Apoptotic stimuli are divided into two broad categories, extrinsic and intrinsic. Extrinsic signals are receptor-mediated and are independent of the mitochondria. Intrinsic signals are mitochondrial dependent and generally activated by stress such as starvation, chemotherapeutic drugs, viral infections, and ionizing irradiation (1, 2). The intrinsic pathways converge on the mitochondria to regulate the Bcl-2 family. Bcl-2 protects cells by maintaining the integrity of the mitochondrial barrier and preventing the release of proapoptotic contents such as cytochrome c and apoptosis-inducing factor (AIF). Release of cytochrome c activates a cascade of caspases that ultimately commit cells to apoptosis, whereas AIF promotes apoptosis in a caspase-independent manner (2). Bcl-2 is regulated by forming complexes with members of the Bcl-2 family, some of which are protective complexes whereas others are inhibitory complexes. The balance between the Bcl-2 bound in protective complexes and the Bcl-2 bound in inhibitory complexes is thought to be critical in determining whether a cell will survive or undergo apoptosis.

Raf-1 protects cells from apoptosis, independently of its signals to MEK and ERK, by translocating to the mitochondria where it binds Bcl-2 and displaces BAD. However, the answer to the question of how Raf-1 is normally lured to the mitochondria and becomes activated remains elusive. p21-activated protein kinases (Paks) are serine/threonine protein kinases that phosphorylate Raf-1 at Ser-338 and Ser-339. Here we elucidate the molecular mechanism through which Pak1 signals to BAD through a Raf-1-activated pathway. Upon phosphorylation by Pak1, Raf-1 translocates to mitochondria and phosphorylates BAD at Ser-112. Moreover, the mitochondrial translocation of Raf-1 and the interaction between Raf-1 and Bcl-2 are regulated by Raf-1 phosphorylation at Ser-338/Ser-339. Notably, we show that formation of a Raf-1-Bcl-2 complex coincides with loss of an interaction between Bcl-2 and BAD. These signals are specific for Pak1, because Src-activated Raf-1 only stimulates the MAP kinase cascade. Thus, our data identify the molecular connections of a Pak1-Raf-1-BAD pathway that is involved in cell survival signaling.

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Shenghao Jin, Ya Zhuo, Weining Guo, and Jeffrey Field‡
From the Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, 19104

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MATERIALS AND METHODS

Reagents and Plasmids—Dulbecco’s modified Eagle’s medium and fetal bovine serum were from Invitrogen. The FuGENE 6 transfection reagent and complete protease inhibitor mixture tablets were from Roche Applied Science. Rabbit polyclonal antibodies against BAD, Ser(P)-112, Ser(P)-136, Bcl-xL, ERK, and phospho-ERK were from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibodies against Raf-1 and Bcl-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-Ser(P)-338 Raf-1 antibody, Raf-1 proteins, and an anti-Src monoclonal antibody were purchased from Upstate Biotechnology. The mouse monoclonal anti-Cox4 antibody and the ApoAlert cell fractionation kit were from BD Biosciences Clontech. Anti-phospho-Raf-1 Tyr-340 and the Raf-1 inhibitors GW5074 (5-iodo-3-(3,5-dibromo-4-hydroxyphenyl)methylene)-2-indoline) and BAY43-9006 as well as rapamycin were from Calbiochem. PD98059, H89, H-33342, a mouse monoclonal anti-FLAG antibody, an anti-FLAG M2 affinity gel, and a FLAG peptide were purchased from Sigma. Alexa Fluor 488 goat anti-mouse IgG and MitoTracker Red 580 were from Molecular Probes. Glutathione-SepharoseTM 4B was purchased from Amersham Biosciences. Plasmids expressing a Myc-tagged wild-type (WT), a kinase-dead (K299R), and a kinase-activated (T423E) version of Pak1 cloned into the pCMV6 vector have been described elsewhere (27). The plasmids used to generate GST-BAD (amino acids 104–141) fusion proteins, namely GST-BAD WT, GST-BAD S112A, GST-BAD S136A, and GST-BAD S112A/S136A (all cloned into the pOEX-4T-1), were provided by Dr. Stanley Korsmeyer. pBAC-his-hPak1, which expresses a Raf-1 WT or a S338A/S339A mutant (1 μg) by using FuGENE 6 as recommended. 8 h after the DNA mixture was added, cells were trypsinized and seeded into a Nunc chamber-coated with fibronectin. 24 h after transfection, cells were washed twice with phosphate-buffered saline (PBS) and the buffer and medium replaced with 5% calf serum. Pak1 or amino acids 104–141 and Raf-1 mutants were obtained by site-directed mutagenesis (Stratagene). The GeneBlocker Bcl-2 siRNA vector mix used to knock down Bcl-2 was purchased from BioVision (Mountain View, CA).

Cell Culture and Transfection—HEK 293T and NIH 3T3 cells were grown at 37 °C in 5% CO2, and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were transfected using FuGENE 6 (Roche Applied Science) and collected 48 h after transfection. Stable transfected cells were selected by Zeocin (150 μg/ml) for 2 weeks and confirmed by Western blotting.

Immunoblot and Immunoprecipitation—Cells were transfected with pCMV6-Pak1 and pcDNA-FLAG-Raf-1; after 24 h the cells were starved for 16 h with starvation medium. Cellular fractionation was performed using the ApoAlert cell fractionation kit (Clontech) in accordance with the manufacturer’s instructions. Briefly, cell pellets were collected by centrifugation at 600 × g for 15 min at 4 °C, washed once with wash buffer, and suspended in 0.8 ml of ice-cold fractionation buffer containing protease inhibitor mixture and dithiothreitol. After incubation on ice for 10 min, cells were homogenized with 60 strokes using a 1-ml Dounce tissue homogenizer. Samples were then transferred to microfuge tubes and centrifuged at 700 × g for 10 min at 4 °C to separate nuclei and unbroken cells, followed by centrifugation of the supernatant at 10,000 × g for 25 min at 4 °C to obtain a mitochondrial-enriched fraction. The cytoplasmic fractions were obtained by centrifugation of the supernatant at 100,000 × g for 1 h. Fractions were then analyzed using SDS-PAGE and Western blot assays.

RESULTS

Pak1 Induces Raf-1 Translocation to the Mitochondria—As targeting active Raf-1 to the mitochondria is necessary for cell protection and the phosphorylation of BAD, we tested to determine if Pak1 phosphorylation of Raf-1 stimulates its translocation to mitochondria. We isolated a mitochondria-enriched fraction after transfection with various Raf-1 and Pak1 plasmids (Fig. 1A). K375W is a kinase dead Raf-1 mutant, and S338A/S339A (S338A/S339A) in Fig. 1A) has the Pak phosphorylation sites mutated (26). The expression levels of the Pak1 and Raf-1 mutants were assessed using an anti-Myc or anti-Raf-1 antibody, whereas the mitochondrial protein Cox4 was used as a marker for the mitochondria. We found that the constitutively activated Pak1 (T423E) stimulated mitochondrial localization of Raf-1 (Fig. 1A, lane 5). Because Pak1 phosphorylates Raf-1 at serine 338, we also probed blots with a phospho-specific Ser-338 antibody and found that the mitochondrial associated Raf-1 was heavily phosphorylated at Ser-338 (Fig. 1A, lane 5). We also noted that Pak1 stimulated mitochondrial translocation of kinase dead Raf-1, K375W (Fig. 1A, lane 7). However, mitochondrial localization of Raf-1 was prevented by serine to alanine mutations at Ser-338 and Ser-339, the Pak phosphorylation sites. These data show that Pak1 stimulates translocation of Raf-1 to mitochondria and that, because the kinase dead Raf-1 was also translocated, Raf-1 activity is not necessary for translocation. To confirm that Pak1 phosphorylation translocated Raf-1 to mitochondria, we carried out immunofluorescence experiments and found that cells expressing activated Pak1 had higher levels of Raf-1 localizing to a perinuclear region.
where mitochondria are often found as compared with cells expressing wild type Pak1 (Fig. 1C). When cells were stained with the phosphoserine 338 antibody, the phosphorylated Raf-1 was highly enriched in this region, which we identified as mitochondria with MitoTracker dye (Fig. 1A). These results suggest that phosphorylation of Ser-338/Ser-339, but not of Tyr-340/Tyr-341, is required for Raf-1 mitochondrial localization.

**Pak1 Phosphorylation of Ser-112 on BAD Requires Raf-1—**

Transfection of Pak1 or mitochondrial targeting of Raf-1 can stimulate BAD phosphorylation (12, 22). Thus, we tested the possibility that Pak1 stimulates BAD phosphorylation through Raf-1. Cells were co-transfected with pEBG-BAD and an empty vector, kinase dead Pak1, or Pak1T423E, starved to reduce basal phosphorylation, and then probed with a phosphoserine 112 antibody. The expression level of BAD was assessed by Western blotting. Pak1T423E stimulation of Ser-112 phosphorylation was not inhibited by the protein kinase A inhibitor H89, the p70S6K inhibitor rapamycin, or the MEK inhibitor PD98059 (Fig. 2A). However, the Raf-1 inhibitors BAY43-9006 and GW5074 (5-iodo-3-[(3,5-dibromo-4-hydroxyphenyl)methylene]-2-indolinone) (data not shown for GW5074) reduced phosphorylation of BAD at Ser-112 to control levels (Fig. 2A, lane 4). We also assessed the expression and phosphorylation of ERK, a downstream target of the Raf-1 cascade. Pak1 weakly stimulated ERK phosphorylation, whereas ERK phosphorylation was inhibited by both BAY43-9006 and PD98059 (Fig. 2A, lanes 3–5). To exclude the possibility that Raf-1 inhibitors interfere with the protein kinase A pathway, we tested the effect of the Raf-1 inhibitor GW5074 on protein kinase A-induced Ser-112 phosphorylation. HEK 293T cells were transfected with pEBG-BAD for 24 h, starved for 16 h, and pretreated with vehicle (Me2SO), a protein kinase A inhibitor (H89), or the Raf-1 inhibitor for 30 min and then stimulated with forskolin to activate adenylyl cyclase.

**FIG. 1. Pak1 stimulates Raf-1 translocation to mitochondria.** A, Pak1 phosphorylation of Raf-1 stimulates its mitochondrial localization. HEK 293T cells were co-transfected with expression vectors encoding Raf-1 and Pak1 as indicated under “Materials and Methods.” 24 h after transfection, cells were collected, and a cellular fractionation was performed to isolate a mitochondria-enriched fraction (Mito.). Equal loading of the mitochondrial pellet was assessed with an anti-Cox4 antibody (Cyto.). B, phosphorylated Raf-1 co-localizes with mitochondria. NIH 3T3 stable cell lines expressing WT Pak1 or Pak1T423E were transfected with either WT Raf-1 or the S338A/S339A (S338A/S339A) mutant. 24 h after transfection, cells were stained with an anti-Ser(P)-338 Raf-1 (p-Ser338) antibody and MitoTracker Red 580 (B) or anti-FLAG (−Flag) antibody alone (C). The fields shown were analyzed independently by fluorescence microscopy at the appropriate wavelength for fluorescein isothiocyanate (Ser(P)-338) and anti-Tyr(P)-340/Tyr(P)-341 (pY340) antibodies. Western blots were performed. We found that activated Src did not stimulate Raf-1 translocation to mitochondria compared with Raf-1 alone, whereas activated Pak1 did (Fig. 1D). The translocation stimulated by Pak1 to the mitochondria was also accompanied by reduced levels of Raf-1 in the cytosol. In this experiment Src phosphorylated Tyr-340 as expected, whereas Pak did not stimulate phosphorylation at this site (Fig. 1D).
Western blots showed that forskolin stimulated Ser-112 phosphorylation and that the phosphorylation was reduced by H89 but not by the Raf-1 inhibitor (data not shown). This result demonstrates that the Raf-1 inhibitor does not interfere with protein kinase A phosphorylation of BAD on Ser-112. Together, these experiments implicated Raf-1 as the mediator of the signal from Pak1 to BAD phosphorylation.

BAY43-9006 can inhibit vascular endothelial growth factor receptor kinase activity (29), whereas GV5074 is not well characterized. Therefore, as an independent means of testing the role of Raf-1, we tested several dominant negative Raf-1 mutants (Fig. 2B). K375W plus S338A/S339A is a kinase dead mutant that cannot be phosphorylated by Pak1. We found that the wild-type Raf-1 alone did not stimulate Ser-112 phosphorylation. However, when Raf-1 was co-transfected with Pak1T423E, there was a slight stimulation of Ser-112 phosphorylation over that seen with Pak1T423E alone. Importantly, each of the Raf-1 mutants significantly reduced phosphorylation of Ser-112 by Pak1T423E. The S338A/S339A mutant was more effective than the kinase dead Raf-1, whereas the K375W plus S338A/S339A triple mutant was the most effective. Thus, these results further confirm that Pak1 stimulation of BAD phosphorylation requires Raf-1.

**Pak Specifically Stimulates Raf-1 Phosphorylation of BAD—**Targeting active Raf-1 to the outer mitochondrial membrane protects cells from apoptosis and stimulates phosphorylation of BAD, although the mechanism of BAD phosphorylation remains controversial. Although one study suggested that BAD is phosphorylated directly by Raf-1 (12), others found that BAD was a poor substrate for Raf-1 in vitro (14). One possibility is that cofactors direct Raf-1 toward BAD. To assess if Pak1 may be a cofactor responsible for determining the specificity of Raf-1 toward BAD, we tested the effects of Pak1 on Raf-1 phosphorylation of BAD in transfections and in vitro. We found that when Pak1 and Raf-1 were co-transfected into cells and Raf-1 was immunoprecipitated, the immunoprecipitated Raf-1 stimulated phosphorylation of BAD at serine 112 (Fig. 3A, lane 3, top section). However, BAD phosphorylation was diminished when Raf-1 mutants were tested. We also observed stimulation of ERK phosphorylation (Fig. 3A, bottom section) when ERK phosphorylation was measured in a coupled MEK/ERK assay, confirming previous reports that Pak cooperates with Raf-1 in the ERK cascade (25, 27). On the other hand, Raf-1 is also activated by tyrosine kinases such as Src through phosphorylation of Tyr-340 and Tyr-341 (30). Thus, we tested whether Src-stimulated Raf-1 phosphorylates BAD at Ser-112. When Raf-1 was treated with Src, only MEK phosphorylation was stimulated (Fig. 3B,
We also performed assays in vitro using recombinant proteins. When we measured BAD phosphorylation with the Ser-112 phospho-specific antibody, phosphorylation was very weak and almost undetectable when either Raf-1 or Pak1 was tested alone (Fig. 3C, lanes 2 and 3). Detailed in vitro studies confirmed that Pak1 phosphorylated BAD poorly at Ser-112 and preferred other sites (data not shown). However, in the presence of both Raf-1 and Pak1 a robust phosphorylation was observed (Fig. 3C, lane 4). To compare the relative activities of Pak1 and Src in this system, we determined the stoichiometry of phosphorylation of a Raf-1-derived peptide, RPRGQRDSSYYWEIE, containing Ser-338/Ser-339 and Tyr-340 (Fig. 3E). Pak1 stimulated incorporation of 1.2 mol of phosphate per mol of peptide, whereas Src stimulated incorporation of 0.84 mol of phosphate per mol of peptide (Fig. 3E, lanes 3 and 5). Pak1 failed to phosphorylate a control peptide with Ser-338/Ser-339 mutated to alanines, whereas Src phosphorylated the peptide as well as it phosphorylated the wild type peptide (Fig. 3E). To mimic Src activation, we tested to determine if an active Raf-1 mutant lacking residues 1–306 and containing Y340D/Y341D mutations can phosphorylate BAD in vitro. This active Raf-1 stimulated MEK/ERK phosphorylation (Fig. 3D) but not BAD phosphorylation (Fig. 3C, lane 5). Again, however, a robust phosphorylation of Ser-112 was detected when the mutant was tested in combination with Pak1 (Fig. 3D, lane 6), suggesting that Pak1 phosphorylation of Ser-338 and Ser-339 is required for Raf-1 phosphorylation of BAD at Ser-112, whereas Src does not contribute to Raf-1 phosphorylation of BAD.

**Pak1 Promotes Bcl-2 Protective Complexes**—One of the mitochondrial proteins associated with protective signals from Raf-1 is Bcl-2, which forms a complex with Raf-1 although it is not phosphorylated by Raf-1. Because Pak1 stimulated mitochondrial localization of Raf-1, we reasoned that Pak1 might be stimulating the formation of complexes between Raf-1 and Bcl-2 at the mitochondria. To test this possibility, we transfected HEK 293T cells with activated Pak1 and WT Raf-1. After transfection, cells were lysed and fractionated to isolate the mitochondria-enriched fractions. We performed immunoprecipitations with the anti-FLAG antibody-conjugated beads (M2 beads) and probed the blots for Bcl-2 and Bcl-X<sub>L</sub>. We found that Bcl-2 but not Bcl-X<sub>L</sub> was precipitated with the mitochondrial Raf-1 (Fig. 4A, lanes 2 and 4). No signal was seen with a control of M2 beads pre-treated with FLAG peptides before immunoprecipitation (Fig. 4A, lanes 1 and 3). We also did the reverse experiment, using an anti-Bcl-2 antibody and probing for Raf-1. As expected, the Bcl-2 antibody, but not the control rabbit IgG, immunoprecipitated Raf-1 (Fig. 4A, lanes 5 and 6). To address whether phosphorylation of Raf-1 by Pak1 is necessary for its association with Bcl-2, we co-transfected cells with Raf-1 and Pak1 or Src, performed the immunoprecipitations with anti-Bcl-2 antisera with whole cell lysates, and probed for Raf-1. We found that neither kinase dead Pak1 nor wild-type Pak1 affected complex formation (Fig. 4B, lanes 2 and 3). However, the active Pak1 strongly stimulated a complex between Bcl-2 and Raf-1 (Fig. 4B, lane 4). No complex was detected with the S338A/S339A Raf-1 mutant (Fig. 4B, lane 5) or when Src was tested with Raf-1 (lane 6). Finally, we utilized RNA interference technology to knock down Bcl-2 in cells. We found that translocation of Raf-1 to the mitochondria was reduced in the knock-down cells (Fig. 4C). Together, these experiments suggest that Pak1 stimulates translocation of Raf-1 to the mitochondria, where it forms a complex with Bcl-2.

**FIG. 4. Pak-1 stimulates Raf-1 association with Bcl-2.** A, Raf-1 associates with Bcl-2 at the mitochondria. HEK 293T cells were co-transfected with Raf-1 and Pak1T423E. Mitochondria were isolated 24 h after transfection, lysed, and then subjected to immunoprecipitations (IP) with an anti-Bcl-2 antibody or M2 beads against the FLAG tag on Raf-1. Samples were run on gels and then probed with the indicated antibodies. IB, immunoblotting. B, Pak-1 phosphorylation of Raf-1 at Ser-338 and Ser-339 stimulates its association with Bcl-2. HEK 293T cells were co-transfected with plasmids as indicated. 24 h after transfection, cells were lysed and immunoprecipitations were performed with anti-Bcl-2 antibody. Samples were then subjected to immunoblotting with anti-Raf-1 antibody. KD, kinase dead; S338/9A, S338A/S339A. C, knock down of Bcl-2 reduces the translocation of Raf-1. HEK 293T cells were transfected with Bcl-2 siRNA vectors using FuGENE 6 reagent. 12 h after transfection, cells were re-transfected with Pak1 and Raf-1. 2 days later, cells were harvested, and mitochondrial fractions (Mit.) were isolated. Samples were subject to analysis by immunoblotting. D, the Raf-1-Bcl-2 interaction correlates with disruption of the BAD-Bcl-2 association. GST pull-down and immunoprecipitations with anti-Bcl-2 antibody were performed using the same whole cell extracts before immunoblotting for Raf-1 (top section) or Bcl-2 (second section from top).

The data we have shown to date indicate that Pak1 phosphorylation of Raf-1 stimulates Raf-1 translocation to mitochondria, phosphorylation of BAD, and Raf-1 binding to Bcl-2. Unphosphorylated BAD can form heterodimers with Bcl-2 and Bcl-X<sub>L</sub> to stimulate apoptosis. Phosphorylation of BAD renders it unable to bind to Bcl-2 and Bcl-X<sub>L</sub>. Therefore, we assessed whether the interaction of Raf-1 and Bcl-2 correlates with the disruption of a Bcl-2-BAD complex. HEK 293T cells were co-transfected with GST-BAD, Raf-1 and Pak1 plasmids. GST pull-down and immunoprecipitation with an antibody to Bcl-2 were performed using the same cell lysates and then Western blotted for associated Raf-1 or Bcl-2 (Fig. 4D). The results show that when Pak1 stimulates the binding of Raf-1 to Bcl-2 it disrupts the association of Bcl-2 with BAD (compare Fig. 4D, lane 3 in the top two sections). Conversely, the S338A/S339A Raf-1 mutant binds poorly to Bcl-2 and enhances the association of Bcl-2 with BAD (lane 4). These data further support a model in which phosphorylated Raf-1, operating at mitochondria, binds to Bcl-2 and phosphorylates BAD, resulting in disruption of the Bcl-2-BAD complex.
Pak1 Protection of Cells Requires Raf-1—To assess the physiological importance of Raf-1 in signals from Pak1 to BAD, we designed studies to determine whether Raf-1 was required for Pak1 to protect cells from apoptosis. Untransfected HEK 293T cells or HEK 293T cells developed to express either empty vector or Pak1T423E were serum-starved in the presence of vehicle (Me2SO), PD98059, or the Raf-1 inhibitor for 36 h. Cells were collected and stained with H-33343 to identify apoptotic cells. The activated Pak1T423E protected cells from serum starvation-induced apoptosis (Fig. 5A). We also found that the cells were weakly influenced by PD98059. However, when the cells were treated with the Raf-1 inhibitor, Pak1 no longer protected the cells. The Raf-1 inhibitor had no effect on its own, but it prevented Pak1 from protecting the cells. We also found that co-transfecting mutant Raf-1 but not WT Raf-1 reduced protection by Pak1 (Fig. 5B). These data support a role of Raf-1 in cell protection pathways mediated by Pak1.

DISCUSSION

Pak1 stimulates BAD phosphorylation in several cell culture models. The mechanism was reported to be by direct phosphorylation at serines 112 or 136, but the studies were done with highly sensitive phospho-specific antibodies. A quantitative examination of BAD phosphorylation, however, found that no phosphorylation could be detected at Ser-136, and only a weak phosphorylation of Ser-112 was found; instead, the major site was Ser-111.2 Thus, we sought to determine how Pak1 stimulates BAD phosphorylation at Ser-112, a widely reported in vivo site. Multiple lines of data point to Pak1 phosphorylation of BAD proceeding through Raf-1. We found that blocking Raf-1 with either a pharmacologic inhibitor or kinase dead Raf-1 mutants markedly reduced Pak1 from stimulating BAD phosphorylation. This activity was independent of MEK, because it was not affected by the MEK inhibitor PD098059. Furthermore, Raf-1 phosphorylation of BAD in vitro was greatly stimulated by Pak1, suggesting that Raf-1 phosphorylates BAD directly at Ser-112. Cell fractionation and immunofluorescence experiments suggested that Pak1 phosphorylation enhanced Raf-1 translocation to the mitochondria. Bcl-2 is the strongest candidate for the mitochondrial targeting protein because its complexes with Raf1 were stimulated by Pak1, whereas RNA interference knock down of Bcl-2 reduced Raf1 mitochondrial localization. A model describing these results is shown in Fig. 5C.

Raf-1 is widely studied for its role in cell proliferation as a Ras effector. It has one well documented target, MEK, a protein kinase that, in turn, phosphorylates ERK. Initially, MEK was its only known target. But, increasingly, MEK-independent signals have been identified that promote cell survival. The first study suggesting a survival role was the observation that Bcl-2 could form a complex with Raf-1 to direct it to the mitochondria (12). Moreover, forcing Raf-1 to the mitochondria with a targeting sequence also protected cells through mechanisms independent of MEK and ERK. Some of the strongest data suggesting a role for Raf-1 in cell survival comes from studies with knock-out mice. Two of the knock-outs, B-Raf and Raf-1, are embryonic lethals. Interestingly, cells from both B-Raf and Raf-1 knock-out mice display relatively normal proliferative rates but have substantially increased rates of apoptosis (13, 15, 31–34). On the other hand, cells from knock-outs of MEK1 display no increases in apoptosis (35). Cells from the two Raf1 knock-outs also have wild type levels of ERK activation. Again, these data suggest a role for Raf-1 in cell survival, a role independent of MEK and the MAPK pathway. Efforts to find a target in the mitochondria have been elusive. In the original study BAD was suggested as a target, but others later showed poor phosphorylation in vitro (14). Our data suggest that this may be because Pak is required as a Raf-1 coactivator. Pak does this by phosphorylating amino acids 338 and 339 on Raf-1. The role of the MAPK pathway in cell protection was also confusing, because other studies have found that Raf-1 protection is sensitive to MEK inhibition.
A recent study found that some of the confusion over the role of Raf-1 in cell survival signaling may be accounted for by multiple pathways utilizing Raf-1 in distinct ways (28). This study traced a signal from the basic fibroblast growth factor (bFGF) through Pak and Raf-1 that protected cells. The signal was independent of MEK, suggesting that there was a novel target. In contrast, another growth factor, VEGF (vascular endothelial growth factor), signaled through Src to Raf-1 independently of Pak but dependent on MEK. This latter pathway may be the predominant pathway utilized by B-Raf, which has acidic residues at amino acids 340 and 341, does not need tyrosine phosphorylation for activation, and protects cells via MEK and MAPK (36). In striking support of this model, we find that Src does not stimulate mitochondrial apoptosis from extrinsic signals by blocking the initiator caspase 8 in addition to a BAD-dependent signal (45). These studies support the expanding role of Pak in cell survival signals through multiple mechanisms. The significance of protective signals described here through Raf-1 are highlighted by the observation that cells from Raf-1 knock-out mice have normal proliferative rates and ERK activation, but high rates of apoptosis (13, 15, 32, 33).

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Shenghao Jin, Ya Zhuo, Weining Guo and Jeffrey Field

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