The Akt Proto-oncogene Links Ras to Pak and Cell Survival Signals*

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The Ras oncogene regulates cellular proliferation, differentiation, transformation, and survival through multiple downstream signals. Ras signals through its effector phosphoinositide 3 (PI3) kinase to the Pak protein kinase (p65Wc), but the steps from Ras to Pak remain to be elucidated. PI3 kinase can stimulate the small G protein, Rac, a direct activator of Pak, as well as the Akt proto-oncogene, a serine-threonine protein kinase. We found that activated Akt stimulated Pak, whereas a dominant negative Akt inhibited Ras activation of Pak in transfection assays. Akt stimulation of Pak was not inhibited by dominant negative mutants of either Rac or Cdc42 suggesting that Akt activated Pak through a GTPase-independent mechanism. We also developed a novel cell-free system to study Ras activation of Pak. In this system Ras activated Pak only in the presence of a crude cell extract but failed to activate Pak when Akt was immunodepleted from the extract. Akt protects cells from apoptosis through phosphorylation of downstream targets such as the Bcl-2 family member, Bad. We found that activated Pak decreased apoptosis and increased phosphorylation of Bad, whereas dominant negative Pak increased apoptosis and decreased phosphorylation of Bad. These studies define a new oncogene-mediated cell survival signal.

Cell transformation requires modulation of signals from oncogenes to stimulate anchorage-independent proliferation, rearrange the actin cytoskeleton, and promote cell survival. This is achieved through the coordinated regulation of multiple signals. The Ras oncogene, a small G protein, can promote these three events through downstream targets such as Raf and phosphoinositide 3 (PI3) kinase (1–3). PI3 kinase stimulates the small G protein, Rac, a direct activator of Pak, as well as the Akt proto-oncogene, a serine-threonine protein kinase. We found that activated Akt stimulated Pak, whereas a dominant negative Akt inhibited Ras activation of Pak in transfection assays. Akt stimulation of Pak was not inhibited by dominant negative mutants of either Rac or Cdc42 suggesting that Akt activated Pak through a GTPase-independent mechanism. We also developed a novel cell-free system to study Ras activation of Pak. In this system Ras activated Pak only in the presence of a crude cell extract but failed to activate Pak when Akt was immunodepleted from the extract. Akt protects cells from apoptosis through phosphorylation of downstream targets such as the Bcl-2 family member, Bad. We found that activated Pak decreased apoptosis and increased phosphorylation of Bad, whereas dominant negative Pak increased apoptosis and decreased phosphorylation of Bad. These studies define a new oncogene-mediated cell survival signal.

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§§ The abbreviations used are: PI3, phosphoinositide 3; MBP, myelin basic protein; p70SRK, p70 ribosomal protein S6 kinase.

promotes cell survival through the Akt proto-oncogene (also known as PKBα) (4–7). Akt is a serine-threonine kinase that promotes cell survival by regulating factors such as Bad, caspase-9, forkhead, and nuclear factor κB (8–12). Another family of effectors downstream of PI3 kinase are the serine-threonine kinases p70SRK (Pak), which are direct targets of both Rac and Cdc42 (13–15). In many cells Pak mediates signals directly from Ras through PI3 kinase to sustain cell transformation, but the steps from PI3 kinase to Pak remain poorly understood (16–18). Here we show that Akt is a key intermediate between Ras and Pak. In addition, activated Pak decreases apoptosis, whereas dominant negative Pak increases apoptosis. These studies define a new Akt survival signal.

EXPERIMENTAL PROCEDURES

Transfection Assays for Kinase Activity—Rat-1 cells were transfected with 1 μg of Pak, 1.5 μg of each test plasmid, and 2.5–4 μg of pUC19 plasmid, as required, to bring the total to 5 μg of total DNA. The plasmids and immune kinase assays to measure Pak activity have been described elsewhere (6, 16, 18, 19). Cell-free Activation of Pak—H-Ras was purified as a glutathione S-transferase fusion protein. K-Ras 4B-D12 was purified from a baculovirus expression system using DE-52 chromatography and a gel filtration column as described (20). Cell lysates were prepared by washing cells with cold phosphate-buffered saline followed by lysis with 40 mM HEPES (pH 7.4), 1% Nonidet P-40, 100 mM NaCl, 1 mM EDTA, 25 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotonin. Samples were then centrifuged at 12000 × g for 20 min at 4 °C, and the supernatants were collected and frozen until needed. Ras was activated by incubating 2 μg of Ras in 1 mM GTP, 50 mM EDTA for 1 h followed by treatment with 50 mM MgCl2. Pak was prepared from Rat-1 cells transfected with Myc-tagged Pak1 and incubated with 9E10 antibody and protein A-agarose for 2 h at 4 °C. Samples were mixed for 30 min on a rotating incubator at 4 °C and washed three times with lysis buffer and twice with 2× phosphorylation buffer (10 mM MgCl2, 40 mM HEPES, pH 7.4). Samples were then incubated with 5 μg of peptide p47SRK^234–331 (21) for 5 min on ice. Kinase assays were initiated by the addition of 10 μCi of [32P]ATP (3000 Ci/mmol) and 20 μM (final concentration) ATP following incubation for 20 min at 22 °C. Next, 25 μl of the reaction samples were loaded onto SpinZyme™phosphocellulose units (Pierce), centrifuged through the membrane into collection tubes (for 20 s), washed twice with 500 μl of wash solution (75 mM phosphoric acid), and filters were counted.

RESULTS

Several proteins are regulated by PI3 kinase including the Akt protein kinase and the p70 ribosomal protein S6 kinase (p70SRK). To test whether Akt signals to Pak, we co-transfected Akt into Rat-1 cells and assayed Pak in an immune kinase assay using MBP as a substrate. Akt stimulated Pak about 10-fold over basal levels, whereas activated Pak failed to stimulate Akt (Fig. 1; data not shown for Akt kinase assays). Pak stimulation was seen following overexpression of two different Akt constructs, wild-type and membrane-targeted Akt but not K179M, a kinase-deficient mutant. In most experiments stimulation by Akt was about half the maximum observed with Rac and about the same as that observed with PI3 kinase. The p70SRK inhibitor, rapamycin, had no effect (data not shown). These data suggest that Akt but not p70SRK is upstream of Pak.

To determine whether Akt was required to mediate the signal from Ras to Pak we tested K179M, a dominant negative Akt mutant. K179M inhibited both Ras and PI3 kinase activation of Pak but not Rac activation of Pak (Fig. 2a). LY294002, a specific PI3 kinase inhibitor, inhibited both Ras and PI3 kinase activation of Pak but did not block Akt or Rac activation of Pak.
These experiments place Akt downstream of Ras and PI3 kinase but upstream of, or perhaps parallel to, Rac. To place Akt relative to Rac and Cdc42 we tested the effects of the dominant negative mutants RacN17 and Cdc42N17 on Akt activation of Pak. Although both dominant negative mutants partially blocked Ras activation of Pak, neither mutant inhibited Akt activation of Pak (Fig. 2c). Together these data suggest that Akt is an essential mediator of Ras activation of Pak but stimulates Pak independently of Rac and Cdc42.

To confirm the transfection studies we developed a cell-free system as an independent assay for Ras activation of Pak. In this system purified recombinant H-Ras or K-Ras (K-Ras 4B-D12) is incubated with extracts from cells expressing Myc-tagged Pak, Pak is then immunoisolated, and its activity is measured using MBP as a substrate (Fig. 3a). Pak was activated with Ras-GTP, but not Ras-GDP or GTP alone. Hence, only the GTP-activated Ras activates Pak. We modified the cell-free system by employing a rapid filter binding assay to measure phosphorylation of a specific Pak substrate, a peptide derived from the p47phox phosphorylation site (21). With this modified assay, the three key components, Ras-GTP, Pak (immunoisolated from transfected Rat-1 cells), and the cell lysate (prepared from untransfected cells), are added separately, which allowed us to rapidly screen different cells. Extracts from Rat-1 cells, MCF-7 cells, Schwann cells, pig brain, and pig spleen all supported substantial levels of Pak activation, but NIH 3T3 cells showed minimal complementation (Fig. 3b; data not shown). In addition, Pak activation was not detectable in extracts from Rat-1 cells (data not shown). Thus the Ras to Pak signal is common to many cell types and multiple Pak isoforms.

Next, to test whether Akt was required in the cell-free system, we depleted the Rat-1 extracts with anti-Akt antibodies (Fig. 3c, inset). Extract incubated with Akt antibodies was almost devoid of activity, whereas extracts incubated with either a nonspecific control antibody or with an antibody against p70S6K still supported robust activation (Fig. 3c). We obtained similar results when we substituted Pak purified from a baculovirus expression system for the immunoisolated Pak in these experiments (data not shown). These experiments support data from the transfection assays that suggest that Akt, but not p70S6K, mediates Ras activation of Pak.

Akt mediates signals from Ras and PI3 kinase to promote survival in part by phosphorylating and inactivating apoptotic factors such as Bad (22, 23). Because Pak is downstream of Akt, we determined whether Pak affects cell survival signals. We tested PI3 kinase-dependent survival by inducing apoptosis by serum starvation or serum starvation combined with 20 μM LY294002. This concentration was chosen because it inhibits Ras activation of Pak about 80% in transfection assays (18) and produces a moderate amount of cell death, which allows detection of both increases and decreases in apoptosis (data not shown). Cells treated under both conditions displayed changes characteristic of apoptosis, including cell shrinkage, membrane blebbing, nuclear condensation, and DNA fragmentation. To test the role of Pak, we utilized G418-selected mass cultures of Rat-1 cells stably expressing Pak mutants. The cell lines that we generated express Pak at levels about 2-fold above the endogenous Pak1. The constructs used in these studies were...
Akt is required to activate Pak in a cell-free system. For MBP assays Ras-GTP was added to crude cell extracts from cells transfected with Pak1, and the kinase assays were performed. a. Ras activation of Pak using MBP as a substrate. B. Ras activation of Pak using p47phox-derived peptide (YRRNNSVFR) as a substrate. c. Akt depletion using p47phox as a substrate. Antibody depletion was performed by incubating 5 μg of antibody (anti-Akt antibody NEB, catalog number 9272, from New England Biolabs, p70S6K, catalog number 230, from Santa Cruz Biotechnology) per 100 μl of Rat-1 cell lysate with protein A-agarose on a rotating incubator at 4 °C for 3 h. The cell lysate was then centrifuged at 14000 g for 5 min to separate the protein A-agarose, and the depleted extracts were analyzed by Western blot (see inset). The data are expressed as mean ± S.D. from 3 repetitions of each data point. Similar results were observed in more than three independent experiments and with 2 preparations of K-Ras.

We determined the levels of phosphorylated Bad in the cell lines expressing various mutant Pak constructs were plated into 35-mm plates in 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium. 3 h later, cells were washed 3 times with serum-free Dulbecco’s modified Eagle’s medium and incubated in the presence of 20 μM LY294002 for the indicated time period. Cell nuclei were stained with Hoechst 33342 and inspected for chromosome condensation. For each sample, about 500 cells were scored. A, survival curves. Data are expressed as a percent of cumulative cell death as a function of time. Similar results were obtained following serum removal alone (data not shown). B, effect of Pak kinase-deficient mutants on Akt protection. The indicated plasmids were transfected along with β-galactosidase into Rat-1 cells. After 12 h cells were switched to serum-free medium for 72 h to induce apoptosis. Cells were stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and scored as apoptotic or normal. The data are expressed as the mean ± S.E. of 3 independent experiments. C, levels of Bad and Serine 136-phosphorylated Bad in cell lines expressing Pak mutants. Western blots of cell extracts from the cell lines used in each panel were probed with an antibody to Bad (lower panel) or an antibody that recognizes Bad once it is phosphorylated on Serine 136 (upper panel). D-F, effect of Pak on Bad phosphorylation in a transfection assay. Bad phosphorylation was detected by co-transfecting 1 μg of pEBG-MBad (New England Biolabs) and 2 μg of each test plasmid into cells and measuring phosphorylation with a phosphospecific antibody (New England Biolabs). The top blot in each panel was probed with phosphoserine 136 antibody, and the lower blot was probed for Bad protein expression.

One of the downstream targets of Akt associated with cell survival is Bad, a pro-apoptotic Bel-2 family protein that is inactivated by phosphorylation. Pak also phosphorylates Bad in vivo and in vitro at the key regulatory residues, Serines 112 and 136, which correlates with a cell-protective function (24). We determined the levels of phosphorylated Bad in the cell lines expressing Pak using a phosphospecific antibody for Serine 136 of Bad (Fig. 4c). In parental cells and cells transfected with kinase-inactive Pak mutants we were unable to detect any phosphorylated Bad. However, significantly more phosphorylated Bad was observed in cells expressing both wild-type and the activated Pak1L83,L86. All of the cell lines expressed comparable levels of Bad, suggesting that Pak does not alter the levels of Bad expressed in cells. We found that the levels of Bad expressed in our cells were too low to reliably measure, so we developed a transfection assay to test the role of Pak relative to Akt in Bad signaling. This assay measured phosphorylation of transfected Bad. As observed in the stable cell lines, we found that transfection of Pak1L83,L86 stimulated Bad phosphorylation (Fig. 4d), whereas the two dominant negative Pak1 mutants did not. Wild-type Pak also stimulated phosphorylation of
Bad but not as effectively as Pak1\textsuperscript{L83,L86}. Furthermore, the two dominant negative Pak1 mutants inhibited Ras-, PI3 kinase-, and Akt-stimulated phosphorylation, whereas dominant negative Akt did not inhibit Pak-stimulated phosphorylation of Bad (Fig. 4, e and f). These data suggest that Pak can mediate signals from Akt to Bad.

**DISCUSSION**

We provide four lines of evidence that Akt mediates the signal from Ras to Pak. First, overexpression of wild-type Akt or an activated Akt mutant stimulates Pak; second, a kinase-deficient Akt mutant inhibits Ras activation of Pak; third, extracts depleted of Akt fail to support Ras activation of Pak in a cell-free system; and fourth, we placed Pak downstream of Akt in signals to Bad. Together, these data suggest a Ras→PI3 kinase→Akt→Pak→Bad signal (Fig. 5). This signal promotes cell survival, because expression of activated Pak delays apoptosis, whereas expression of two dominant negative mutants of Pak enhances apoptosis. A total of three apoptosis paradigms that utilize Ras signaling pathways yielded similar results. Together, these suggest that Pak promotes cell survival by inhibiting apoptosis. Bad is one target that may mediate the cell survival function of Pak, but other potential survival targets include members of the extracellular signal-regulated kinase cascade (16, 25, 26).

Akt activation of Pak occurs downstream of PI3 kinase, because kinase-deficient Akt blocks both Ras and PI3 kinase activation of Pak. Interestingly, unlike almost all known activators of Pak including Ras and PI3 kinase, neither Rac nor Cdc42 are required for Akt activation of Pak. The simplest model is that Akt phosphorylates Pak to stimulate its activity, because activation of Pak by Akt requires a functional kinase activity. However, Akt is activated by PI3 kinase through direct and indirect mechanisms, and the Akt regulatory kinase, PDK1, can phosphorylate Pak, so the stimulation of Pak by Akt may come about through multiple mechanisms (27).\textsuperscript{2} Although the Akt pathway is sufficient for activation, our data also suggest, however, that signals from both Akt and Rac/Cdc42 contribute to Ras activation of Pak in vivo. Hence, Pak activation may be analogous to Ras activation of Raf where a small G protein and protein kinases coordinate signals. Although Ras can bind and activate Raf, sustained activation is achieved after Ras recruits Raf to the membrane where it is phosphorylated by several protein kinases including Src and Pak (25).

Recent studies have suggested both pro-apoptotic and anti-apoptotic roles for Pak. In *Xenopus* oocytes Pak activation is anti-apoptotic (29). However, another member of the Pak family, Pak2, is cleaved and activated by caspase 3. Caspase-activated Pak2 induces a number of morphological features associated with apoptosis including cell shrinkage, externalization of phosphatidylserine, and formation of apoptotic bodies (30, 31). Pak1 is not a substrate for caspases; therefore, depending on the susceptibility to caspase cleavage, different members of the Pak family may either perform pro-apoptotic or anti-apoptotic functions. This is similar to Bcl-2 family members that switch from anti-apoptotic to pro-apoptotic roles following caspase cleavage (32).

In sum, our studies provide evidence for a novel survival signaling pathway through Pak (Fig. 5). Therefore agents that inhibit Pak, like those that inhibit Ras processing (28), may both reduce cell proliferation and shrink tumors by promoting apoptosis.

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\textsuperscript{2}Gary Bokoch, personal communication.
