

The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal

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Serum and certain growth factors have the ability to inhibit programmed cell death (apoptosis) and promote survival. The mechanism by which growth factors deliver an anti-apoptotic signal and the mechanism by which this survival signal is uncoupled from mitogenesis are not clear. We studied five downstream effectors of growth factor receptors—Ras, Raf, Src, phosphoinositide 3-kinase (PI 3-kinase), and Akt (PKB)—for their abilities to block apoptosis. Activated forms of Ras, Raf, and Src, although transforming, were not sufficient to deliver a survival signal upon serum withdrawal. In contrast, inhibition of PI 3-kinase accelerated apoptosis, and an activated form of the serine/threonine kinase Akt, a downstream effector of PI 3-kinase, blocked apoptosis. The ability of Akt to promote survival was dependent on and proportional to its kinase activity. In Rat1a fibroblasts, activated Akt did not alter Bcl-2 or Bcl-X_L expression but inhibited Ced3/ICE-like activity. Thus, the PI 3-kinase/Akt (PKB) signaling pathway transduces a survival signal that ultimately blocks Ced3/ICE-like activity. These results suggest that uncoupling of survival and mitogenesis can be explained by differing abilities of distinct mitogens to efficiently induce the PI 3-kinase/Akt signaling pathway.

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Programmed cell death and apoptosis have a fundamental role in animal development and tissue homeostasis (Ellis et al. 1991; Korsmeyer 1995; Steller 1995). Growth factors, cytokines, and serum provide both mitogenic and anti-apoptotic signals to cells and therefore play an important role in maintaining the homeostatic balance between cell proliferation and cell death (Raff et al. 1993). The mitogenic effects of these factors have been well described, and a growing body of literature has demonstrated that their withdrawal can induce programmed cell death in several cell systems (Koury and Bondrant 1990; Yonish-Rouach et al. 1991; Askew et al. 1993; Raff et al. 1993; Harrington et al. 1994). Although much is known about the signaling events involved in the mitogenic pathway, little is known about the paths leading to inhibition of programmed cell death.

Growth factors and cytokines vary in their abilities to induce proliferation and survival, but they all act by activating their respective receptor or receptor-activated tyrosine kinases that in turn activate signal transduction pathways. However, the ability of certain growth factors to induce proliferation is not necessarily correlated with their ability to promote survival, and some growth factors and cytokines promote survival of postmitotic cells (Fairbairn et al. 1993; Raff et al. 1993; Harrington et al.

1994). It was therefore suggested that the mechanism by which certain growth factors promote survival is different from the mechanism by which they induce mitogenesis. Moreover, mutagenic analysis of the insulin-like growth factor-I (IGF-I) receptor showed that mitogenesis, transformation, and survival-promoting properties of IGF-IR can be dissociated (Miura et al. 1995; Resnicoff et al. 1995a,b).

In fibroblasts and other cells of mesenchymal origin, serum deprivation induces apoptosis that can be accelerated dramatically by ectopic expression of the *c-myc* proto-oncogene. Cell death can be inhibited by serum and certain purified growth factors, including IGF-I, insulin, and platelet-derived growth factor (PDGF), but not epidermal growth factor (EGF) or fibroblast growth factor (Harrington et al. 1994). EGF is a potent mitogenic factor and a relatively poor survival factor in these cells, whereas IGF-I and PDGF are good survival and not potent mitogenic factors.

In addition to the well-established effects of deregulated mitogenic signals on tumorigenesis, deregulation of a survival signal transduced by growth factors and cytokines has also been suggested to be a critical step toward tumorigenesis. The IGF-I receptor was shown to contribute to tumorigenesis in part by promoting survival both in tissue culture and in animals (Resnicoff et al. 1995a). In pancreatic islet cells and hepatocytes, expression of IGF-II increases cell survival and promotes tumorigenesis. Furthermore, tumors develop with reduced malignancy and with a higher incidence of apoptosis in mice

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that are deficient in IGF-II (Christofori et al. 1994; Ueda and Ganem 1996).

To investigate the mechanisms by which certain growth factors mediate survival, we have analyzed several downstream effectors of growth factor receptors for their ability to promote survival. We found that signaling through Ras, Raf, and Src pathways was not sufficient to transduce the survival signal. Previous results employing mutant PDGF receptor and inhibitors of PI 3-kinase suggested that PI 3-kinase can deliver a survival signal (Yao and Cooper 1995). Because growth factors that are potent activators of a downstream effector of PI 3-kinase, the serine/threonine kinase Akt/protein kinase B (PKB), are also potent survival factors in fibroblasts (Harrington et al. 1994; Franke et al. 1995), we examined the ability of Akt to deliver a survival signal. We found that an activated Akt was sufficient to promote survival upon growth factor withdrawal. The ability of Akt to promote survival was dependent on its kinase activity and on the activity of its upstream activator, PI 3-kinase. These results may explain why some growth factors that are mitogenic and not effective inducers of the PI 3-kinase/Akt pathway are also poor survival factors.

The apoptosis pathway in mammalian cells, as in *Caenorhabditis elegans*, requires coordination of positive and negative regulators. Members of the Bcl-2 family in mammalian cells, homologs of the *C. elegans ced9* gene, are suppressors of cell death, whereas mammalian homologs of the *ced3* gene of *C. elegans* that encode the Ced3/ICE [interleukin-1 β -converting enzyme]-like proteases can actively kill cells (for review, see Ellis et al. 1991; Korsmeyer 1995; Steller 1995). Here we show that, at least in fibroblasts, Akt does not induce the expression of Bcl-2 or Bcl-X_L but inhibits the activities of Ced3/ICE-like proteases that specifically cleave the poly(ADP-ribose) polymerase (PARP). Thus, one mechanism by which the PI 3-kinase/Akt pathway promotes survival is by inhibiting Ced3/ICE-like activity upon growth factor withdrawal. Because there are multiple potential downstream effectors of Akt, there may be multiple mechanisms by which Akt can deliver the survival signal.

It is possible that the ability of the PI 3-kinase/Akt pathway to mediate survival has a more global role in embryonic development and in the longevity of adult tissues. Interestingly, a homolog of the mammalian PI 3-kinase catalytic subunit has been implicated recently in mediating longevity in *C. elegans* (Morris et al. 1996). Because several Akt homologs were found in *C. elegans* (Waterston et al. 1992), it remains to be determined whether Akt will also have a role in longevity.

Results

Activated Ras, Raf, and Src, although tumorigenic, cannot override apoptosis induced by serum withdrawal in conjunction with induced Myc activity

Serum and growth factors activate various signaling pathways upon binding to their cognate growth factor

receptors. The best characterized downstream effectors of growth factor receptors are the Ras, Raf, and Src molecules. We therefore wanted to test whether these molecules are involved in transmitting the survival signal induced by growth factors. We first examined the ability of activated Ras, Raf, and Src to block apoptosis induced by serum withdrawal in conjunction with ectopically expressed Myc.

A conditionally active Myc (MycER), which enables induction of Myc activity upon addition of β -estradiol (E2) (Eilers et al. 1989), was introduced into Rat1a fibroblasts by retroviral infection. Upon activation, Myc transforms Rat1a cells in the presence of serum (Eilers et al. 1989), but accelerates apoptosis in its absence (Evan et al. 1992). A single clone of Rat1a cells expressing high levels of MycER [Rat1a (MycER)] was isolated and then infected with retroviruses encoding V12H-Ras, Δ Raf1, or v-Src, the constitutively active forms of the H-Ras, Raf1, and Src signaling molecules. For comparable quantitation of cell death, the *bcl-2* gene, which was shown previously to protect cells from Myc-mediated apoptosis (Bissonnette et al. 1992; Fanidi et al. 1992; Wagner et al. 1993), was also introduced into Rat1a (MycER) cells. Pools of several hundred clones infected with the second retrovirus were maintained as cell lines. MycER was expressed at similar levels in all cell lines, and expression of Bcl-2, V12H-Ras, Δ Raf1, and v-Src in the different cell lines was verified by Western analysis (Fig. 1A).

To demonstrate that these proteins were functionally active, we assayed the transforming activities of the constitutively active Ras, Raf, and Src alleles by the abilities of the corresponding cell lines to grow in soft agarose (Fig. 1B). In the presence of E2 the parental Rat1a (MycER) cells formed between 100 and 200 colonies ≥ 0.5 mm in diameter (data not shown). In the absence of E2, however, the MycER cells formed abortive minicolonies that averaged only 0.1–0.2 mm in diameter with fewer than five colonies ≥ 0.5 mm. This growth was most likely attributable to basal activity of the MycER protein in the absence of E2. Rat1a (MycER/Bcl-2) cells formed slightly larger colonies (0.2–0.3 mm) and only 20–25 colonies ≥ 0.5 mm, consistent with previous reports (Koskinen et al. 1995), presumably because Bcl-2 enables longer survival. The cell lines expressing the constitutively activated forms of Ras, Raf, and Src formed significantly more colonies >0.5 mm in the absence of E2 (Fig. 1B). These results demonstrate that the activated Ras, Raf, and Src are functionally active and are able to transform Rat1a cells independent of high levels of Myc activity.

To determine whether activated Ras, Raf, or Src could provide the protection from apoptosis elicited by growth factors, we examined the effect of their expression on apoptosis induced by serum withdrawal. The cell lines were incubated in 0.5% fetal calf serum (FCS) in the presence or absence of 1 μ M E2. The percentage of apoptotic cells was assessed 16 hr following Myc induction by staining with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI). Fragmented and condensed nuclei were scored as apoptotic (Fig. 1C). These nuclei also scored

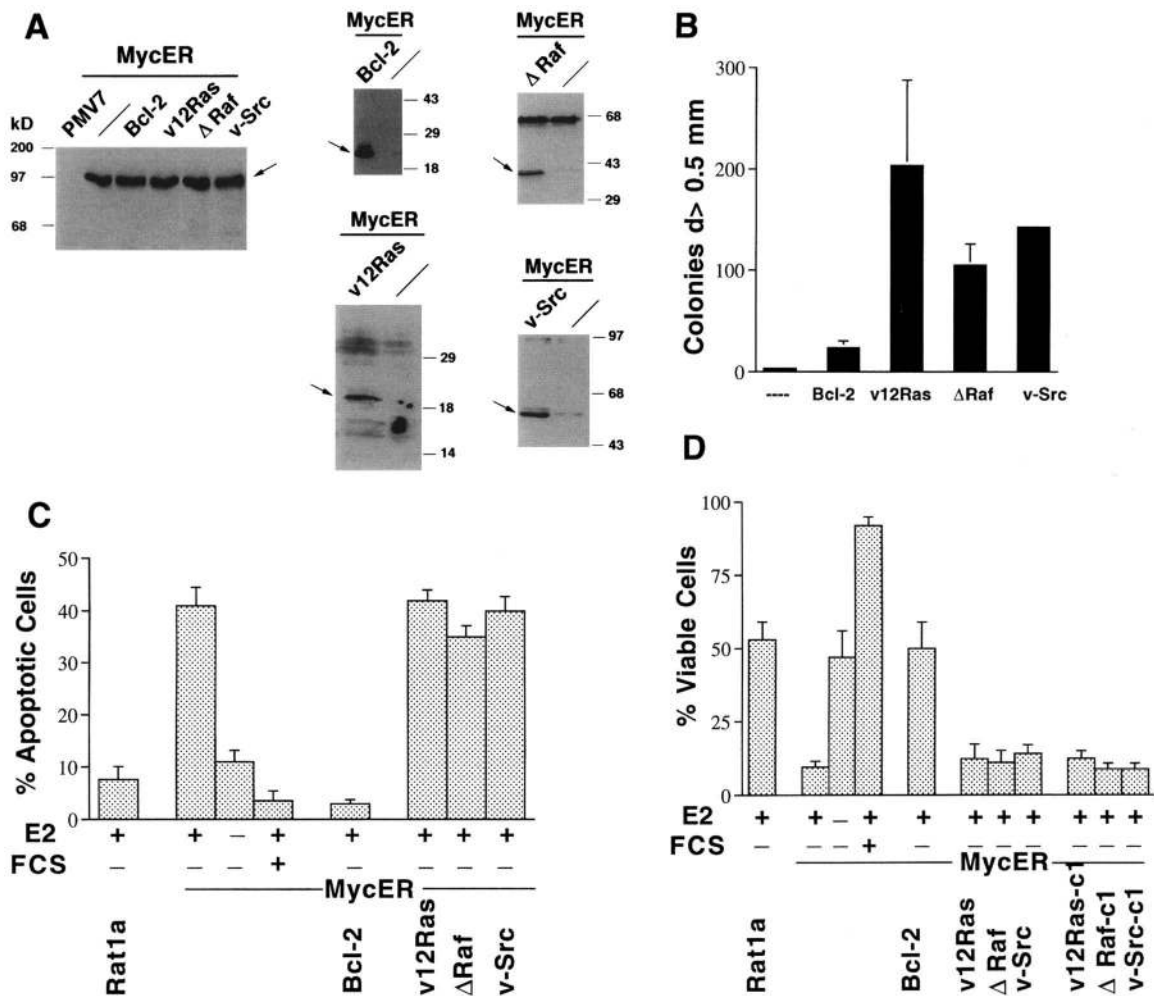


Figure 1. Constitutively active forms of H-Ras, Raf-1, and Src do not block apoptosis mediated by serum deprivation. (A) Immunoblot analysis of ectopically expressed proteins in established cell lines. (Left) Expression of MycER; (right) expression levels of ectopically expressed Bcl-2, V12H-Ras, ΔRaf-1, and v-Src. Relevant proteins are indicated by arrows. Molecular size markers (in kD) are indicated. (B) Quantitation of soft agarose colonies with diameter >0.5 mm. Five thousand Rat1a (MycER) cells expressing the indicated genes were plated in soft agarose in the absence of E2; colonies were scored after 24 days. Bars represent an average of two experiments ± standard error of the mean (S.E.). (C) Percentage of apoptotic cells with or without MycER activation (± E2) in 10% (+) or 0.5% (-) FCS. Apoptosis was scored by DAPI staining after 16 hr. Averages (± S.E.) of at least 500 cells from four independent experiments are shown. (D) Percentage of live cells scored by PI/FDA staining at 48 hr. Cells were treated as in C. Individual clones (indicated as C1) isolated from soft agarose were also examined. Averages (± S.E.) of at least 200 cells from four independent experiments are shown.

positive for the terminal deoxynucleotidyl transferase-mediated deoxyuridine nick end-labeling (TUNEL) reaction (not shown; see also Fig. 2).

Cell death was also measured 48 hr after MycER activation in 0.5% FCS, using propidium iodide (PI) and fluorescein diacetate (FDA) staining (Favaron et al. 1988). Live cells hydrolyze FDA with intracellular esterases to produce yellow-green fluorescence. Loss of membrane integrity of dead cells enables PI to interact with DNA and to yield red fluorescence. After 48 hr, cells were stained with PI/FDA and the percentages of live (FDA-stained) and dead (PI-stained) cells were determined (Fig. 1D).

In the presence of 10% FCS, only 4% of cells were scored as apoptotic after 16 hr (Fig. 1C), and 8% after 48 hr (Fig. 1D) of MycER activation. Rat1a (MycER) cells in 0.5% FCS and in the absence of E2 exhibited a baseline level of cell death that was slightly higher than that observed for Rat1a cells following serum deprivation (Fig. 1C,D). This is likely to be a result of the basal MycER activity that was also observed in the soft agarose assay (Fig. 1B). No significant differences in the levels of cell death were observed between the parental Rat1a (MycER) cell line and the Rat1a (MycER/V12H-Ras), (MycER/ΔRaf), and (MycER/v-Src) cell lines. Rat1a (MycER/Bcl-2) cells, however, were significantly protected from

Myc-accelerated apoptosis. As assessed by DAPI staining after 16 hr of MycER activation, 36%–46% of the cells were apoptotic in all cell lines except the MycER/Bcl-2 cells, which exhibited <4% cell death (Fig. 1C). After 48 hr, all cell lines, with the exception of Rat1a (MycER/Bcl-2), showed 86%–91% cell death as measured by the PI/FDA assay (Fig. 1D). Similar results were obtained for individual clones isolated from soft agarose (Fig. 1D). Thus, although stably expressed, activated Ras, Raf, and Src can transform these cells, each is insufficient to deliver the anti-apoptotic signal generated by serum.

Inhibition of PI 3-kinase overrides the survival signal induced by serum

It was shown previously that wortmannin, a selective inhibitor of PI 3-kinase, blocks growth factor-induced survival of PC12 cells by a Ras-independent mechanism (Yao and Cooper 1995). We therefore tested the effect of wortmannin on Myc-accelerated apoptosis. Rat1a (MycER) cells were grown for 24 hr in 1.5% FCS, then treated with 1 μ M E2 for 1 hr for activation of MycER. Cells were rinsed and placed in DMEM with 0.01% DMSO or 200 nM wortmannin for 20 min; then 5% FCS was added back to the cells, which were stained with DAPI and TUNEL 4 hr later. In the absence of wortmannin, very little cell death was observed (Fig. 2A). As shown in Figure 2B, wortmannin induces cell death in the presence of serum and enhances apoptosis induced by MycER activation. The Rat1a (MycER/Bcl-2) cells, however, were refractory to wortmannin treatment (Figure 2C,D).

Quantitative analysis showed a 3- to 4-fold increase in the percentage of apoptotic Rat1a (MycER) cells after treatment with 200 nM to 2 μ M wortmannin for 4 hr in the presence of 5% FCS regardless of MycER activity (Fig. 2D), and similar results were obtained with Rat1a (MycER/V12H-Ras), (MycER Δ Raf), and (MycER/v-Src cells) (data not shown). Acceleration of cell death was detected at concentrations as low as 20 nM (Fig. 2D). The concentrations of wortmannin that accelerated apoptosis in these experiments have been reported to be specific for inhibition of PI 3-kinase and do not inhibit PI 4-kinase, PKA, PKC, or PKG (Ui et al. 1995; Wymann et al. 1996). These results, therefore, suggest that either PI 3-kinase or other members of this family of proteins (Hunter 1995) that are inhibited by wortmannin are required to deliver a survival signal mediated by serum and growth factors.

A downstream effector of PI 3-kinase, the serine/threonine kinase Akt/PKB, transduces a survival signal that is correlated with its kinase activity

One downstream effector of PI 3-kinase is the serine/threonine kinase Akt/PKB, the cellular homolog of the v-Akt oncoprotein (Bellacosa et al. 1991; Burgering and Coffey 1995; Franke et al. 1995). v-Akt is identical to its cellular homolog, c-Akt, except that it is fused in-frame to the viral Gag protein, targeting Akt to the plasma

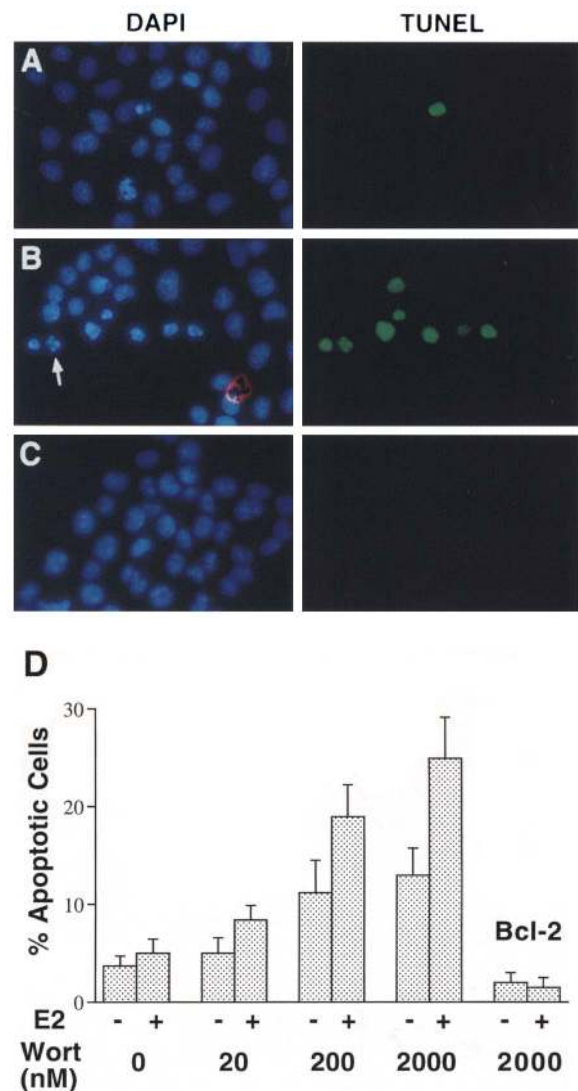


Figure 2. Wortmannin overrides serum-mediated survival and accelerates apoptosis. Representative fields of DAPI (left) and TUNEL (right) staining 4 hr following MycER activation in the absence or presence of wortmannin. Nuclei scored positive by TUNEL have condensed chromatin as shown by DAPI staining. (A) Activation of MycER in the presence of 5% FCS and the absence of wortmannin. Only one apoptotic cell was observed in this representative field. (B) Activation of MycER in the presence of 5% FCS and 200 nM wortmannin in Rat1a (MycER) cells. Apoptosis is accelerated by treatment with wortmannin. A typical apoptotic nucleus at a late stage of apoptosis and with condensed chromatin is indicated by the arrow. (C) Activation of MycER in the presence of 5% FCS and wortmannin in Rat1a (MycER/Bcl-2) cells. Bcl-2 blocks acceleration of apoptosis by wortmannin. (D) Quantitation of DAPI-stained apoptotic Rat1a (MycER) or Rat1a (MycER/Bcl-2) cells treated with varying concentrations of wortmannin in the presence of 5% FCS and with or without MycER activation (\pm E2). Bars represent means of four independent experiments (\pm S.E.). Cells were treated (as described above) with DMSO or varying concentrations of wortmannin and assayed by DAPI staining 4 hr following activation of MycER.

membrane (Bellacosa et al. 1991). This targeting activates Akt (Bellacosa et al. 1991; Burgering and Coffey 1995; Franke et al. 1995), yielding a kinase activity in the absence of serum almost as high as c-Akt kinase activity in 20% FCS (see below).

Akt is activated preferentially by growth factors that promote survival (Harrington et al. 1994; Franke et al. 1995), and, as shown above (Fig. 2), PI-3 kinase inhibition by wortmannin resulted in apoptosis in the presence of serum. We therefore tested the possibility that Akt is involved in mediating the survival signal transduced by serum and growth factors. Akt and v-Akt were introduced into Rat1a (MycER) cells by retroviral infection. A second activated form of Akt with the Src myristoylation signal fused in-frame to the c-Akt coding sequence (MyrAkt) (Franke et al. 1995) was also introduced into the cells. Several hundred clones expressing c-Akt, v-Akt, and MyrAkt were pooled and maintained as cell lines. Individual clones expressing v-Akt were also isolated and maintained as cell lines. The expression of c-Akt, v-Akt, MyrAkt, and MycER in the established cell lines was verified by Western analysis (Fig. 3A). Cells were then tested for susceptibility to apoptosis in 0.5% FCS and 1 μ M E2. MyrAkt and v-Akt significantly protected cells from apoptosis induced by serum withdrawal and MycER activation, whereas minimal protection from cell death was observed in the c-Akt-overexpressing cells (Fig. 3B).

To assess whether Akt is associated with general inhibition of apoptosis induced by serum withdrawal, wild-type Rat1a cells were also examined. Rat1a cells exhibit a significant level of programmed cell death when plated at low density in the absence of serum (0% FCS). Both MyrAkt and v-Akt protected Rat1a cells from apoptosis following serum withdrawal, whereas ectopic expression of c-Akt elicited only modest protection (Fig. 3C). The difference in the abilities of the various Akt forms to elicit survival is correlated with their kinase activities (Fig. 3D). Rat1a cells expressing c-Akt have a basal kinase level slightly higher than that of control Rat1a cells, and they demonstrated only minimal protection from apoptosis. However, cells expressing v-Akt or MyrAkt have further elevated kinase activities (2- and 4-fold, respectively) and demonstrated a proportionally higher level of protection from apoptosis (Fig. 3C,D).

To verify further that the ability of Akt to deliver a survival signal is correlated with its kinase activity, we assayed Akt kinase activity in lysates of c-Akt and v-Akt-overexpressing cells in the absence of serum and upon serum stimulation (Datta et al. 1995; Franke et al. 1995). In Rat1a (c-Akt) cells, Akt kinase activity was dramatically induced 15 min after addition of 20% FCS to serum-deprived cells and was inhibited completely by wortmannin (Fig. 4A). This activity peaked 15–30 min after addition of serum and declined by 3 hr to a basal level of activity observed in proliferating cells (data not shown). In contrast to c-Akt-overexpressing cells, Rat1a (v-Akt) cells exhibited a higher basal kinase activity in 0% FCS that was only slightly lower than that of c-Akt expressing cells 15 min following addition of 20% FCS (Fig. 4A).

The Akt kinase activity of Rat1a (v-Akt) cells can be superinduced by 20% FCS. This induction was reduced by wortmannin to levels similar to that observed in 0% serum (Fig. 4A). Because the v-Akt kinase activity in 0% serum was comparable to that in the presence of serum and wortmannin, we tested whether v-Akt would still protect cells when PI 3-kinase activity was blocked. As shown in Figure 4B, cells expressing v-Akt are resistant to wortmannin-induced cell death to the same extent as they are to serum deprivation-induced cell death. Thus, in conditions of low Akt kinase activity (serum starvation or wortmannin treatment), cells are susceptible to apoptosis, whereas in conditions of higher kinase activity (presence of serum or activated Akt) cells are resistant.

To demonstrate that the endogenous Akt delivers the survival signal mediated by growth factors, we employed a mutant of Akt that contains a point mutation in the ATP binding site (Akt K179M) that was shown previously to abolish its kinase activity (Franke et al. 1995). This kinase-inactive Akt mutant, which previously has been suggested to act in a dominant-negative manner (Kohn et al. 1995), was toxic to Rat1a cells and could not be stably expressed. Low levels of the kinase-inactive c-Akt could be expressed, however, in Rat1a cells overexpressing Bcl-2 [Rat1a (Bcl-2) cells] (Fig. 5B, lane 2), but decreased the ability of Bcl-2 to inhibit cell death upon serum deprivation (Fig. 5C), and accelerated apoptosis in low serum concentrations (Fig. 5C,D). Although Bcl-2 is able to protect cells from wortmannin treatment for 4 hr in the presence of serum (Fig. 2B), it cannot override the effect of the Akt kinase-deficient mutant for a prolonged period of serum deprivation or at low serum concentrations (Fig. 5C,D). Thus, it is likely that the toxicity of the kinase-deficient mutant is a result of inhibition of endogenous Akt, further supporting our observation that Akt kinase activity is required for the anti-apoptotic signaling pathway induced by growth factors and serum.

The acceleration of apoptosis by the kinase-deficient Akt that was observed in the absence of serum (Fig. 5D) is possibly a result of inhibition of endogenous basal Akt kinase activity. This basal level of Akt kinase activity may reflect intracellular levels of PtdIns(3)P, a product of PI 3-kinase that was shown to activate Akt by binding to its pleckstrin homology (PH) domain (Franke et al. 1995) but whose level does not change significantly upon growth factor stimulation (Kapeller and Cantley 1994).

Taken together, these results strongly suggest that Akt is able to deliver a survival signal induced by growth factors and serum, and that this ability is dependent on its kinase activity. In addition, the ability of activated Akt to override the apoptotic effect of wortmannin in the presence of serum suggests that wortmannin induces apoptosis by inhibiting the ability of PI 3-kinase to activate Akt.

Akt does not elevate Bcl-2 and Bcl-X_L expression but inhibits PARP protease activity

The apoptotic pathway in mammalian cells, as in *C. elegans*, is coordinated by negative regulators that are

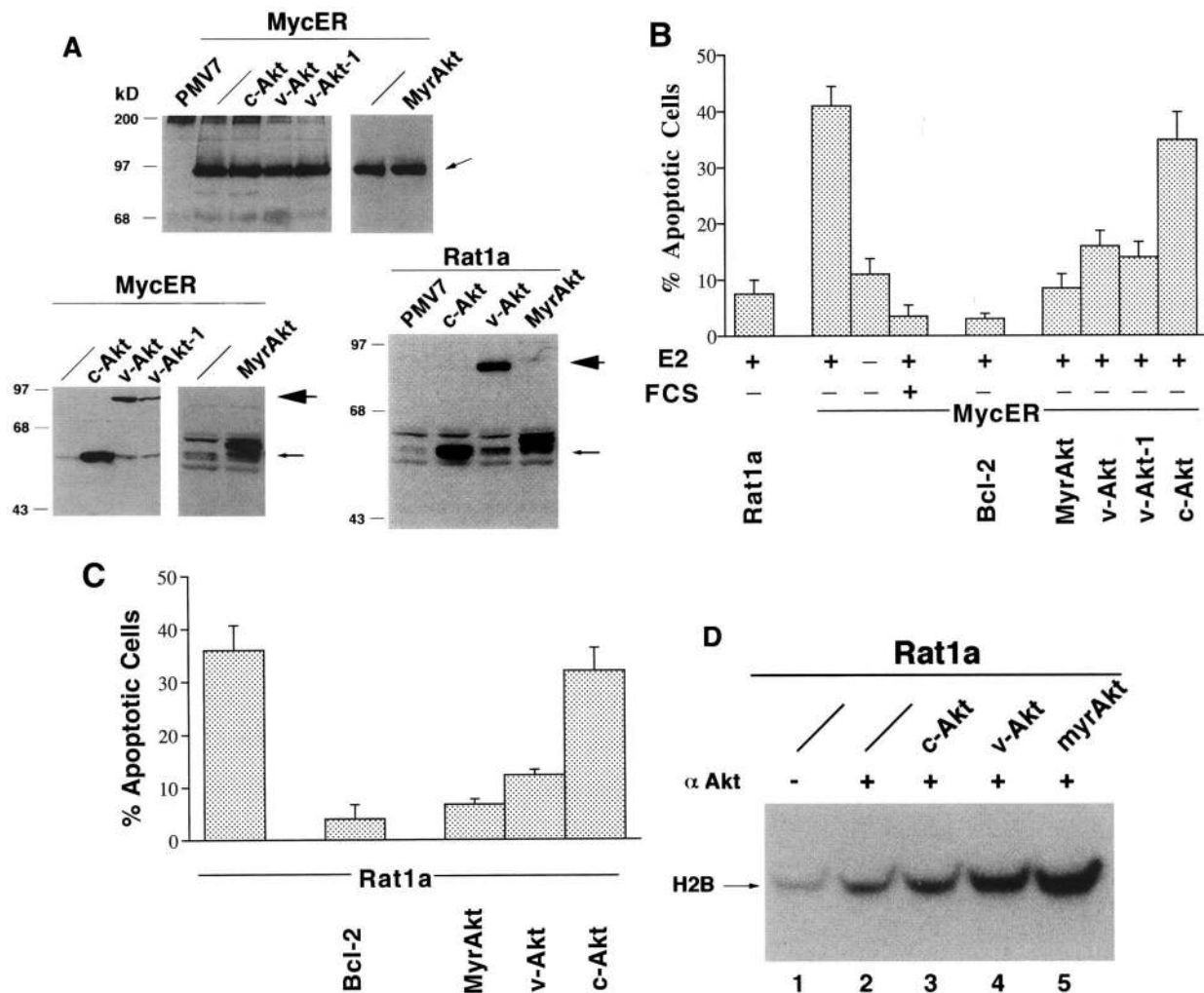


Figure 3. Activated Akt protects from Myc and non-Myc-accelerated apoptosis. (A) Immunoblot analysis of ectopically expressed proteins in Rat1a (MycER) and parental Rat1a cells. (Top) Expression of MycER; arrow indicates migration of MycER. (Bottom) Expression of Akt proteins; v-Akt and MyrAkt/c-Akt are indicated by large and small arrows, respectively. Molecular size markers (in kD) are indicated. The reduced mobility of the MyrAkt is a result of HA epitope tag in this construct. Expression in a pool of v-Akt expressing cells and in an individual clone (v-Akt-1) is shown. (B) Percentage of apoptotic cells scored by DAPI staining of Rat1a (MycER) nuclei 16 hr following MycER activation (\pm E2) in the presence of 10% FCS (+) or 0.5% FCS (-). The data presented here and in Fig. 1C were obtained simultaneously. (C) Percentage of apoptotic cells scored by DAPI staining of Rat1a nuclei 16 hr following incubation in 0% FCS. Rat1a cells were plated at 50,000 cells/3-cm-diam. plate and stained with DAPI. (D) Basal Akt kinase activity of Rat1a cells (lanes 1,2) and Rat1a cells expressing the indicated Akt proteins measured by histone 2B (H2B) phosphorylation. Cells were starved in 0% FCS overnight. Extracts were then immunoprecipitated with rabbit IgG (lane 1) or α -Akt (lanes 2–5). Akt kinase assay was performed, with the immunoprecipitants and histone H2B used as a substrate, and was quantitated with the AMBIS system.

members of the Bcl-2 family that promote survival, and by positive regulators that are the members of the Ced3/ICE family of proteases that can actively kill cells. Thus, potential mechanisms by which Akt promotes survival may include activation of the Bcl-2 family members or/and inactivation of the Ced3/ICE family of proteases (Takahashi and Earnshaw 1996). As shown in Figure 6, A and B, we did not observe any change in the steady-state levels of Bcl-2 or Bcl-X_L after serum deprivation regardless of activated Akt expression. We then examined the activity of the PARP protease (Tewari et al. 1995), (Fig. 6C), which can specifically cleave PARP to yield an 85-

kD fragment and has a similar substrate specificity as the *C. elegans* Ced3 protease (Xue et al. 1996). We found that upon serum withdrawal, caspase activity that cleaves PARP is induced (Fig. 6C, lanes 1–6,13), whereas in cells that constitutively express activated Akt the induction of this activity is inhibited (Fig. 6C, lanes 7–12,14). Similar results were obtained when a fluorogenic substrate DEVD-AFC, which mimics a site on PARP, was used as a cleavage substrate for cell lysates made at different time points following induction of apoptosis (Fig. 6D,E). By using a fluorogenic substrate, YVAD-AFC, which mimics a site on pro-IL-1 β , we found

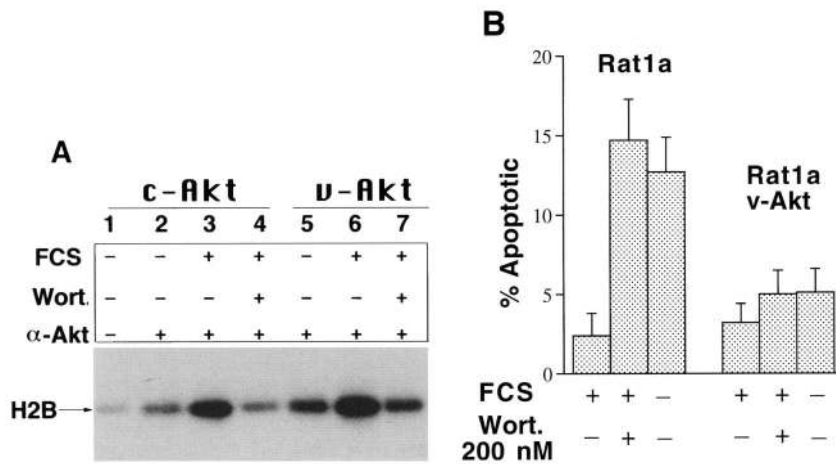


Figure 4. Akt-mediated survival signal is dependent on Akt kinase activity. (A) Akt kinase activity of c-Akt- and v-Akt-overexpressing Rat1a cells in various cell culture conditions. Cells were starved overnight in 0% FCS, then rinsed twice and placed in 20% serum (\pm FCS) with or without cotreatment of 200 nM wortmannin (\pm Wort.). After 15 min, cell lysates were incubated with rabbit IgG ($-\alpha$ -Akt) or anti-Akt antibodies ($+\alpha$ -Akt) and Akt kinase activity was measured in the immunoprecipitants. A representative kinase assay is shown. (B) Percent apoptotic Rat1a cells and Rat1a (v-Akt) cells following serum starvation and wortmannin treatment. Cells were starved overnight in 0.5% FCS then rinsed twice and placed in 0% serum \pm 200 nM wortmannin. FCS (5%) was added after 30 min, and after an additional 3.5 hr nuclei were DAPI stained. Bars represent averages (\pm S.E.) of four independent experiments.

no induction of this enzymatic activity following serum deprivation (Fig. 6D,E). The induction of caspase activity that cleaves PARP was more pronounced in cells that were deprived of serum in conjunction with MycER activation, but the activity was also observed in Rat1a cells deprived of serum (Fig. 6C, lanes 13,14; Fig. 6E). The inhibition of the caspase activity that cleaves PARP by activated Akt is only slightly less than the inhibition observed by ectopically expressed Bcl-2 (Fig. 6D). We therefore concluded that a caspase activity that cleaves PARP [which is likely to be mediated by CPP32 (Tewari et al. 1995)], and not ICE-like activity, is induced after serum deprivation. This protease activity is dramatically inhibited by activated Akt and by Bcl-2.

Discussion

The anti-apoptotic properties of certain growth factors and cytokines are not necessarily coupled to cellular proliferation (Fairbairn et al. 1993; Raff et al. 1993; Harrington et al. 1994). Here we show that the Ras/Raf and Src signaling pathways, although mitogenic, are not sufficient to promote survival upon growth factor withdrawal. However, although signal transduction through the PI 3-kinase/Akt pathway can activate a survival signal, we found that activated Akt is not sufficient to induce serum-deprived cells to enter the cell cycle, as measured by bromodeoxyuridine (BrdU) incorporation (S.G. Kennedy and N. Hay, unpubl.). These results may explain why some mitogenic growth factors, such as EGF, which are not effective inducers of the PI 3-kinase/Akt signaling pathway, are also poor survival factors in cells of mesenchymal origin (Harrington et al. 1994; Franke et al. 1995).

The inhibition of apoptosis elicited by activated Akt is similar to or higher than the level of inhibition promoted by IGF-I, insulin, and PDGF (Harrington et al. 1994). How-

ever, these anti-apoptotic effects are about twofold lower than the serum-mediated block of apoptosis, suggesting that serum may activate multiple signaling pathways that inhibit apoptosis. These may include distinct PI 3-kinases and Akt proteins that are induced by multiple extracellular signals or other unidentified pathways. These signals may also include activation of atypical PKCs and/or NF- κ B (Diaz-Meco et al. 1996; Liu et al. 1996).

It was shown previously that activation of Akt is not induced by stably expressed activated Ras and is not affected in cells that express dominant-negative Raf or cells that are deficient in Src (Burgering and Coffey 1995; Franke et al. 1995). These results are consistent with the observations presented here. However, both Ras and Src have been shown to activate PI 3-kinase (Pleiman et al. 1994; Rodriguez-Viciana et al. 1994). One possible explanation for this apparent paradox is that the levels of stably expressed activated Ras or Src, although able to transform cells in the presence of serum, are not sufficient to activate the PI 3-kinase/Akt signaling pathway in the absence of serum. However, transient high-level expression of both activated Ras and Src activates Akt (Datta et al. 1996). Another possible explanation is that the ability of Ras and Src to activate PI 3-kinase is cell type-specific.

The ability of activated Akt to promote survival is not limited to fibroblasts, as we found that activated Akt protects PC12 pheochromocytoma cells from undergoing apoptosis upon growth factor withdrawal (S.G. Kennedy and N. Hay, unpubl.) and a neuronal cell line from undergoing apoptosis following induction of differentiation (E. Eves, A. Bellacosa, P.N. Tsichlis, M.R. Rosner, and N. Hay, unpubl.). However, Akt might not be a general inhibitor of apoptosis as we have found that activated Akt does not effectively block etoposide- or UV-induced apoptosis in the presence of serum (S.G. Kennedy and N. Hay, unpubl.).

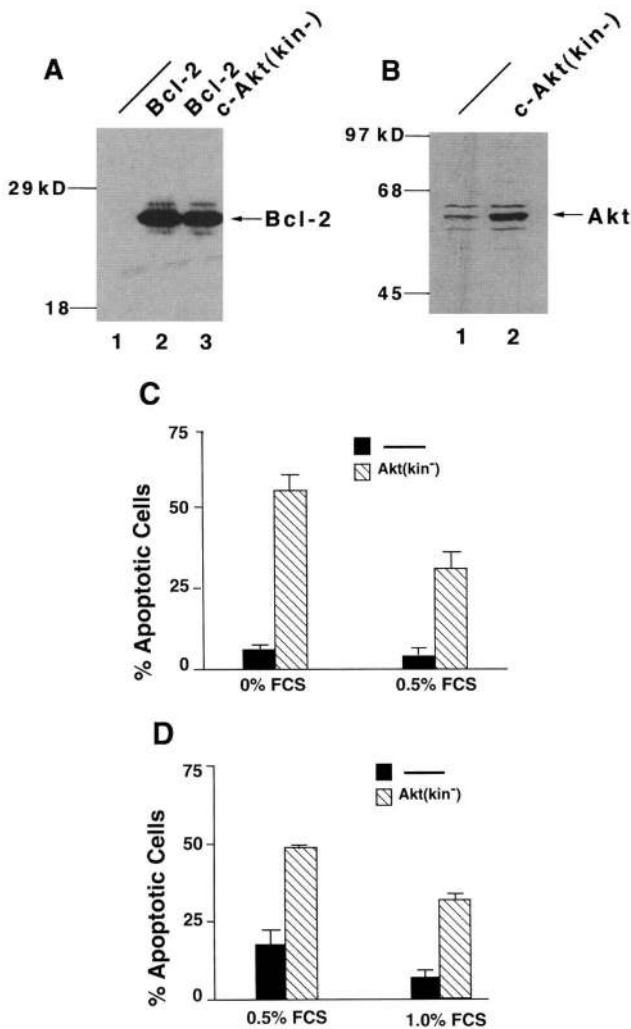


Figure 5. A dominant-negative Akt mutant containing a point mutation in the ATP binding site accelerates apoptosis. (A) Expression levels of ectopically expressed human Bcl-2 in Rat1a (Bcl-2) and Rat1a (Bcl-2/Akt kin⁻). (Lane 1) Rat1a cells; (lane 2) Rat1a(Bcl-2) cells; (lane 3) Rat1a (Bcl-2/Akt kin⁻) cells. (B) Expression level of ectopically expressed Akt K179M in Rat1a (Bcl-2) cells. (Lane 1) Rat1a (Bcl-2) cells; (lane 2) Rat1a (Bcl-2/Akt kin⁻) cells. Percent apoptotic Rat1a (Bcl-2) and Rat1a (Bcl-2/Akt kin⁻) cells as measured by DAPI staining after 30 hr (C) or 60 hr (D). Cells were seeded as described in Fig. 3C and then in 0%, 0.5%, or 1.0% FCS for 30 hr or 60 hr. Bars represent averages (\pm S.E.) of three independent experiments.

How does Akt promote survival?

Two major components play a role in the control of the cell death pathway both in *C. elegans* and in mammals: the Bcl-2 family members, which block apoptosis, and the Ced-3/ICE-like protease family, which executes the apoptotic pathway. The PI 3-kinase/Akt signaling pathway might be predicted to act through modulation of Bcl-2 expression, and it was shown that expression of Bcl-2 is induced by IL-2 (Miyazaki et al. 1995). However, we did not detect any significant changes in the steady-

state levels of Bcl-2 and Bcl-X_L in Rat1a cells that constitutively express an activated Akt. In addition, we did not detect any changes in the steady-state level of Bax following serum withdrawal or upon activation of Akt (S.G. Kennedy and N. Hay, unpubl.). It is possible that, in hemopoietic cells, the PI 3-kinase/Akt pathway can induce Bcl-2 expression. This could be an indirect effect of an autocrine loop mediated by PI 3-kinase/Akt that induces secretion of cytokines such as IL-2, which will then elicit both proliferation and survival. We found, however, that the ability of Akt to mediate survival was associated with the inhibition of Ced-3/ICE-like protease(s) that specifically cleave PARP. This cleavage activity is likely to be mediated by CPP32, which recognizes the peptide sequence DEVD (Tewari et al. 1995). Interestingly, upon serum withdrawal, unlike Fas-mediated apoptosis (Enari et al. 1996), the activity of PARP protease is induced without any detectable induction of ICE-like activity that can specifically cleave pro-IL-1 β and recognizes the peptide sequence YVAD. We concluded that, at least in fibroblasts, growth factors and Akt block CPP32-like activity (caspase activity that cleaves PARP) and promote survival by a mechanism independent of changes in Bcl-2 or Bcl-X_L expression. We cannot completely exclude the possibility that an Akt signaling pathway, although not altering the expression of Bcl-2 and Bcl-X_L, may render them hyperactive by a post-translational mechanism (Uhlman et al. 1996). Further support for a possible interplay between Bcl-2 activity and Akt signaling is suggested by our observations that Bcl-2 inhibits wortmannin-induced cell death and that a dominant-negative Akt antagonizes the ability of Bcl-2 to promote survival.

Because there are multiple downstream effectors of Akt, it is not completely clear how the survival signal is transduced. It is possible that multiple pathways contribute synergistically to the anti-apoptotic function of Akt. The inhibition of Ced-3/ICE-like activities (caspase activity that cleaves PARP) could rely on known potential downstream effectors of Akt or an unknown pathway. A model illustrating multiple potential mechanisms by which Akt delivers a survival signal is shown in Figure 7.

One potential downstream effector is TOR/FRAP (target of rapamycin/FKBP rapamycin-associated protein) (Brown and Schreiber 1996). TOR is conserved between yeast and man, and TOR kinase activity can be inactivated by the drug rapamycin (Barbet et al. 1996; Kunz and Hall 1993). Genetic experiments in yeast as well as experiments employing rapamycin in mammalian cells have shown that deletion of TOR or inactivation by rapamycin leads to G₁ arrest (Barbet et al. 1996). However, rapamycin was also shown to accelerate apoptosis in several cell systems subjected to different types of insult (Shi et al. 1995). Thus, depending on the level of the insult, the inactivation of TOR may mediate either growth arrest or apoptosis, similar to activation of p53 (Bates and Voudsen 1996). It is therefore possible that constitutive activation of TOR (presumably by an activated Akt) is analogous to inactivation or deletion of

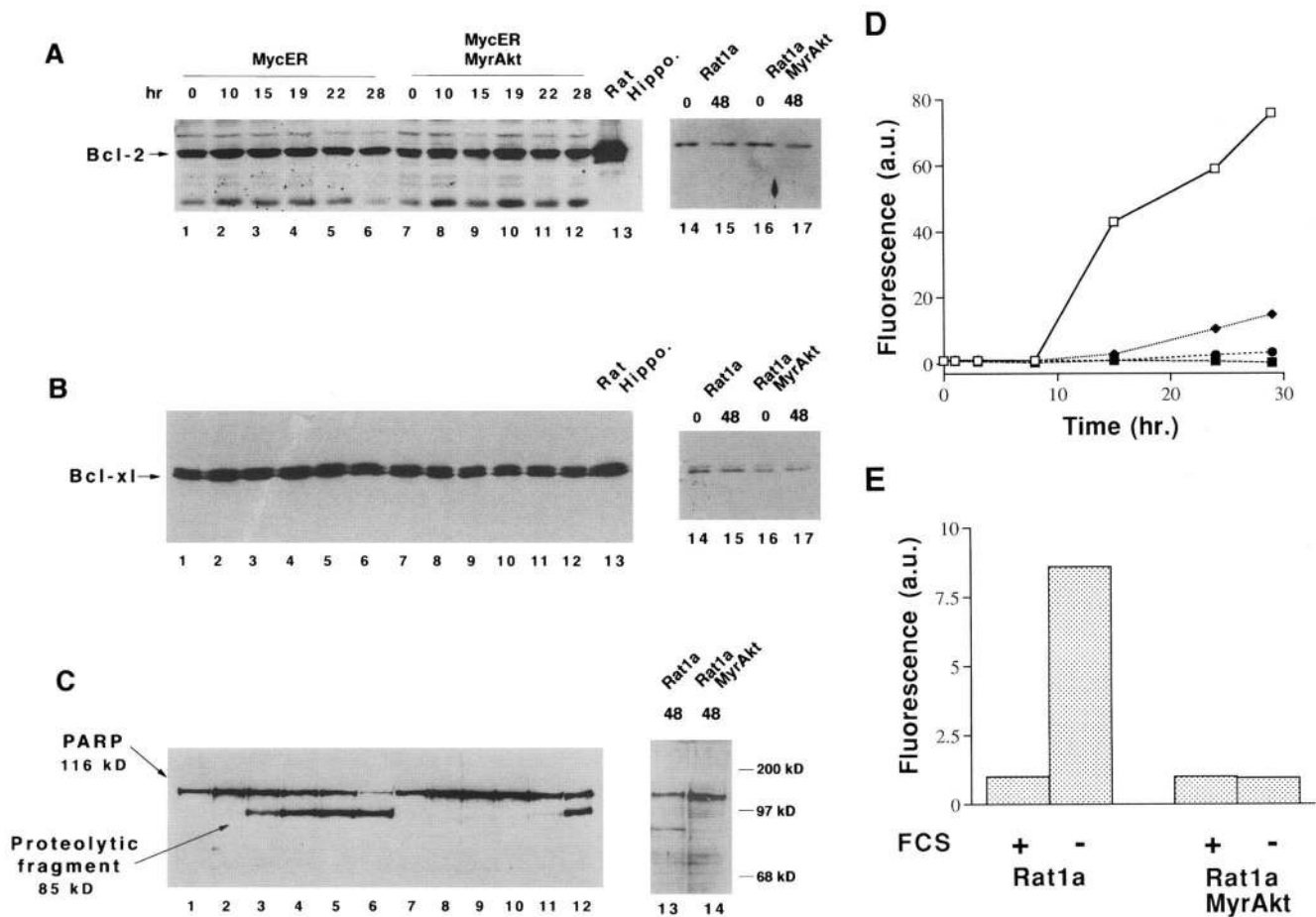


Figure 6. Akt does not elevate the expression of Bcl-2 or Bcl-X_L, but inhibits activation of a PARP protease upon serum withdrawal. Rat1a(MycER) and Rat1a (MycER/MyrAkt) cells were serum-starved (0.5% FCS) and treated with 1 μ M E2 for the indicated times. Rat1a and Rat1a(MyrAkt) cells were serum-starved (0% FCS) for 48 hr. (A) Immunoblot analysis of Bcl-2 expression. Expression of Bcl-2 in Rat1a(MycER) cells following serum withdrawal and activation of Myc (lanes 1–6). Expression of Bcl-2 in Rat1a (MycER/MyrAkt) cells following serum withdrawal and activation of Myc (lanes 7–12). Bcl-2 expression in Rat1a and in Rat1a(MyrAkt) cells following serum withdrawal (lanes 14–17). Extract from rat hippocampal neurons was used as a control for the endogenous rat Bcl-2 protein (lane 13). (B) Immunoblot analysis of Bcl-X_L expression. Expression of Bcl-X_L in Rat1a(MycER) cells following serum withdrawal and activation of Myc (lanes 1–6). Expression of Bcl-X_L in Rat1a (MycER/MyrAkt) cells following serum withdrawal and activation of Myc (lanes 7–12). Bcl-X_L expression in Rat1a and in Rat1a(MyrAkt) cells following serum withdrawal (lanes 14–17). Extract from rat hippocampal neurons was used as a control for the endogenous rat Bcl-X_L protein (lane 13). (C) Immunoblot analysis of PARP cleavage. PARP cleavage in extracts made from cells treated as described in A and B (lanes 1–12). PARP cleavage in Rat1a and Rat1a(MyrAkt) 48 hr following serum withdrawal (lanes 13,14). The full-length PARP (116 kD) and its proteolytic fragment (85 kD) are indicated by arrows. (D) Kinetics of CPP32-like and ICE-like protease activity in Rat1a cell lines with DEVD-AFC (MycER, □; MycER/MyrAkt, ◆; and MycER/Bcl-2, ●) and Rat1 cell line MycER with YVAD-AFC (■) at different time points following serum withdrawal. (E) Activity of CPP32-like protease in Rat1a and Rat1a (MyrAkt) cell lysates extracted after 48 hr in 0% serum.

p53. Alternatively (or additionally), apoptosis may be inhibited by acceleration of protein translation, which can be mediated by the ability of Akt, either directly or in conjunction with TOR, to activate p70 S6-kinase or/and to inhibit 4E-BP1 (Burgering and Coffey 1995; Beretta et al. 1996). The activation of p70 S6-kinase by Akt might not be sufficient, however, to promote survival because we found that a dominant-negative Akt inhibited S6-kinase activity by only 30% (S.D. Conzen, S.G. Kennedy, and N. Hay, unpubl.), whereas the effect of the dominant-negative Akt on apoptosis was much more pronounced. Further experiments are required to verify this

hypothesis and to identify downstream targets for this enhanced translation.

Another downstream effector of Akt/PKB is glycogen synthetase kinase 3 (GSK-3) (Cross et al. 1995). GSK-3 activity is inhibited by growth factors and by Akt (Welsh et al. 1994; Cross et al. 1995). GSK-3 was also shown to play a role in cell fate determination in both invertebrates and vertebrates (Briscoe and Firtel 1995). Whether and how GSK-3 activity is associated with programmed cell death remains to be determined. In adult tissues, it is possible that activation of GSK-3 leads to activation of the adenomatous polyposis coli (APC) protein, which

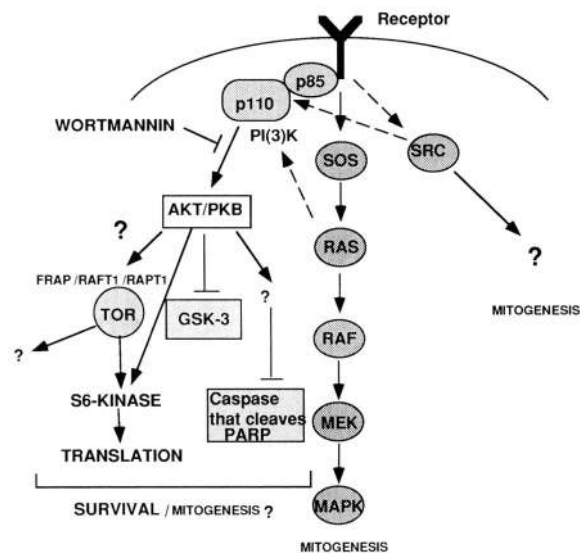


Figure 7. Model illustrating paths leading from growth factor receptor to mitogenesis and survival pathways. According to this model, activation of Ras and Src will lead mainly to mitogenesis, whereas activation of Akt will lead to survival. The ability of Akt to mediate survival is likely to be dependent on downstream effectors such as p70 S6-kinase and protein translation, activation of FRAP/TOR, and inhibition of GSK-3. According to this model, if TOR can be constitutively activated by Akt, it will promote survival in a manner analogous to inactivation of p53 (for more details see Discussion). These downstream effectors of Akt or other independent Akt-induced activity may lead to inhibition of the caspase activity that cleaves PARP.

may then accelerate apoptosis by degradation of β -catenin and inhibition of adhesion. This hypothesis is based on recent observations that placed APC downstream of GSK-3 and upstream of β -catenin (Matsumine et al. 1996; Peifer 1996; Rubinfeld et al. 1996), and on a recent report that showed that ectopically expressed wild-type APC accelerates apoptosis (Morin et al. 1996). Accordingly, it is feasible that inactivation of GSK-3 by Akt can contribute to the block of apoptosis. It is worth noting that increased adhesion by extracellular matrix has been shown to block ICE activity and inhibit apoptosis, and that fibronectin can inhibit apoptosis upon serum withdrawal (Boudreau et al. 1995; S.G. Kennedy and N. Hay, unpubl.). Interestingly, fibronectin has also been shown to activate Akt (W.G. King, M.D. Mattaliano, and J.S. Brugge, pers. comm.).

Stress-inducible kinases, which are modestly activated by wortmannin-induced inhibition of PI 3-kinase (Kharbanda et al. 1995; S.G. Kennedy and N. Hay, unpubl.), were shown previously to be associated with the induction of apoptosis (Verheij et al. 1996). Thus, the PI 3-kinase/Akt signaling pathway might inhibit activation of stress-inducible kinases and, in this manner, inhibit a pro-apoptotic signal from the stress kinases. However, we have not been able to detect any reduced activity of the stress-inducible kinase JNK in cells that constitu-

tively express activated Akt, following UV irradiation (S.G. Kennedy and N. Hay, unpubl.).

We have demonstrated that the PI 3-kinase/Akt pathway has an important role in mediating cell survival. It is also possible that, by mediating survival, the PI 3-kinase/Akt pathway has a more global role in embryonic development and in longevity of adult tissues. A recent report has implicated a *C. elegans* homolog of p110, the catalytic subunit of the mammalian PI 3-kinase, as a mediator of longevity (Morris et al. 1996). Because Akt homologs were found in *C. elegans* (Waterston et al. 1992), it is conceivable that Akt also plays a role in longevity.

Further delineation of the PI 3-kinase/Akt signaling pathway may yield attractive targets for cancer chemotherapy. Many current chemotherapy regimens rely on induction of apoptosis (Thompson 1995), and many tumors have evaded cell death by activation of survival pathways such as Bcl-2 overexpression (Wagner et al. 1993) or by up-regulation of expression of potential survival factors (Christofori et al. 1994). Interestingly, Akt has been shown to be overexpressed in some human tumors (Staal 1987; Bellacosa et al. 1995). Specific inhibitors of kinases in these survival pathways may be therapeutic by themselves or, if used in combination with current chemotherapy modalities, may enhance tumor cell sensitivity. Our findings suggest that interfering with the Akt-mediated survival signal, by a dominant-negative Akt mutant, abrogates both serum- and growth factor-mediated survival and survival induced by Bcl-2 overexpression. Similarly, specific activators of this pathway would be expected to promote cell survival and might potentially be used for treatment of degenerative diseases that result from inappropriately increased cell death.

Materials and methods

Cell culture and viral infection

Cell culture, retrovirus stock production by transient transfection of retroviral vectors into BOSC23 cells, and retroviral infection were performed as described previously (Wagner et al. 1994). The retroviral vectors used were pMV12 for MycER, pMV7 or pMV12 for Bcl-2 (Wagner et al. 1993), pZIPNeo for V12H-Ras (Yao and Cooper 1995), pLNC for Δ Raf-1 (Samuels et al. 1993), pMX2122 for v-Src (gift of M. McMahon, DNAX, Palo Alto, CA), pLXSN for v-Akt and c-Akt, and SR- α for MyrAkt (Franke et al. 1995).

Protein analysis

Total cell protein from 1×10^5 cells was denatured in 2 \times Laemmli buffer. Extracts were electrophoresed in 8% or 10% SDS-polyacrylamide gels. Following electrophoresis, proteins were blotted onto nitrocellulose membranes by semidry transfer and incubated with the following primary antibodies: H222 rat monoclonal anti-ER (gift of G. Greene, University of Chicago, IL), rabbit polyclonal anti-Bcl-2 (Santa Cruz n-19), mouse monoclonal anti-v-Src (LA074, Quality Biotech), rabbit polyclonal anti-Raf-1 (C-12, Santa Cruz Biotech, Inc.), anti-H-Ras (259, Santa Cruz Biotech, Inc.), anti-c-Akt (Franke et al. 1995),

and mouse monoclonal anti-PARP c2-10 (gift of G. Poirier, Sante-foy, Quebec, Canada). For the PARP protease assay cells were resuspended in lysis buffer containing 6 M urea and were sonicated before loading on gel.

Soft agarose assays

Five thousand cells were plated in 0.35% Sea Plaque agarose on top of a layer of 0.7% Sea Plaque agarose in DMEM with 10% FCS. Colonies were allowed to grow for 24 days. Plates were projected onto a white board and the number of colonies >0.5 mm in diameter were counted.

Apoptosis assays

For DAPI staining, cells were seeded at 1.5×10^5 cells/3-cm-diameter plate and allowed to grow in 10% FCS for 24 hr. Cells were rinsed once in PBS and 2 ml of DMEM containing 0.5% FCS and 1 μ M of E2 was added; 2 ml of DMEM without FCS was added to Rat1a cells without MycER. After 16 hr, 400 μ l of formaldehyde was added to each well for 30 min. Cells were then rinsed with PBS and cold 0.1% Triton X-100/0.1% sodium citrate in PBS was added for 2 min. Cells were rinsed with PBS and 1 μ M DAPI in PBS was added for 1 min. Cells were rinsed twice with PBS and air-dried. Ten microliters of mounting solution (50% glycerol-TBS at pH 9.0, and 1 mg/ml of phenylene diamine) was added, overlaid with a coverslip, and sealed with nail polish. Three fields per plate containing a minimum of 100 cells were scored, using an ultraviolet microscope, for percentage of apoptotic cells. DAPI staining experiments described in Figures 1C and 3B were performed simultaneously.

For PI/FDA staining, cells were seeded at 150,000 cells/3-cm diameter plate in 10% FCS for 24 hr. Cells were rinsed once in PBS and 2 ml of DMEM containing 0.5% FCS \pm 1 μ M of E2 was added for 48 hr. Five hundred microliters of 10 μ g/ml of FDA and 30 μ g/ml of PI in PBS was added to each well. After 5 min, cells were photographed under UV illumination. Each field contained a minimum of 200 cells. Slides were projected onto a white board and live (FDA stained) and dead (PI stained) cells were counted.

The TUNEL assay was a modification of the DAPI assay. Cells were seeded onto single well Costar glass slides. Following addition of 0.1% Triton X-100/0.1% sodium citrate (see above), slides were rinsed twice with PBS. Slides were air-dried, then 50 μ l of TUNEL solution (Boehringer Mannheim) was added. Slides were placed in a humidified chamber at 37°C for 1 hr, then rinsed twice with PBS and counterstained with 1 μ M DAPI in PBS. Slides were allowed to dry, mounted, and fixed as described above.

Akt kinase assay

Rat1a cells (3×10^5) were plated on 6-cm diameter plates and grown overnight, then starved overnight in 0% FCS. Immunoprecipitation and kinase assays were done according to Franke et al. (1995). Protein concentration was determined by Bradford assay and equal amounts of protein were used for immunoprecipitation. Control immunoprecipitations were done with rabbit IgG pre-bound to protein A/G-agarose beads. Quantitation of phosphorylated H2B was determined by radio-imaging of dried gels on an AMBIS system.

In vitro assay for aspartase activity (ICE-like and CPP32-like activities)

Cells were seeded as described above for the apoptosis assays. Cells were rinsed in cold PBS and scraped into 1 ml buffer of 25

mM HEPES (pH 7.5), 5 mM EDTA, 1 mM EGTA, 5 mM MgCl₂, 5 mM DTT, 10 μ g/ml of pepstatin/leupeptin, and 1 mM PMSF. Cells were sonicated on ice. Cell lysates were centrifuged for 30 min at 160,000g and supernatants were stored at -80°C. Protein concentration was quantitated by Bradford assay. Lysates (10 μ g of protein) in 1 ml of 25 mM HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, and 10 mM DTT were incubated with fluorescently conjugated peptides DEVD-AFC (15 μ M) and YVAD-AFC (25 μ M) (Enzyme System Products, Dublin, CA) at 37°C for 30 min. Fluorescence of cleaved peptides was quantified in a fluorometer (excitation 400 nm, emission 505 nm).

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