

rons. Surprisingly, this enzyme was effective in enhancing survival (Fig. 3 *B*). The enzymatic activity of this kinase was necessary, since the activated PI 3-kinase did not promote survival if cells were maintained in the presence of LY294002 and since a kinase-dead version of the construct did not promote survival (Figs. 3 and 4).

More recently, Akt kinase (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991) has been shown to be stimulated by the products of PI 3-kinase (Franke et al., 1995, 1997; Klippel et al., 1997) after activation of receptor tyrosine kinases. When expressed in SCG neurons, wtAkt and vAkt were both protective after NGF withdrawal. Franke et al. (1995) found a low level of Akt kinase activity in the absence of PDGF after transfection with wtAkt into NIH 3T3 cells. Perhaps even in the absence of NGF there is sufficient PI 3-kinase activity to stimulate wtAkt kinase when Akt protein levels are high. Alternatively, overexpression of the protein could lead to an increase in membrane-localized Akt, which may lead to enhanced activity (Ahmed et al., 1993; Kulik et al., 1997). Interestingly, we find that K179MAkt, which has been shown to act as a dominant negative protein (Dudek et al., 1997), does not induce apoptosis in the presence of NGF, again suggesting that this pathway is not obligatory in the presence of NGF.

Dudek et al. (1997) have carried out experiments on cerebellar granule neurons (CGN) whose survival in serum-free, low K^+ medium could be partially supported by insulin-like growth factor 1 (IGF-1). LY294002 treatment of these neurons resulted in their death, suggesting that IGF-1 conferred protection via PI 3-kinase signaling. Transfection of CGN with Akt kinase expression vectors promoted their survival in the absence of IGF-1, suggesting that Akt kinase was important in the survival mediated by this growth factor. However, analogous to the situation we describe with NGF and SCG neurons, PI 3-kinase was not an obligatory survival pathway when cells were grown in serum plus high K^+. Since the submission of this paper, Miller et al. (1997) have also described survival mediated by PI 3-kinase in CGN, maintained in IGF-1 or by depolarization in low serum (Miller et al., 1997).

We also investigated the effect on survival of $p70^{S6}$ kinase thought to be downstream of PI 3-kinase and Akt kinase (Downward, 1994). Rapamycin, a potent inhibitor of $p70^{S6}$ kinase activation (Price et al., 1992), did not induce apoptosis itself and did not inhibit the survival-promoting action of Akt kinase. Thus our results suggest that in SCG neurons PI 3-kinase signals downstream to Akt kinase and that Akt kinase is not working via $p70^{S6}$ kinase. It is unclear what molecules Akt is signaling to in these neurons. A known substrate for Akt is glycogen synthase kinase 3 (GSK3), which is inhibited upon phosphorylation by Akt. GSK3 was initially described as negatively regulating glycogen synthase, the rate-limiting enzyme in glycogen synthesis, but is now known to phosphorylate many other proteins (Welsh et al., 1996), and it may be the case that molecules downstream of GSK3 play a role in apoptosis.

To investigate further how PI 3-kinase can inhibit death, we also looked at the level and phosphorylation state of the transcription factor c-Jun. We had previously shown that upon the withdrawal of NGF from SCG neurons, c-Jun increases in level and becomes phosphorylated, leading to its activation (Ham et al., 1995; A. Watson, personal communication). In addition, the c-Jun NH_2-terminal protein kinase (JNK) pathway had been implicated in apoptosis of PC12 cells (Xia et al., 1995). We therefore examined by immunofluorescence the level of c-Jun and of phospho–c-Jun in cells microinjected with activated PI 3-kinase. PI 3-kinase did not prevent the accumulation of nuclear c-Jun or phospho–c-Jun in neurons after NGF withdrawal (Fig. 5). This suggests that PI 3-kinase is acting to inhibit the death pathway downstream of c-Jun or independently of it. PI 3-kinase did not induce c-Jun expression itself when injected in the presence of NGF (data not shown). Importantly, these data also indicate that the kinases involved in c-Jun regulation, such as JNK, are not directly affected by the PI 3-kinase pathway, since phosphorylation of c-Jun followed the normal pattern after NGF withdrawal. It is possible therefore that PI 3-kinase is a cytoplasmic survival effector that is able to mediate NGF-induced survival independently of new transcription or protein synthesis.

In summary, we have demonstrated a potential role for PI 3-kinase and Akt kinase in the survival of SCG neurons after NGF withdrawal. Since inhibition of PI 3-kinase does not induce apoptosis in the presence of NGF, it is likely that there are other survival pathways stimulated by NGF. In addition we have shown that the protective effect of PI 3-kinase is not due to prevention of c-Jun activation. Stimulation of the PI 3-kinase pathway, with its survival-promoting effects, could therefore lead to novel therapies for a range of degenerative disorders associated with neuronal apoptosis.

We would like to thank Dr. C. Bazenet for critical reading of this manuscript, Ms. K. Ferguson for editorial assistance, and the laboratories that generously supplied antibodies and plasmids.

Received for publication 2 April 1997 and in revised form 13 August 1997.

References

Ahmed, N.N., T.F. Franke, A. Bellacosa, K. Datta, M.E. Gonzalez-Portal, T. Taguchi, J.R. Testa, and P.N. Tsichlis. 1993. The proteins encoded by c-akt and v-akt differ in post-translational modification, subcellular localization and oncogenic potential. *Oncogene.* 8:1957–1963.

Bellacosa, A., J.R. Testa, S.P. Staal, and P.N. Tsichlis. 1991. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science (Wash. DC).* 254:274–277.

Borasio, G.D., J. John, A. Wittinghofer, Y.A. Barde, M. Sendtner, and R. Heumann. 1989. ras p21 protein promotes survival and fiber outgrowth of cultured embryonic neurons. *Neuron.* 2:1087-1096.

Borasio, G.D., A. Markus, A. Wittinghofer, Y.A. Barde, and R. Heumann. 1993. Involvement of ras p21 in neurotrophin-induced response of sensory, but not sympathetic neurons. *J. Cell Biol.* 121:665–672.

Burgering, B.M., and P.J. Coffer. 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature (Lond.).* 376:599–602.

Carpenter, C.L., and L.C. Cantley. 1996. Phosphoinositide kinases. *Curr. Opin. Cell Biol.* 8:153–158.

Carter, A.N., and C.P. Downes. 1995. Molecular mechanisms of regulation of phosphoinositide 3-kinase by insulin, insulin-like growth factor I and nerve growth factor in PC12 cells. *Biochem. Soc. Trans.* 23:148–152.

Choi, D.W. 1996. Ischemia-induced neuronal apoptosis. *Curr. Opin. Neurobiol.* 6:667–672.

Chou, M.M., and J. Blenis. 1995. The 70-kD S6 kinase: regulation of a kinase with multiple roles in mitogenic signalling. *Curr. Opin. Cell Biol.* 7:806–814.

Chung, J., C.J. Kuo, G.R. Crabtree, and J. Blenis. 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell.* 69:1227–1236.

Coffer, P.J., and J.R. Woodgett. 1991. Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. *Eur. J. Biochem.* 201:475–481.

Deckwerth, T.L., and E.M. Johnson. 1993. Neurotrophic factor deprivation-induced death. *Ann. NY Acad. Sci.* 679:121–131.

Downward, J. 1994. Signal transduction. Regulating S6 kinase. *Nature (Lond.).* 371:378–379.

Dudek, H., S.R. Datta, T.F. Franke, M.J. Birnbaum, R. Yao, G.M. Cooper,

R.A. Segal, D.R. Kaplan, and M.E. Greenberg. 1997. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science (Wash. DC).* 275:661–665.

Ernhardt, P., and G.M. Cooper. 1996. Activation of the CPP32 apoptotic protease by distinct signaling pathways with differential sensitivity to Bcl-X_L. *J. Biol. Chem.* 271:17601–17604.

Estus, S., W.J. Zaks, R.S. Freeman, M. Gruda, R. Bravo, and E.M. Johnson. 1994. Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. *J. Cell Biol.* 127: 1717–1727.

Fantl, W.J., D.E. Johnson, and L.T. Williams. 1993. Signaling by receptor tyrosine kinases. *Annu. Rev. Biochem.* 62:453–481.

Franke, T.F., S.I. Yang, T.O. Chan, K. Datta, A. Kazlauskas, D.K. Morrison, D.R. Kaplan, and P.N. Tsichlis. 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell.* 81:727–736.

Franke, T.F., D.R. Kaplan, L.C. Cantley, and A. Toker. 1997. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-biphosphate. *Science (Wash. DC).* 275:665–668.

Garcia, I., I. Martinou, Y. Tsujimoto, and J.C. Martinou. 1992. Prevention of programmed cell death of sympathetic neurons by the bcl-2 proto-oncogene. *Science (Wash. DC).* 258:302–304.

Ham, J., C. Babij, J. Whitfield, C.M. Pfarr, D. Lallemand, M. Yaniv, and L.L. Rubin. 1995. A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron.* 14:927–939.

Hara, K., K. Yonezawa, H. Sakaue, A. Ando, K. Kotani, T. Kitamura, Y. Kitamura, H. Ueda, L. Stephens, T.R. Jackson et al., 1994. 1-Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells. *Proc. Natl. Acad. Sci. USA.* 91:7415–7419.

Hawrot, E., and P.H. Patterson. 1979. Long-term culture of dissociated sympathetic neurons. *Methods Enzymol.* 58:574–584.

Hu, Q., A. Klippel, A.J. Muslin, W.J. Fantl, and L.T. Williams. 1995. Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. *Science (Wash. DC).* 268:100–102.

Jackson, T.R., I.J. Blader, L.P. Hammonds-Odie, C.R. Burga, F. Cooke, P.T. Hawkins, A.G. Wolf, K.A. Heldman, and A.B. Theibert. 1996. Initiation and maintenance of NGF-stimulated neurite outgrowth requires activation of a phosphoinositide 3-kinase. *J. Cell Sci.* 109:289–300.

Jing, S., P. Tapley, and M. Barbacid. 1992. Nerve growth factor mediates signal transduction through trk homodimer receptors. *Neuron.* 9:1067–1079.

Jones, P.F., T. Jakubowicz, F.J. Pitossi, F. Maurer, and B.A. Hemmings. 1991. Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. *Proc. Natl. Acad. Sci. USA.* 88:4171–4175.

Kaplan, D.R., and R.M. Stephens. 1994. Neurotrophin signal transduction by the Trk receptor. *J. Neurobiol.* 25:1404–1417.

Kauffmann-Zeh, A., P. Rodriguez-Viciana, E. Ulrich, C. Gilbert, P. Coffer, J. Downward, and G. Evan. 1997. Suppression of c-Myc-induced apoptosis by Ras signaling through PI(3)K and PKB. *Nature (Lond.).* 385:544–548.

Kido, Y., Y. Okabayashi, T. Okutani, Y. Sugimoto, K. Sakaguchi, and M. Kasuga. 1995. EGF-induced activation of 70-kDa S6 kinase in CHO cells expressing human EGF receptors. *Biochem. Biophys. Res. Commun.* 209:131–138.

Kimura, K., S. Hattori, Y. Kabuyama, Y. Shizawa, J. Takayanagi, S. Nakamura, S. Toki, Y. Matsuda, K. Onodera, and Y. Fukui. 1994. Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. *J. Biol. Chem.* 269:18961-18967.

Klippel, A., J.A. Escobedo, Q.Hu, and L.T. Williams. 1993. A region of the 85-kilodalton (kDa) subunit of phosphatidylinositol 3-kinase binds the 110-kDa catalytic subunit in vivo. *Mol. Cell. Biol.* 13:5560-5566.

Klippel, A., W.M. Kavanaugh, D. Pot, and L.T. Williams. 1997. A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Mol. Cell. Biol.* 17:338–344.

Koch, C.A., D. Anderson, M.F. Moran, C. Ellis, and T. Pawson. 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science (Wash. DC).* 252:668–674.

Kulik, G., Klippel, A., and Weber, M.J. 1997. Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. *Mol. Cell. Biol.* 17:1595–1606.

Miller, T.M., Tansey, M.G., Johnson, E.M., and Creedon, D.J. 1997. Inhibition of phosphatidylinositol 3-kinase activity blocks depolarization and insulin-like growth factor I-mediated survival of cerebellar granule cells. *J. Biol. Chem.* 272:9847–9853.

Minshall, C., S. Arkins, G.G. Freund, and K.W. Kelley. 1996. Requirement for phosphatidylinositol 3′-kinase to protect hemopoietic progenitors against apoptosis depends upon the extracellular survival factor. *J. Immunol.* 156: 939–947.

Nicholson, D.W. 1996. ICE/CED3-like proteases as therapeutic targets for the control of inappropriate apoptosis. *Nat. Biol.* 14:297–301.

Nobes, C.D., and A.M. Tolkovsky. 1995. Neutralizing anti-p21ras Fabs suppress rat sympathetic neuron survival induced by NGF, LIF, CNTF and cAMP. *Eur. J. Neurosci.* 7:344–350.

Nobes, C.D., J.B. Reppas, A. Markus, and A.M. Tolkovsky. 1996. Active p21Ras is sufficient for rescue of NGF-dependent rat sympathetic neurons. *Neuroscience.* 70:1067–1079.

Obermeier, A., R. Lammers, K.H. Wiesmuller, G. Jung, J. Schlessinger, and A. Ullrich. 1993. Identification of Trk binding sites for SHC and phosphatidylinositol 3′-kinase and formation of a multimeric signaling complex. *J. Biol. Chem.* 268:22963–22966.

Obermeier, A., R.A. Bradshaw, K. Seedorf, A. Choidas, J. Schlessinger, and A. Ullrich. 1994. Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLC γ. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1585–1590.

Okada, T., L. Sakuma, Y. Fukui, O. Hazeki, and M. Ui. 1994. Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. *J. Biol. Chem.* 269:3563–3567.

Oppenheim, R.W. 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* 14:453–501.

Park, E.K., S.I. Yang, and S.S. Kang. 1996. Activation of Akt by nerve growth factor via phosphatidylinositol-3 kinase in PC12 pheochromocytoma cells. *Mol. Cells.* 6:494–498.

Price, D.J., J.R. Grove, V. Calvo, J. Avruch, and B.E. Bierer. 1992. Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. *Science (Wash. DC).* 257:973–977.

Rukenstein, A., R.E. Rydel, and L.A. Greene. 1991. Multiple agents rescue PC12 cells from serum-free cell death by translation- and transcription-independent mechanisms. *J. Neurosci.* 11:2552–2563.

Scheid, M.P., R.W. Lauener, and V. Duronio. 1995. Role of phosphatidylinositol 3-OH-kinase activity in the inhibition of apoptosis in haemopoietic cells: phosphatidylinositol 3-OH-kinase inhibitors reveal a difference in signalling between interleukin-3 and granulocyte-macrophage colony stimulating factor. *Biochem. J.* 312:159–162.

Segal, R.A., and M.E. Greenberg. 1996. Intracellular signaling pathways activated by neurotrophic factors. *Annu. Rev. Neurosci.* 19:463–489.

Serve, H., N.S. Yee, G. Stella, L. Sepp-Lorenzino, J.C. Tan, and P. Besmer. 1995. Differential roles of PI3-kinase and Kit tyrosine 821 in Kit receptor-mediated proliferation, survival and cell adhesion in mast cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:473–483.

Smeal, T., B. Binetruy, D.A. Mercola, M. Birrer, and M. Karin. 1991. Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. *Nature (Lond.).* 354:494–496.

Soltoff, S.P., S.L. Rabin, L.C. Cantley, and D.R. Kaplan. 1992. Nerve growth factor promotes the activation of phosphatidylinositol 3-kinase and its association with the trk tyrosine kinase. *J. Biol. Chem.* 267:17472–17477.

Stephens, R.M., D.M. Loeb, T.D. Copeland, T. Pawson, L.A. Greene, and D.R. Kaplan. 1994. Trk receptors use redundant signal transduction pathways involving SHC and PLC-γ 1 to mediate NGF responses. *Neuron.* 12:691–705.

Thompson, C.B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science (Lond.).* 267:1456–1462.

Vemuri, G.S., and F.A. McMorris. 1996. Oligodendrocytes and their precursors require phosphatidylinositol 3-kinase signaling for survival. *Development.* 122:2529–2537.

Virdee, K., and A.M. Tolkovsky. 1996. Inhibition of p42 and p44 mitogen-activated protein kinase activity by PD98059 does not suppress nerve growth factor-induced survival of sympathetic neurones. *J. Neurochem.* 67:1801–1805.

Vlahos, C.J., W.F. Matter, K.Y. Hui, and R.F. Brown. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-1 (LY294002). *J. Biol. Chem.* 269:5241–5248.

Welsh, G.I., C. Wilson, and C.G. Proud. 1996. GSK3: a SHAGGY frog story. *Trends Cell Biol.* 6:274–279.

Weng, Q.P., K. Andrabi, M.T. Kozlowski, J.R. Grove, and J. Avruch. 1995. Multiple independent inputs are required for activation of the p70 S6 kinase. *Mol. Cell. Biol.* 15:2333–2340.

Weng, G., M.A. Markus, A. Markus, A. Winkler, and G.D. Borasio. 1996. p21ras supports the survival of chick embryonic motor neurones. *Neuroreport.* 7:1077–1081.

Wood, K.W., and T.M. Roberts. 1993. Oncogenes and protein kinases in neuronal growth-factor action. *Biochim. Biophys. Acta.* 1155:133–150.

Yano, H., S. Nakanishi, K. Kimura, N. Hanai, Y. Saitoh, Y. Fukui, Y. Nonomura, and Y. Matsuda. 1993. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. *J. Biol. Chem.* 268:25846–25856.

Yao, R., and G.M. Cooper. 1995. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science (Wash. DC).* 267:2003-2006.

Yao, R., and G.M. Cooper. 1996. Growth factor-dependent survival of rodent fibroblasts requires phosphatidylinositol 3-kinase but is independent of pp70S6K activity. *Oncogene.* 13:343–351.

Xia, Z., M. Dickens, J. Raingeaud, R.J. Davis, and M.E. Greenberg. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science (Wash. DC).* 270:1326–1331.