The Ras/Phosphatidylinositol 3-Kinase and Ras/ERK Pathways 
Function as Independent Survival Modules Each of Which Inhibits a Distinct Apoptotic Signaling Pathway in Sympathetic Neurons*

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Ras promotes robust survival of many cell systems by activating the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway, but little is understood about the survival functions of the Ras/ERK pathway. We have used three different effector-loop mutant forms of Ras, each of which activates a single downstream effector pathway, to dissect their individual contributions to survival of nerve growth factor (NGF)-dependent sympathetic neurons. The PI3-kinase pathway-selective protein RasVal-12T35S was as powerful as oncogenic RasVal-12 in preventing apoptosis induced by NGF deprivation but conferred no protection against apoptosis induced by cytosine arabinoside. Identical results were obtained with transfected Akt. In contrast, the ERK pathway-selective protein RasVal-12P35S had no protective effects on NGF-deprived neurons but was almost as strongly protective as RasVal-12 against cytosine arabinoside-induced apoptosis. The protective effects of RasVal-12T35S against cytosine arabinoside were completely abolished by the ERK pathway inhibitor PD98059. RasVal-12E37G, an activator of RafGDS, had no survival effect on either death pathway, similar to RasS17N, the full survival antagonist. Thus, Ras provides two independent survival pathways each of which inhibits a distinct apoptotic mechanism. Our study presents one of the few clear-cut cases where only the Ras/ERK, but not the Ras/PI3K/Akt pathway, plays a dominant survival signaling role.

One of the central problems posed by degenerative disorders involving postmitotic cells is how to prevent cell death. We have demonstrated previously that p21Ras (Ras) protein plays a pivotal role in mediating survival by nerve growth factor (NGF)† and other cytokines in rat sympathetic (SCG) neurons (1–3). However, the mechanisms used by Ras to protect the neurons from apoptosis are not yet fully understood. Active Ras associates with multiple downstream targets to exert its biological effects (4, 5) including Raf-1 kinase, the catalytic subunit of a phosphatidylinositol 3-kinase (PI3K), and the Ras guanine nucleotide dissociation stimulator (RalGDS) (6). PI3K, which is directly stimulated by Ras (7) and promotes survival through PKB/Akt in numerous cell systems (8), is persistently activated by NGF in SCG neurons (9) and has been shown to mediate survival in sympathetic (9) and sensory neurons (10) as well as robust neurite outgrowth (9). Moreover, expression of active PI3K (11) or PKB/Akt (9, 12) is sufficient to protect SCG neurons from apoptosis induced by NGF withdrawal. In contrast, whereas p42 and p44 mitogen-activated protein kinases (ERKs), which mediate the Ras/Raf-1 pathway, are strongly and persistently activated by NGF (13, 14), ERK activity is not required for survival support by NGF or other cytokines (14, 15). A third Ras-interacting protein, RalGDS, contributes to cell transformation (4), but its functions in neurons are still unclear. In PC12 cells its overexpression inhibited neurite outgrowth induced by NGF suggesting a dominant-negative effect (16).

Recently, we uncovered a putative role for ERK activity in SCG neuron survival by demonstrating that the MEK inhibitor PD98059, which abolishes ERK activity (14, 15), dramatically increased apoptosis induced by araC treatment in the presence of NGF (17). These experiments raised the possibility that besides the Ras/PI3K pathway, the Ras/ERK pathway might also protect against apoptosis of NGF-deprived neurons. There is great interest in understanding the role of the Ras/ERKs pathway in survival since it is becoming increasingly clear that there exist PI3K/Akt-independent survival signaling pathways and that PI3K/Akt activity induced by some cytokines is not being utilized for survival (18–20), although the identity of the alternative survival signals was not determined. However one study (20) excluded ERK activity from being the alternative pathway mediating Akt-independent survival. In PC12 cells it was suggested that ERKs promoted survival by inhibiting c-Jun N-terminal kinase/p38 stress kinases (21), but in SCG neurons we found no obligate relationship between the two processes (13). In cardiomyocytes ERK activity was induced by oxidative stress and was shown to limit damage by inducing cyclooxygenase-2 expression and production of prostacyclin (22), but the relationship to apoptosis was not clear. Moreover, ERK activity is not induced by oxidative signals in SCG neurons. The role of the Ras/ERK signaling pathway in suppression of apoptosis thus remains largely unresolved.

In this study we investigate the relative importance of each of the signal pathways downstream of p21Ras to determine whether these pathways are synergistic in protection against apoptosis, whether there are limits to protection by the PI3K pathway, and whether there are circumstances in which the ERK pathway might play a predominant role in survival. To this end, we have studied the effects of three different effector-loop domain point mutants of Ha-Ras(G12V) (RasVal-12) which
interact with different single effectors in mammalian cells (4–7, 23). Ras(12V,35S) and Ras(12V,40C) binds to Raf-1 and activates the mitogen-activated protein kinase pathway with about 20% efficiency of Ras(Val-12) (7). It shows no interaction with PI3-kinase p110α subunit but interacts with RalGDS very slightly; Ras(12V,37G) (Ras(Val-12)37G) binds to RalGDS but not to Raf-1 or p110α; Ras(12V,40C) (Ras(Val-12)40C) binds and activates PI3-kinase p110α but not Raf-1 or RalGDS. Previously, it was suggested that Ras(Val-12)40C but not RasVal-1235S suppresses c-Myc-induced apoptosis in fibroblasts (23). By using these constructs, we demonstrate that RasVal-12, 40C is as efficient as Ras Val-12 in protecting against the death of NGF-deprived neurons. In contrast, only RasVal-12,35S could protect against apoptosis induced by araC, the Ras/Pi3K pathway playing no role in this protection. These results were corroborated using active Akt and the ERK pathway inhibitor PD98059. The RalGDS-selective proteins RasVal-12,37G had no protective effects. Thus unlike cycling cells where the three Ras pathways are synergistic for transformation (5), in sympathetic neurons the two Ras downstream pathways that are implicated in survival are neither additive nor synergistic. Rather, they function as independent mechanisms to support neuron survival by suppressing two different mechanisms of apoptotic induction.

EXPERIMENTAL PROCEDURES

MATERIALS—Plasmids pDCE-Ha-Ras (G12V,35S), pDCE-Ha-Ras (G12V,37G), and pDCE-Ha-Ras (G12V,40C) in which HA-tagged proteins were expressed under the CMV promoter were generous gifts from Dr. Michael H. Wigler (Cold Spring Harbor Laboratory). pPK5-Myc-Ha-Ras (G12V) and pEXV-Myc-Ha-Ras (S17N) cDNA and the rat monoclonal anti-Ras antibody Y13-259 were kindly provided by Professor Alan Hall (MRC-LMCB, UCL, UK). Ras(S17N) cDNA was subcloned into the pPKS-Myc expression vector that contains a CMV promoter (Ras17N), pCMV-m/p-RAF-PRBo (a membrane-targeted form of PKB/Akt containing sites for myristoylation/palmitoylation (m/p-Akt)) was provided by Dr. Brian Hemmings (Friedrich Miescher-Institut, Basel, Switzerland). After amplification, all constructs were fully sequenced to confirm fidelity of mutations. LipofectAMINE was purchased from Life Technologies, Inc. Monoclonal antibodies to hemagglutinin (HA) and c-Myc from Babco. Antibody to phospho-Akt was from New England Biolabs. Anti-mouse IgG conjugated with Cy3 was from Jackson ImmunoResearch Laboratories. Cytosine arabinoside (araC) and Hoechst 33342 were from Sigma. PD98059 was purchased from Calbiochem.

Cell Culture—Superior cervical ganglia (SCG) were dissected from 1-day-old rat pups, and sympathetic neurons were extracted as described previously (13). Briefly, SCG were digested for 40 min at 37 °C in 0.1% trypsin, and a single-cell suspension was obtained by triturating the digested ganglia through a narrow-bore flame-polished Pasteur pipette. To purify the neurons, cells were preplated twice for about 1 h each onto collagen-coated culture dishes in L15-CO2 medium containing 5% rat serum under 5% CO2, 37 °C, and nonadhering neurons were collected by centrifugation. HeLa cells were cultured with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1 mM glutamine, penicillin (100,100 IU/ml), and streptomycin (100,100 μg/ml) in 5% CO2 at 37 °C.

Transfection and Induction of Apoptosis—Transfection was carried out following the protocol from Life Technologies, Inc., with some modifications. Briefly, newly isolated neurons were plated onto poly-L-lysine/laminin-coated glass coverslips in 24-well plates and cultured for 3 h in L15-CO2 medium containing 3% rat serum and 20 ng/ml NGF. After one wash with serum-free medium without antibiotics, the cells were incubated for 4 h in the same medium containing 3 μl of LipofectAMINE and 0.15 pmol of plasmid DNA per well. Transfections were terminated by replacing the transfection mixture with the culture medium. After 24 h of transfection, cells were washed twice with medium lacking NGF and incubated in the same medium for 20 h in the presence or absence of NGF and/or 1 mM araC. In some cases, 80 μM PD98059 was added. HeLa cells were passaged 1 day before transfection and transfected using the same protocol used for neurons. After 1 day of expression, the cells were washed with Dulbecco’s modified Eagle’s medium, serum-starved for 20 h to remove endogenous signaling through Ras, and then lysed for Western blot analysis.

**RESULTS**

**Confirmation of Functional Specificity of Ras Constructs**—Newly isolated rat sympathetic neurons were transfected with five different mutant forms of human Ha-Ras as follows: RasVal-12, which is constitutively active but nonselective; RasVal-12,35S, which couples almost exclusively to the Raf/ERK pathway; RasVal-12,40C, which couples exclusively to the Pi3K/Akt pathway; RasVal-12,37G, which couples to RalGDS; and Ras17N, which is dominant-negative. Since the number of transfected sympathetic neurons was not high enough for a biochemical analysis by Western blotting, we first examined the fidelity of gene expression and pathway coupling by our constructs by using transfected HeLa cells (Fig. 1). Antibodies detecting either the c-Myc or HA tag epitopes fused to the N termini of the various Ras mutant proteins recognized two major bands of around 25 kDa in the transfected cells but not in control cells (Fig. 1, A and B). The lower bands represent the mature posttranslationally modified form of the transfected Ras proteins, and the upper bands consist of the unmodified forms of transfected Ras that can accumulate during overexpression. To confirm the specificity of the various mutated forms of Ras for the signaling pathways, we probed the HeLa cell samples with antibodies to active phospho-ERKs, which are downstream of Raf-1, and phospho-Akt, which is downstream of PI3K (Fig. 1C). Fig. 1C shows that RasVal-12 strongly enhanced phosphorylation of both Akt and ERKs in the transfected HeLa cells; RasVal-12,35S only increased ERK phosphorylation whereas RasVal-12,40C only increased Akt phosphorylation thus confirming the lack of cross-reactivity between the signals induced by the two loop effector mutants. Neither Ras17N nor RasVal-12,37G had any activatory effect on either kinase, confirming that these mutants do not activate either pathway.

**Expression of Mutant Forms of Ras in SCG Neurons**—To assess the contribution of the various Ras effector pathways to neuronal survival, we scored the number of living and apoptotic...
neurons expressing different forms of Ras by fluorescence microscopy after staining with the DNA dye Hoechst 33342. Expression of the transfectant Ras proteins was revealed by immunocytochemical staining of the c-Myc or HA tags. The extent of survival of Ras-overexpressing neurons was compared with that of the total population of cells in the same samples. As shown in Fig. 2 (a, c, e, g, and i), all the different forms of tagged Ras proteins were localized primarily to the plasma membrane, as expected of the mature protein, although there was also staining in the Golgi region, where the pre-processed form of Ras is accumulated. Staining was distributed in both neurosoma and neurites. Since these neurons were transfectant prior to neurite outgrowth, Ras proteins must have been exported into the growing neurites, presumably by being included within membrane vesicles that provide neurite precursor building blocks. During the initial stages of apoptosis, neurites were collapsed, and Ras staining in the fragmented neurites was usually seen as small dots around the neurosoma (Fig. 2c, lower neuron). Occasionally, overexpression of Ras proteins caused nuclear deformation that showed a crescentic or lobulated pattern of nuclei containing uncondensed DNA masses (Fig. 2d, upper neuron). Deformation seemed to be due to the abundance of protein accumulated in the Golgi prior to export. When these neurons had healthy neurites they were scored as surviving cells.

**Ras17N Abolishes NGF-mediated Survival**—Expression of RasVal-12 had no deleterious effects on neurons maintained in the presence of NGF (Fig. 3a and b; Fig. 4) (survival of RasVal-12-positive cells 91 ± 2.6% compared with 95.4 ± 0.8% in the total population) and completely blocked apoptosis induced by 20 h of NGF deprivation (survival of RasVal-12-positive cells 87 ± 4% compared with 69 ± 3.5% in the total population of neurons, p < 0.001 – NGF/total versus –NGF/RasVal-12). Most RasVal-12 expressing cells showed a healthy profile with robust neurites despite the absence of NGF (compare Figs. 2a and 3a). In contrast, Ras17N caused massive neuronal apoptosis in the presence of NGF (survival being reduced from 96 ± 0.6 to 41 ± 7.6%), there being a significant increase (by 28%; p < 0.005) in the percentage of cells with apoptotic nuclei compared with the value measured in the total population of NGF-deprived neurons (Fig. 2c and d; Fig. 4). Little further apoptosis was
observed in the Ras17N-expressing neurons that were also NGF-deprived (35 ± 3.3% survival) thus supporting the idea that Ras17N is only dominant-negative in the context of a counter-signal induced by NGF. Thus, most of the apoptosis that occurred in Ras17N-expressing neurons occurred prior to NGF deprivation. Furthermore, no proper neurite outgrowth could be observed in the Ras17N-positive neurons maintained in the presence of NGF (Fig. 2e). Thus, constitutively active transfected RasVal12 was sufficient to support neuron survival and rescue neurons from NGF deprivation, whereas dominant inactive Ras17N kills neurons by completely blocking survival signaling from NGF, implying that Ras activity is necessary for NGF-mediated survival.

The Ras/PI3K but Not the Ras/ERK or Ras/RalGDS Pathways Rescues Neurons from NGF Withdrawal—To examine which of the downstream pathways of Ras contribute to rescue from NGF withdrawal, SCG neurons were deprived of NGF after transfection with specific effector-loop domain mutant Ras constructs. In the absence of NGF, expression of RasVal12/40C (which activates the PI3K pathway) completely inhibited neuronal death induced by 20 h of NGF withdrawal (91.3 ± 1.1% survival, Fig. 4; p < 0.001 compared with −NGF/total) and did not reduce survival in the presence of NGF (93 ± 0.9%). Furthermore, RasVal12/40C (like RasVal12) promoted effusive outgrowth of healthy and long neurites in both presence or absence of NGF (Figs. 2g and 3e). Thus, the activity of the Ras-linked PI3K pathway fully blocks the apoptotic signal and is sufficient for supporting neuronal survival in the absence of a neurotrophic factor. In contrast to RasVal12/40C, RasVal12/35S expression (which activates the Raf-1/ERK pathway) did not block neuronal death during NGF deprivation, the rate of survival of RasVal12/35S-positive cells (60 ± 5%) being similar to that of NGF-deprived cells in the total population (69 ± 3.5%) (Figs. 3, c and d and Fig. 4). In the presence of NGF, RasVal12/35S-overexpressing neurons underwent as much apoptosis (69 ± 2.4% survival) as the NGF-deprived neurons, suggesting that it may have some dominant-negative effects when the NGF survival signaling is active. Compared with RasVal12-positive neurons, surviving RasVal12/35S-positive cells exhibited a thinner and less dense profile of neurites (Fig. 2, a and c, and Fig. 3, a and c). Thus the Ras/ERK pathway does not appear to deliver a protective signal against NGF withdrawal-induced neuronal death.

To address the function of RalGDS pathway, RasVal12/37G was expressed in SCG neurons. As shown in Fig. 2i, RasVal12/37G seemed to suppress neurite outgrowth in the presence of NGF, and many expressing cells did not display HA-stained neurites. In addition, a low proportion of survival (64 ± 3%) was scored in RasVal12/37G-positive neurons in the presence of NGF (Fig. 4) suggesting a slight dominant-negative effect of RasVal12/37G on neuronal survival signals. This interpretation was supported by the lack of further reduction in survival (57 ± 7%) when neurons were deprived of NGF for 20 h.

The Ras/ERK but Not the Ras/PI3K Pathway Protects Neurons from Apoptosis Induced by araC—Treatment with 1 mM araC can induce sympathetic neuron apoptosis in the presence of NGF as efficiently as NGF deprivation (24, 25). Our previous data (17) suggested that ERK activity was involved in the protection against araC-induced apoptosis prompting us to explore the contribution of both the Ras/Raf-1 and Ras/PI3K pathways to this protection using mutant Ras constructs. After 36 h of expression of RasVal12, RasVal12/40C, or RasVal12/35S, the neurons were treated with 1 mM araC in the presence or absence of NGF for 20 h. In the presence of NGF, araC reduced neuronal survival to 70 ± 3% in the total population of neurons (Fig. 6a) and caused similar cell death in neurons expressing
Ras Val-1235S was delivering a survival signal to the latter neurons. To investigate whether Ras Val-1235S has survival effects in the absence of NGF, and whether Ras/ERK pathway reduces araC-induced neuronal death (Fig. 5 and 6b). Remarkably, contrary to its protective role during NGF deprivation, Ras Val-1240C failed to rescue the neurons undergoing araC treatment, and only 18 ± 0.1% survival was observed in the neurons expressing Ras Val-1240C (Figs. 5 and 6b). Ras Val-1237G also failed to protect against araC-induced apoptosis (Fig. 6, a and b; 57 ± 7% or 20 ± 5% survival in the presence or absence of NGF).

The Protection of Ras Val-1235S against araC-Induced Apoptosis Is Dependent on ERK Activity—Although we showed above that Ras Val-1235S promotes ERK (but not Akt) phosphorylation in HeLa cells, these data provide no evidence that Ras Val-1235S is utilizing the ERK pathway in SCG neurons. Moreover, because Ras Val-1235S induced some death in the presence of NGF that was not further enhanced when NGF was withdrawn (Fig. 4), it was still technically possible that Ras Val-1235S was delivering a survival signal to the latter neurons. To investi...
activity triggered by RasVal-1235S contributes to the protection against araC-induced neuronal apoptosis but not against apoptosis induced by NGF deprivation.

Active Akt Does Not Inhibit Apoptosis Induced by araC—The Ras/Pi3K pathway activates Akt in numerous cell systems (8). To address further the role of the Ras/Pi3K pathway signal in araC-induced neuronal apoptosis, we transfected a membrane-targeted Akt construct (m/p-Akt) that we showed previously to block the death of araC-treated neurons. Data presented correspond to the mean of three independent experiments; the error bars are standard deviations (p < 0.01 versus NGF/araC/total versus NGF+araC/PD98059/35S, all other comparisons with and without PD98059 were not significantly different).

Ras/Pi3K virgule Akt and the Ras/ERK pathways are crucial mediators of neuronal survival because there is no overlap in the processes that are targeted by each pathway. Thus, only Ras/Pi3K/Akt prevents death due to NGF deprivation, but the Ras/ERK pathway is the only pathway that can promote neuroprotection when death is induced by araC. Our study reveals one of the first clear-cut cases where the Ras/ERK but not the Ras/Pi3K/Akt pathway plays a dominant survival signaling role.

Ras17N and RasVal-1237G Do Not Activate Survival Pathways—To evaluate the technique of transient transfection, we first examined whether the survival of SCG neurons, which had been inhibited previously using anti-Ras blocking antibodies (2), could also be inhibited by the dominant-negative form of Ras, Ras17N. We found that expression of inactive Ras17N promoted a dramatic increase in apoptosis in the presence of NGF (Figs. 2 and 4), more than double the amount induced by NGF deprivation. The most likely reason for the increase in apoptosis in neurons expressing Ras17N is that these neurons underwent a much longer period of Ras signal deprivation since the dominant-negative effect of Ras17N would have begun from the time of its expression, whereas NGF deprivation began only 36 h after transfection. Further evidence that Ras17N functioned by counteracting active Ras rather than by being directly pro-apoptotic is that there was little further increase in the death of Ras17N-expressing neurons after NGF deprivation (when Ras is no longer active).

Our data also indicate that RasVal-1237G, which was identified by its ability to activate RalGDS (4), has no protective effects, since it blocked survival in the presence of NGF but failed to either reduce cell death after NGF deprivation or to protect against araC-induced apoptosis. In addition to death induction, expression of RasVal-1237G also reduced neurite outgrowth. The roles of RalGDS, which stimulates guanine nucleotide dissociation from Ral, in transmitting signals from Ras...
and its cellular functions are still not clear (30). In PC12 cells, Ral guanine nucleotide exchange factors act opposite to other Ras effectors suppressing cell cycle arrest and neurite outgrowth induced by NGF (16). However, a growth-promoting effect of RaIGDS was observed either when it was expressed alone (31, 32) or in cooperation with the Ras/Raf pathway (4). Thus, if RaIGDS mediates any of the effects of Ras in SCG neurons, it is clear that the Raf pathway is not used by Ras to deliver a survival signal. The negative effect of RasVal-1237G on neurite outgrowth may be due to Raf activity but could also be due to inhibition of the Ras/Raf pathway through its considerable dominant-negative effects since PI3K activity is required for NGF-induced neurite outgrowth in SCG neurons (9).

Only the Ras/Pi3K Pathway Can Mediate Protection by Ras against NGF Deprivation—The Pi3K pathway has been implicated in anti-apoptotic signaling in numerous cell types, including sympathetic neurons (9, 11, 12), and sensory neurons (10, 33). One known effector of Pi3K is Akt, a serine-threonine kinase that is activated in response to NGF stimulation (9, 17) and that was shown recently to be required for SCG neuron survival (9, 12). Although Pi3K is activated directly by Ras (7), it has been suggested that a major proportion of Pi3K activity induced by NGF through TrkA is mediated upstream of Ras (34). In this report, we show that RasVal-1240C reduced neuronal apoptosis due to NGF withdrawal as effectively as RasVal-14 or active Akt. Taken together with similar results obtained with active Pi3K (11), these data support the idea that the Ras/Pi3K Akt pathway may mediate the NGF survival signal and exclude a role in survival for the other Ras-linked effector pathways tested here. Overexpression of RasVal-1235S not only failed to inhibit neuronal apoptosis caused by NGF withdrawal (Figs. 3 and 4) but in the presence of NGF its overexpression reduced neuronal survival to a similar level as that found in the total NGF-deprived neuron population, suggesting that it has mild dominant-negative effects against NGF-induced active Ras. This idea is supported by the findings that like Ras17N, no further decrease in survival was observed in RasVal-1235S-positive cells undergoing NGF deprivation, during which time Ras is not activated. Moreover, the MEK inhibitor PD98059 failed to increase the death of RasVal-1235S-expressing neurons deprived of NGF.

Only the Ras/ERK Pathway Can Mediate Protection by Ras against araC-induced Death—Our finding that the Ras/ERK pathway plays no role during NGF-induced survival can be contrasted with our finding a crucial role for Ras/ERK signaling, but not the Ras/Pi3K signaling, in counteracting araC-induced apoptosis. AraC causes apoptosis in several types of neurons in vitro by mechanisms that are still unclear (35–37). Our previous data showed that araC requires the presence of wild type p53 to induce apoptosis in sympathetic neurons and proposed a potential protective role of ERK against araC toxicity mainly through the observation that the MEK inhibitor PD98059 caused a significant increase in araC-induced apoptosis in the presence of NGF and that ciliary neurotrophic factor, which cannot sustain ERK activity (13), also provided no protection against araC (17). When araC was added to sympathetic neurons expressing RasVal-12, RasVal-1240C, or RasVal-1235S in the presence of NGF (Fig. 6a), there was no significant difference in the loss of survival between non-transfected or the mutant Ras-expressing neurons, the degree of apoptosis in each case being about 30%. For RasVal-12 or RasVal-1240C this is the result predicted because araC does not interfere with NGF signaling (17) so the contribution of the signals from these Ras mutants would be redundant to that induced by NGF. The lack of increased apoptosis in neurons expressing RasVal-1235S, which might have been predicted from its dominant-negative actions in the presence of NGF, was most likely because of its ability to counterbalance this negative input by its survival-promoting effect, revealed most clearly when araC was added in the absence of NGF.

Thus, in the presence of araC but absence of NGF, RasVal-12 and RasVal-1235S reduced neuronal death close to the levels observed in the total population of neurons incubated in presence of NGF and araC (64, 47, and 70%, respectively), against only 13% survival of the total population of neurons. The less substantial survival support by RasVal-1240C compared with RasVal-12 is consistent with its weaker ERK stimulating activity (Fig. 1b (7)). However, the survival of RasVal-1240C- and m/p-Akt-overexpressing cells was negligible, being only 5 and 3%, respectively, higher than that of the non-transfected cells.

We noted that even protection by RasVal-12 did not completely block apoptosis induced by araC, as observed when araC treatment occurs in the presence of NGF. Incomplete protection by NGF or RasVal-12 may be due to multiple signals induced by araC/p53, only one of which is inhibitable by ERK activity. Therefore, araC always provokes a dominant death phenotype, explaining why RasVal-1240C and active Akt fail to exert any protection when neurons are exposed to a combination of NGF deprivation and araC treatment. To demonstrate that RasVal-1235S was utilizing the Ras/ERK pathway to inhibit araC-induced apoptosis, we used the MEK inhibitor PD98059. As predicted, PD98059 completely abolished the survival-promoting effects of RasVal-1235S. Therefore, considering that RasVal-12 and RasVal-1235S both target the Raf/ERK pathway but that RasVal-1240C only activates the Pi3K/Akt pathway, the activation of Raf/ERK but not the PI3K pathway is able to reduce neuronal death induced by araC treatment without synergizing with any other Ras pathway.

The Targets of the Two Survival Pathways Must Be Upstream of Cytochrome c Release—Both NGF deprivation and araC treatment cause very similar apoptotic profiles, cytochrome c translocation from the mitochondria to the cytosol, caspase 3 activity, and poly (ADP-ribose) polymerase cleavage in sympathetic neurons (Ref. 38 and data not shown). Thus, the complete separation of pathways that counteract NGF depri-
vation or araC treatment suggests that the two death stimuli trigger SCG neuronal death using at least two different, independent mechanisms. Since RasVal-1235S and RasVal-1240C contribute their protective effects to the two types of neuronal apoptosis independently, Ras must exert its protective role before the death mechanisms converge onto the mitochondria. The targets of Ras signals that protect neurons from apoptosis through its different effector pathways are still unclear. It has been suggested that Akt, the downstream target of PI3K, inactivates the proapoptotic function of BAD, a member of Bcl-2 family, by phosphorylation of Ser-136 (39, 40). Although evidence for phosphorylation of BAD by Akt in sympathetic neurons is still lacking, this mechanism could potentially explain how Ras rescues the neurons from NGF withdrawal-induced apoptosis. If so, then the pathway that is inhibited by the Ras/ERK pathway must regulate cytochrome c release by another mechanism. It is now of major interest to find out what its targets might be.

The summary scheme in Fig. 9 shows that the power of p21Ras as a neuroprotective signal generator lies in its ability to promote signaling through the PI3K and ERK pathways simultaneously but not redundantly to inhibit two independent mechanisms of apoptosis. The results presented here not only provide new insights into the mechanisms of Ras functions but also identify the kind of therapeutic agents that might be effective in preventing neurodegeneration caused by neurotoxin deprivation or external stimulation.

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Addendum—After this paper was submitted, a study by Hetman et al. (1999) Science 286, 1801–1805 provided new insights into the mechanisms of Ras functions but also identify the kind of therapeutic agents that might be effective in preventing neurodegeneration caused by neurotoxin deprivation or external stimulation.

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