Cell, Vol. 87, 629–638, November 15, 1996, Copyright ©1996 by Cell Press

Bcl-2 Targets the Protein Kinase Raf-1 to Mitochondria

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Summary

A green fluorescent protein (GFP)–Raf-1 fusion protein was used to show that Bcl-2 can target this kinase to mitochondria. Active Raf-1 fused with targeting sequences from an outer mitochondrial membrane protein protected cells from apoptosis and resulted in phosphorylation of BAD, a proapoptotic Bcl-2 homolog. Plasma membrane–targeted Raf-1 did not protect from apoptosis and resulted in phosphorylation of ERK-1 and ERK-2. Untargeted active Raf-1 improved Bcl-2-mediated resistance to apoptosis, whereas a kinase-inactive Raf-1 mutant abrogated apoptosis suppression by Bcl-2. Bcl-2 can therefore target Raf-1 to mitochondrial membranes, allowing this kinase to phosphorylate BAD or possibly other protein substrates involved in apoptosis regulation.

Introduction

The Bcl-2 family of proteins consists of both inhibitors and promoters of programmed cell death and apoptosis. Many of these proteins can interact with each other through a complex network of homo- and heterodimers (Sato et al., 1994; Sedlak et al., 1995). The relative ratios of pro- and antiapoptotic Bcl-2 family proteins determine the ultimate sensitivity or resistance of cells to a wide variety of apoptotic signals (Oltvai and Korsmeyer, 1994). The precise biochemical mechanisms by which Bcl-2 family proteins exert their influence on cell life and death, however, remain far from clear, though several theories have been advanced, including regulation of organellar Ca^{2+} homeostasis, reactive oxygen species production, protein transport, cysteine protease activation, and mitochondrial permeability transition (Baffy et al., 1993; Kane et al., 1993; Hockenbery et al., 1993; Krajewski et al., 1993; Ryan et al., 1994; Boulakia et al., 1996; Zamzami et al., 1996).

Bcl-2 and most of its homologs contain hydrophobic stretches of amino acids near their C-termini (transmembrane [TM] domains) that allow for their insertion into intracellular membranes. Though varying among different cell types and particular members of the Bcl-2 family, in general, a large proportion of these protein molecules are associated with the outer mitochondrial membrane (Krajewski et al., 1993; González-Garcia et al., 1994; Yang et al., 1995). Several observations suggest an important role for mitochondria in the control of apoptosis. For example, removal of mitochondria from cytosolic extracts of Xenopus laevis oocytes undergoing follicular atresia abolishes their ability to induce apoptosis-like destruction of exogenous nuclei (Newmeyer et al., 1994). Similarly, mitochondria isolated from cells induced to undergo apoptosis can stimulate apoptosis-like destruction of naive nuclei, whereas mitochondria purified from Bcl-2 overexpressing cells do not (Zamzami et al., 1996). Interestingly, chemical inducers of mitochondrial megapore opening (permeability transition) can induce normal mitochondria derived from healthy cells to liberate factors that result in apoptosis-like destruction of nuclei (Zamzami et al., 1996). Moreover, release of cytochrome C from mitochondria occurs during apoptosis and can promote activation of proteases of the interleukin-1β-converting enzyme (ICE) family (Liu et al., 1996). Overexpression of Bcl-2 has also been reported to prevent the dumping of mitochondrial Ca^{2+} into the cytosol of cells treated with uncouplers of oxidative phosphorylation (Baffy et al., 1993), a known stimulus for inducing permeability transition in mitochondria (Bernardi et al., 1994). Finally, mutant versions of Bcl-2 lacking their C-terminal TM domain inefficiently associate with mitochondria and are impaired in their ability to prevent cell death (Tanaka et al., 1993; Nguyen et al., 1994), whereas substitution of a TM domain from the yeast outer-mitochondrial membrane protein Mas70p restores targeting to mitochondria and returns full antiapoptotic function (Nguyen et al., 1994).

Bcl-2 family proteins contain up to four conserved domains: BH1, BH2, BH3, and BH4. All antiapoptotic members of the Bcl-2 protein family contain the BH4 domain (Zha et al., 1996), which is typically located near the N-terminus of these proteins and corresponds to the first amphipathic α helix in the crystal structure of the Bcl-X_L protein, an antiapoptotic homolog of Bcl-2 (Muchmore et al., 1996). In contrast, the proapoptotic members of the Bcl-2 family lack BH4, with the exception of Bcl-X_S. Deletion mutants of Bcl-2 lacking the BH4 domain exhibit either loss of function or dominant-inhibitory activity, paradoxically promoting apoptosis (Borner et al., 1994; Hanada et al., 1995; Hunter et al., 1996), thus indicating the functional significance of the BH4 domain. BH4 is not, however, required for binding to either Bax or wild-type Bcl-2 protein (Hanada et al., 1995), suggesting that it plays a role in some other aspect of Bcl-2 function.

In addition to dimerizing with other homologous proteins, the Bcl-2 protein has been reported to associate with several other nonhomologous proteins, including the protein kinase Raf-1 (Wang et al., 1994; Blagosklonny et al., 1996). Raf-1 is best known for the role it plays in growth factor receptor–mediated signal transduction pathways involving $p21^{ras}$ family proteins located at the plasma membrane (Daum et al., 1994). When Ras assumes an active conformation through binding of GTP, a subpopulation of the Raf-1 protein molecules in cells translocates from the cytosol to the plasma membrane, via interaction with $p21^{ras}$ (Hall, 1994). The region on Raf-1 that binds to Ras corresponds to a negative regulatory domain located in approximately the

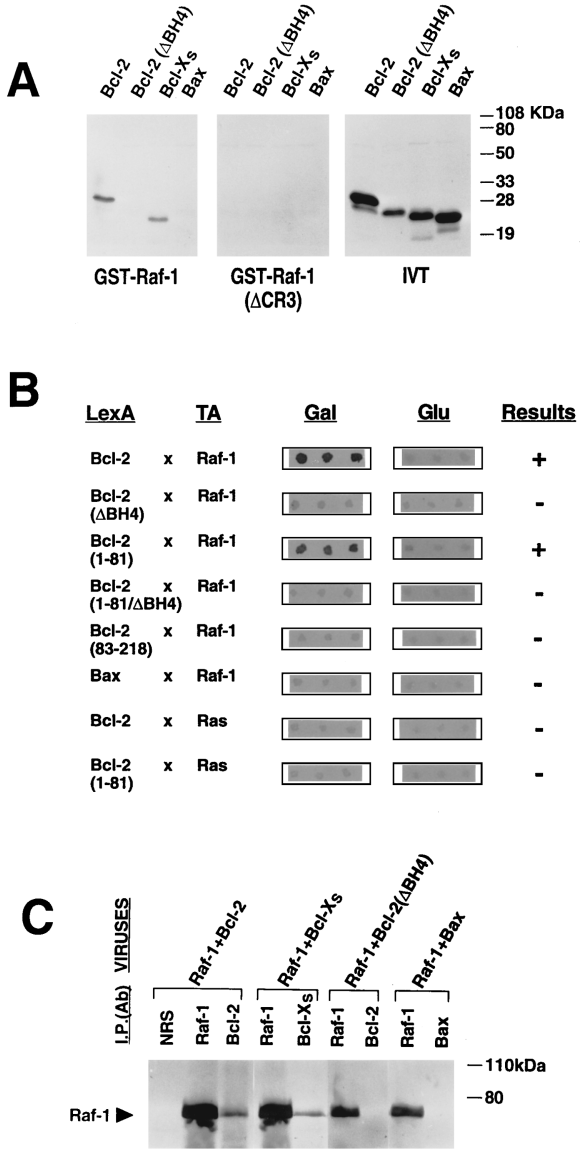

Figure 1. Raf-1 Interaction with Bcl-2 Is Dependent on the BH4 Domain.

(A) Baculovirus-produced GST–Raf-1 (left) or GST–Raf-1(ΔCR3) (center) proteins (~5 μg) were immobilized on glutathione–Sepharose (~20 μl) and then incubated with in vitro translated (IVT) [^{35}S]methionine-labeled Bcl-2, Bcl-2(ΔBH4), Bcl-X_s, or Bax proteins. The bound proteins were analyzed by SDS–PAGE. An equivalent amount of in vitro translated proteins was run directly in the gel (right).

(B) Interactions of pairs of fusion proteins containing either an N-terminal LexA DNA-binding domain (LexA) or B42 *trans*-activation domain (TA) were tested by yeast two-hybrid assay using β-galactosidase filter assays. Three independent transformants were plated on either galactose (Gal)- or glucose (Glu)-containing medium, to either induce or repress, respectively, the Gal-1 promoters in the two-hybrid plasmids.

(C) Coimmunoprecipitation experiments were performed by using lysates prepared from Sf9 cells that had been coinfected with Raf-1 and either Bcl-2, Bcl-2(ΔBH4), Bcl-X_s, or Bax-producing recombinant baculoviruses. Immunoprecipitations were performed using either normal rabbit serum (NRS) or polyclonal rabbit antisera specific for Raf-1, Bcl-2, Bcl-X, or Bax. Western blots were developed with anti-Raf-1 monoclonal antibody (URP30) according to a colorimetric method.

first third of the N-terminal length of the kinase (residues 51–149), upstream of the catalytic domain (residues 330–627) (Zhang et al., 1993). This retargeting of Raf-1 to the plasma membrane both serves as a mechanism for locally activating this kinase and also probably helps to bring Raf-1 into contact with relevant physiological substrates (Stokoe et al., 1994; Leevers et al., 1994). We provide evidence here that the Bcl-2 protein can target Raf-1 to mitochondria, through an interaction that depends on the BH4 domain in Bcl-2 and the catalytic domain in Raf-1, thus promoting resistance to apoptosis and altering the substrate preferences of Raf-1 in cells.

Results

Raf-1 Interaction with Bcl-2 Depends on BH4 Domain

We examined whether removal of the BH4 domain (residues 11–33) from Bcl-2 abolished its ability to interact with Raf-1 by using three approaches: first, binding of in vitro translated ^{35}S-labeled Bcl-2 or Bcl-2(ΔBH4) proteins to GST–Raf-1 fusion protein; second, yeast two-hybrid assays; and third, coimmunoprecipitation experiments where Bcl-2 and Bcl-2(ΔBH4) proteins were coexpressed with Raf-1 in Sf9 insect cells infected with recombinant baculoviruses (Figures 1A–1C, respectively). All three of these approaches demonstrated a BH4 dependence for the interaction of Bcl-2 with Raf-1 and confirmed previous studies showing that the catalytic domain of Raf-1 is necessary and sufficient for this interaction (Wang et al., 1994). The proapoptotic protein Bcl-X_S, which contains BH4 but lacks the BH1 and BH2 domains found in most other Bcl-2 family proteins, also can associate with Raf-1, whereas Bax (which lacks BH4) does not.

Bcl-2 Targets Raf-1 to Mitochondria in a BH4-Dependent Manner in Mammalian Cells

To explore further the interaction of Bcl-2 and Raf-1, experiments were performed in 293 epithelial cells. If Bcl-2 and Raf-1 associate in intact cells, we reasoned that Bcl-2 should target a portion of the Raf-1 molecules from the cytosol to the same membranous locations where Bcl-2 resides. To test this hypothesis, Raf-1 was expressed as a GFP fusion protein in 293 cells, with or without Bcl-2. To simplify interpretation of the data, only the catalytic domain (Cat) of Raf-1 was included, thus removing the Ras-binding domain of Raf-1 and avoiding targeting to membranes as a result of interactions with Ras family proteins.

Analysis of 293 cells by fluorescence confocal microscopy after transfection with an expression plasmid encoding the GFP–Raf-1(Cat) protein revealed a mostly diffuse pattern of fluorescence (Figure 2a). 293 cells cotransfected with expression plasmids encoding the GFP–Raf-1(Cat) protein and Bcl-2(ΔBH4) displayed the same pattern of fluorescence (Figure 2b). In contrast, the fluorescence in 293 cells coexpressing GFP–Raf-1(Cat) and wild-type Bcl-2 was concentrated in punctate

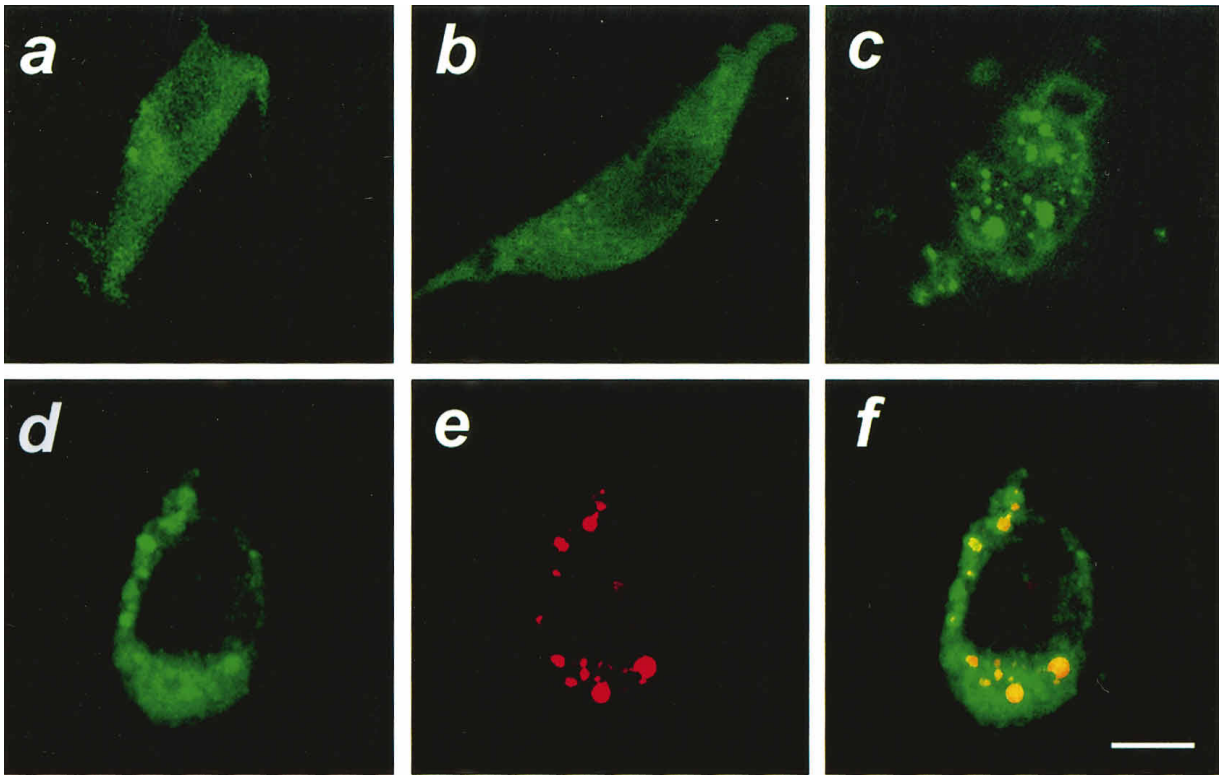

Figure 2. Bcl-2 Protein Targets Raf-1 to Mitochondria

293 cells stably expressing GFP–Raf-1(Δ26–303) were transiently transfected with parental pCI-Neo (a), pCI-Neo-Bcl-2(ΔBH4) (b), or pCI-Neo-Bcl-2 (c–f) plasmid DNA. After ~48 hr, the cells were incubated with MitoTracker dye and analyzed by fluorescence confocal microscopy using appropriate filters for visualization of green (a–d), red (e), or combined (f) fluorescence resulting from the GFP and MitoTracker molecules.

foci in the cytosol, suggestive of association with mitochondria or other organelles (Figure 2c). Two-color analysis using a rhodamine-like dye that is specifically imported into mitochondria (MitoTracker) confirmed that a large portion of the GFP–Raf-1 protein molecules were associated with mitochondria (Figures 2d–2f). In contrast, when the GFP protein was expressed in 293 cells without appended Raf-1 sequences, only a diffuse, mostly cytosolic staining was found; it failed to colocalize with mitochondria, regardless of whether the cells coexpressed Bcl-2 or not (data not shown). Thus, Bcl-2 did not cause nonspecific adsorption of GFP to mitochondria. Moreover, immunoblot analysis of the transiently transfected 293 cells confirmed production of comparable amounts of the wild-type and ΔBH4 mutant Bcl-2 proteins (data not shown), indicating that the failure of GFP–Raf-1(Cat) to associate with mitochondria when coexpressed with Bcl-2(ΔBH4) could not be explained by inadequate production of this mutant protein.

Targeting Raf-1 to Mitochondria Enhances Its Antiapoptotic Activity

The data presented above revealed that Bcl-2 can target Raf-1 to mitochondria but left unanswered the question of the significance of this event for regulation of apoptosis. To address this issue, expression plasmids were constructed that produce Raf-1 fused with the TM domain of the yeast outer mitochondrial membrane protein Mas70p (Hase et al., 1984), with the idea that the Mas70p TM domain should target Raf-1 to the outer mitochondrial membrane without the assistance of Bcl-2. In addition, Mas70p TM–Raf-1 fusion constructs were prepared in which lysine 375 in Raf-1, which is critical for ATP binding, was mutated, thereby producing a protein without kinase activity. Such kinase-inactive mutants of Raf-1 can often function as dominant inhibitors of the endogenous wild-type kinase (Kolch et al., 1991). To avoid complications due to the association of Raf-1 with Ras-family proteins, both the kinase active and inactive Mas70p–Raf-1 fusion proteins were expressed without the N-terminal portions of Raf-1 (residues 26–303) which mediate binding to Ras (hereinafter referred to as M-Raf-1 and M-Raf-1(K375W)). As an additional control, the same Raf-1(Δ26–303) protein lacking the Ras-binding domain was fused with the CAAX box–containing C-terminus of K-Ras, which targets Raf-1 specifically to the plasma membrane (Stokoe et al., 1994; Leevers et al., 1994).

These Raf-1 fusion proteins were stably expressed in 32D.3, a hemopoietic cell that is dependent on interleukin-3 (IL-3) for its growth and survival. Immunoblot analysis of lysates prepared from these cells confirmed the production of the expected ~45 kDa M-Raf-1, M-Raf-1(K375W), and Raf-1–CAAX proteins (Figure 3A, lanes 3–5). Targeting of the M-Raf-1 (Figure 3B) and M-Raf-1(K375W) (data not shown) proteins to mitochondria was verified by subcellular fractionation methods (Figure 3B), in which cytosolic (C), light membrane (LM), and heavy

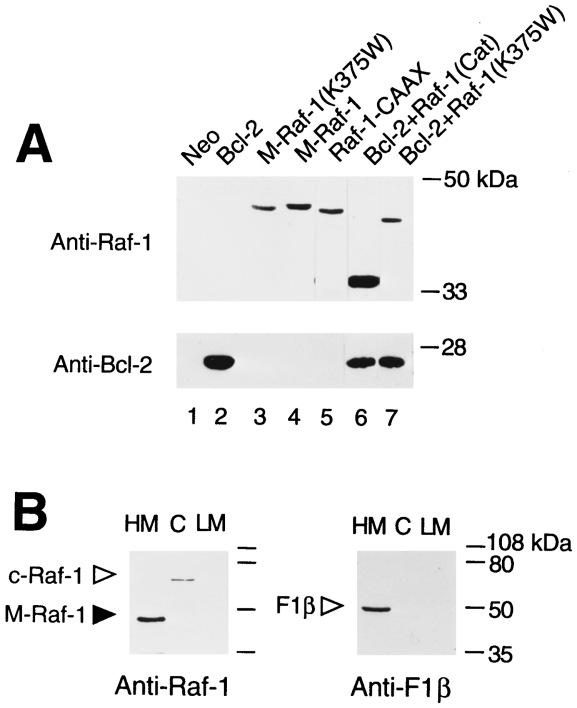

Figure 3. Characterization of 32D.3 Cell Transfectants

(A) Stably transfected 32D.3 cells were analyzed by immunoblotting (20 μg of protein) using anti-peptide antisera specific for human Bcl-2 (bottom) or Raf-1 (top).

(B) Stably transfected 32D.3–M-Raf-1 cells were used to produce HM, cytosolic (C), and LM fractions, which were subjected to SDS–PAGE/immunoblotting assay (20 μg of protein per lane) using antibodies specific for Raf-1 (left) or the mitochondrial protein F1-β-ATPase (right).

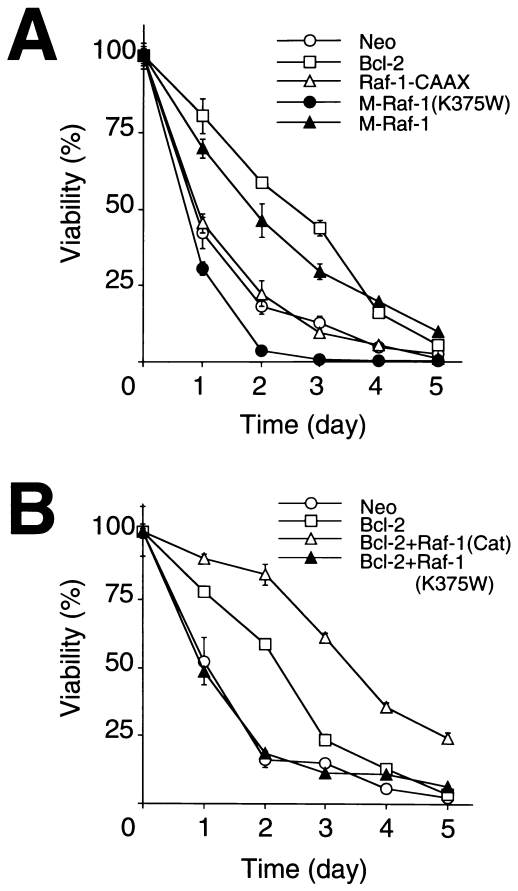

Figure 4. Effects of Mitochondrial Targeting of Raf-1 on Survival of 32D.3 Cells

32D.3 cells (A) or 32D.3–Bcl-2 cells (B) were stably transfected with pcDNA-3 (Neo) or pcDNA-3 encoding active Raf-1 (Δ2–334), kinase-inactive Raf-1 (Δ26–303)(K375W), Raf-1(Δ26–303)-CAAX, M-Raf-1, or M-Raf-1(K375W) as indicated. Cells were cultured without IL-3 for various times before MTT assays (data expressed as percent relative to time 0; mean ± SD; n = 3). Similar results were obtained by trypan blue dye exclusion assay (data not shown).

membrane (HM) fractions were prepared and analyzed for the presence of Raf-1 proteins by immunoblotting. The ~45 kDa M-Raf-1 protein was contained entirely within the HM fraction, where mitochondria reside (Figure 3B). In contrast, the endogenous p72-Raf-1 wild-type protein was located mostly in the cytosolic fraction.

The kinetics of cell death were explored in cultures of these 32D.3 cells expressing the M-Raf-1 and M-Raf-1(K375W) proteins when deprived of IL-3. When compared with 32D.3-Neo cells that received only the parental plasmid as a control, 32D.3 cells expressing the M-Raf-1 protein exhibited a delay in cell death comparable to that seen in transfected 32D.3 cells overexpressing Bcl-2 (Figure 4A). In contrast, 32D.3 cells expressing the M-Raf-1(K375W) protein experienced accelerated cell death, compared with the 32D.3–Neo control cells, when deprived of IL-3 (Figure 4A). 32D.3 cells expressing the Raf-1–CAAX protein, as well as the same cells expressing Raf-1(Cat) protein lacking either a CAAX box or the TM domain of Mas70p (Wang et al., 1994; data not shown), displayed cell death kinetics comparable to those of the control transfectants (Figure 4A). The viability of all transfectants when cultured with IL-3 was ≥95% during the first 3 days of the experiment, but then began to decline in cultures of 32D–Neo, 32D–Raf-1–CAAX, and 32D–M-Raf-1(K375W) cells (data not shown). In addition to MTT assays, the results of these studies where confirmed by flow cytometric DNA content analysis propidium iodide–stained cells (data not shown), where the proportion of cells with subdipolid amounts of DNA, indicative of apoptosis, was determined. This DNA content analysis also showed that despite their prolonged survival in IL-3-deficient medium, neither 32D.3–Bcl-2 nor 32D.3–M-Raf-1 cells continued to cycle in the absence of IL-3, and the preponderance of these cells arrested in G0/G1-phase when deprived of lymphokines. Taken together, therefore, these data are consistent with the hypothesis that targeting active Raf-1 kinase to mitochondrial membranes can specifically enhance cellular resistance to apoptosis, whereas interfering with action of endogenous Raf-1 at mitochondria has the opposite effect.

The M-Raf-1 and M-Raf-1(K375W) Proteins Modulate the Antiapoptotic Activity of Bcl-2

If the interaction of Bcl-2 with Raf-1 plays a role in the regulation of apoptosis by Bcl-2, then coexpressing an

active Raf-1 kinase with Bcl-2 should enhance resistance to apoptosis compared with the resistance in cells transfected with Bcl-2 alone. Conversely, the inactive Raf-1 mutant should potentially reduce the resistance of Bcl-2-expressing cells to apoptosis, by interfering with the interaction of Bcl-2 with the endogenous, wild-type Raf-1 protein. To test this hypothesis, a clone of 32D.3 that had been previously transfected with Bcl-2 by using a hygromycin-resistance marker was stably transfected with pcDNA-3 expression plasmids, producing first an active form of the Raf-1 protein consisting essentially only of the catalytic domain (Cat) of Raf-1(Δ2–334); second, an inactive K375W mutant Raf-1 lacking the Ras-binding domain (Δ26–303); or third, the pcDNA-3 parental vector as a control (contains the G418-resistance gene). It was important to eliminate the Ras-binding domain in the K375W mutant Raf-1 protein so as to avoid interference with Ras-regulated pathways that are required for cell proliferation (Kolch et al., 1991; Porras et al., 1994; Troppmair et al., 1994). Unlike the experiments above, mitochondrial targeting sequences were not included in the active and inactive Raf-1 proteins, thus making the association of these proteins with mitochondrial membranes dependent on interactions with Bcl-2.

Immunoblot analysis of the Bcl-2, Bcl-2–Raf-1(Cat), and Bcl-2–Raf-1(K375W) cells using an antiserum specific for the human Bcl-2 protein demonstrated essentially equivalent amounts of plasmid-derived human Bcl-2 protein in these cells (see Figure 3A), the levels of which were comparable to those in some human tumor lines that overexpress Bcl-2 (data not shown). Incubation of the same blot with an antiserum directed against a peptide corresponding to the C-terminus of Raf-1 demonstrated the presence of the expected ~35 kDa Raf-1(Cat) and ~42 kDa Raf-1(K375W) proteins (see Figure 3A).

When deprived of IL-3, 32D.3 cells coexpressing Bcl-2 and Raf-1(Cat) exhibited prolonged survival compared with cells expressing Bcl-2 alone (Figure 4B). In contrast, 32D.3 cells coexpressing Bcl-2 and Raf-1(K375W) had reduced survival compared with cells expressing Bcl-2 alone. The kinetics of cell death in cultures of 32D–Bcl-2–Raf-1(K375W) cells, for instance, were essentially the same as in the control 32D.3–Neo cells that lacked Bcl-2 (Figure 4B), indicating that this dominant inhibitory form of Raf-1 completely abrogated the protective effect of Bcl-2 on cell survival. Cells transfected with the plasmid encoding the active Raf-1(Cat) protein, without Bcl-2, also displayed cell survival characteristics indistinguishable from those of the Neo control cells (Wang et al., 1994), suggesting that Raf-1 inefficiently participates in the suppression of apoptosis in those cells that contain relatively low levels of endogenous Bcl-2 protein.

Raf-1 Cooperates with Bcl-2 in Suppressing Staurosporine-Induced Apoptosis

The broad-specificity protein kinase inhibitor staurosporine has been shown to induce apoptosis in essentially all types of cells derived from metazoan species and thus has been used extensively as a general tool for exploring the molecular control of apoptosis (Weil et al., 1996). To determine whether the ability of Raf-1 and Bcl-2 to cooperate in the suppression of apoptosis extends beyond the paradigm of IL-3 withdrawal, the various 32D.3 transfectants described above were cultured with 1 μM staurosporine in the presence of IL-3. Relative numbers of viable cells were then determined 1 day later by MTT assay. As shown in Figure 5A, approximately 1.5-fold more 32D.3–Bcl-2 cells survived treatment with staurosporine than did 32D.3–Neo cells ($p < 0.01$ by unpaired t test). The 32D.3 cells expressing the mitochondria-targeted and constitutively active Raf-1 protein M-Raf-1 withstood treatment with staurosporine about as well as the 32D.3–Bcl-2 cells ($p < 0.01$ for M-Raf-1 versus Neo). In contrast, 32D.3 cells transfected with a plasmid encoding the plasma membrane–targeted Raf-1–CAAX protein (Figure 5A) or untargeted Raf-1(Cat) (data not shown) survived no better than the 32D–Neo control cells. In contrast, 32D.3 cells coexpressing the same Raf-1(Cat) protein with Bcl-2 exhibited significantly greater resistance to staurosporine, compared with 32D.3 cells expressing Bcl-2 alone ($p < 0.05$ by unpaired t test). None of the various 32D.3 transfectants, however, was resistant to staurosporine-induced cell cycle arrest (data not shown), implying that the enhanced resistance to this kinase inhibitor cannot be explained by reduced import of the drug or accelerated metabolism to inactive products.

Since Bcl-2 is well known for its ability to inhibit staurosporine-induced apoptosis and because 32D.3 cells expressing the mitochondria-targeted Raf-1 protein exhibited increased resistance to staurosporine-mediated cell death, it was important to determine whether Raf-1 is a staurosporine-resistant or -sensitive kinase. For this purpose, Raf-1 was expressed in Sf9 cells by using a recombinant baculovirus that encoded a mutant version of Raf-1 (259D) that has constitutively high levels of kinase activity (Morrison et al., 1993; Wang et al., 1996). Immunoprecipitates of Raf-1(259D) protein were then prepared from baculovirus-infected Sf9 cells, and the ability of this kinase to phosphorylate the substrate protein GST–MEK in the presence or absence of various concentrations of staurosporine was tested. As shown in Figure 5B, concentrations of staurosporine as high as 5 μM had no inhibitory effect on Raf-1 kinase activity, and even ≥10 μM had only modest effects (<30% inhibition). From these studies, therefore, we conclude first that targeting of activated Raf-1 kinase to mitochondria, either by fusion with the TM domain of Mas70p or through its interaction with Bcl-2, promotes resistance to staurosporine-induced apoptosis, and second, that Raf-1 is a staurosporine-resistant kinase.

Raf-1 Regulates the Phosphorylation of Different Proteins When Targeted to Plasma Membrane versus Mitochondria

Since Bcl-2 can promote association of Raf-1 with mitochondria, whereas Ras induces association of Raf-1 with the plasma membrane, it seems likely that activated Raf-1 protein would encounter different substrates at these two membrane sites. To explore this possibility, the state of phosphorylation of extracellular signal–

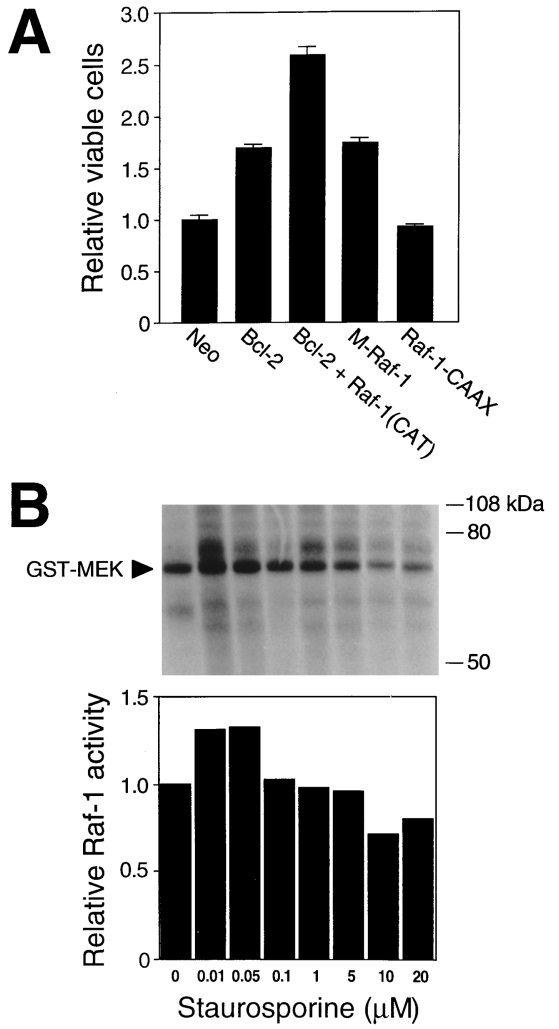

Figure 5. Raf-1 Is a Staurosporine-Resistant Kinase

(A) Stably transfected 32D.3 cells were cultured in medium containing IL-3 and 1 μM staurosporine for 24 hr. The number of viable cells relative to 32D.3-Neo was determined by MTT assay (mean ± SD; n = 3), which was set as 1.0. Similar results were obtained by trypan blue dye exclusion assay (data not shown).

(B) Active Raf-1(259D) kinase was incubated with 1 μg of GST–MEK and various concentrations of staurosporine with 20 μCi of [^{32}P]γATP. Phosphorylation of GST–MEK was analyzed by SDS–PAGE/autoradiography (top), and the results were quantified by β scanning (bottom). Incubation of GST–MEK with anti-Raf-1 antiserum/protein A complexes lacking Raf-1 resulted in no detectable background phosphorylation (Wang et al., 1996; data not shown).

regulated protein kinases 1 and 2 (ERK1 and ERK2) was examined. The ERK1 and ERK2 proteins become phosphorylated in a Ras-dependent manner by MAPK/ERK kinase (MEK), a direct substrate of Raf-1 whose enzymatic activity becomes elevated upon stimulation of cells with various growth factors (Kyriakis et al., 1992). The phosphorylation of ERK1 and ERK2 results in a shift in their mobility upon SDS–polyacrylamide gel electrophoresis (PAGE) to slower-migrating species that can be detected by immunoblot analysis.

The 32D.3 cells expressing the M-Raf-1 and Raf-1–CAAX proteins were therefore deprived of IL-3 for 6 hr and the gel mobility of ERK1 and ERK2 was compared. Cells expressing the Raf-1–CAAX protein contained both the unphosphorylated ~42 kDa ERK1 and ~44 kDa ERK2 proteins as well as the higher molecular weight, phosphorylated forms of ERK1 and ERK2 (Figure 6A). In contrast, 32D.3 cells expressing M-Raf-1 and 32D.3 cells coexpressing Bcl-2 and untargeted Raf-1(Cat) contained only the unphosphorylated forms of ERK1 and ERK2. The differences in ERK1 and ERK2 phosphorylation seen in 32D.3 cells expressing the M-Raf-1 and Raf-1–CAAX proteins cannot be attributed to lower levels of the kinase activity in the former, since both the M-Raf-1 and Raf-1–CAAX proteins lacked the N-terminal regulatory region where Ras binds and were already maximally active. These findings thus support the idea that targeting of Raf-1 to different intracellular membrane compartments through its interactions with Ras (plasma membrane) and Bcl-2 (mitochondrial membranes) promotes the interactions of Raf-1 with different substrate proteins.

What then are the substrates of Raf-1 at mitochondrial membranes? Previously, we observed that while Raf-1 can associate with Bcl-2, it does not normally phosphorylate it (Wang et al., 1994). To explore the possibility that Raf-1 might phosphorylate other Bcl-2 family proteins, baculovirus-produced Raf-1 (259D) protein was tested for its ability to phosphorylate in vitro the bacterially produced fusion proteins GST–Bcl-2, GST–Bcl-X_L, GST–Bcl-X_S, GST–Mcl-1, GST–Bax, GST–Bak, and GST–BAD, making comparisons with the known substrate GST–MEK. Among these various GST fusion proteins, only GST–BAD and GST–MEK were phosphorylated in vitro by Raf-1 (Figure 6B).

To determine whether the BAD protein becomes phosphorylated in intact cells when Raf-1 is targeted to mitochondria, 293 cells were transiently cotransfected with a plasmid producing a hemagglutinin (HA) epitope–tagged human BAD protein and plasmids producing the various versions of Raf-1 described above: M-Raf-1, M-Raf-1(K375W), Raf-1–CAAX, or the parental plasmid (Neo) as a control. After 2 days, HA–BAD was analyzed by immunoblotting using an anti-HA monoclonal antibody. In addition to the ~30 kDa HA–BAD protein that was present in all transfectants, 293 cells expressing the active M-Raf-1 protein, but not cells expressing the M-Raf-1(K375W) or Raf-1–CAAX proteins, also contained a slower-migrating ~31 kDa form of HA–BAD (Figure 6C, lane 4). Treatment of the HA–BAD-containing cell lysates with alkaline phosphatase removed the ~31 kDa form (Figure 6C, lane 8), suggesting that it had resulted from phosphorylation. Labeling cells with $^{32}PO_4$ confirmed that M-Raf-1 induced phosphorylation of the BAD protein in intact cells, whereas M-Raf-1(K375W) and Raf-1–CAAX did not (Figure 6D).

Discussion

The findings reported here suggest a novel role for Raf-1 at mitochondrial membranes (and possibly other intracellular membranes where Bcl-2 resides) as a blocker of apoptotic cell death, as opposed to its more traditionally known role as a promoter of cell proliferation and differentiation within the context of the Ras signaling pathways at the plasma membrane. Several analogies can

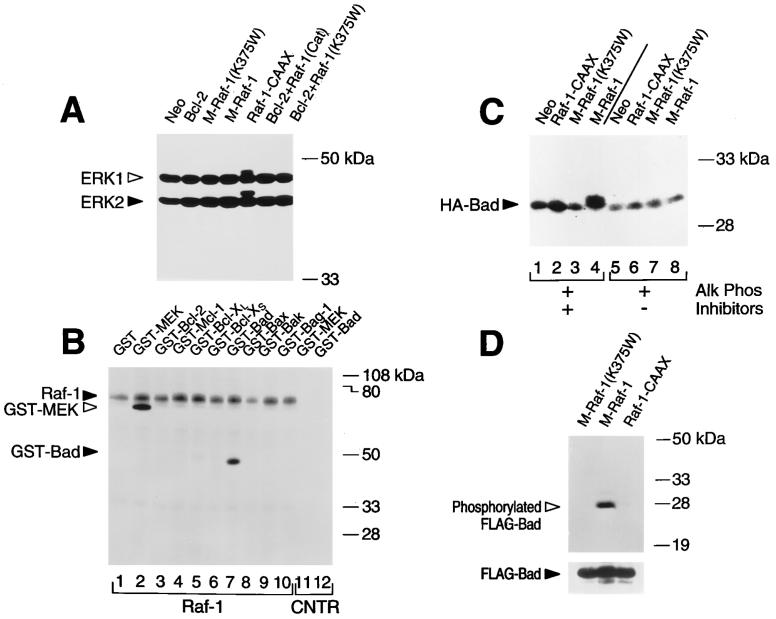

Figure 6. Differential Phosphorylation of ERK1, ERK2, and BAD in 32D.3 Cells Expressing Mitochondria- and Plasma Membrane–Targeted Active Raf-1

(A) Phosphorylation of ERK1 and ERK2 in 32D.3–Raf-1–CAAX, 32D.3–M-Raf-1, and other transfectants was examined based on altered migration of the phosphorylated proteins in gels. Stably transfected 32D.3 cells were cultured in the absence of IL-3 for 6 hr, and lysates were prepared (25 μg per lane). Data represent SDS–PAGE/immunoblotting using anti-ERK rabbit antiserum.

(B) Raf-1(259D) was tested for ability to phosphorylate various GST fusion proteins in vitro as described for Figure 5B. Anti-Raf antiserum/protein A complexes lacking Raf-1(259D) protein were used as a control (CNTL).

(C) 293 cells were transiently transfected with 20 μg of pcDNA3-HA-BAD and 20 μg of pcDNA-3 plasmid (Neo) (lanes 1 and 5) or pcDNA-3 encoding Raf-1-CAAX (lanes 2 and 6), M-Raf-1(K375W) (lanes 3 and 7), or M-Raf-1 (lanes 4 and 8). After 2 days, the cells were cultured in 0.5% serum for 6 hr, and cell lysates (50 μg of total protein) were incubated with 1 U of alkaline phosphatase at 37°C for 4 hr in the presence (plus) or absence (minus) of phosphatase inhibitors prior to SDS–PAGE/immunoblot analysis using anti-HA monoclonal antibody and ECL-based detection. Reprobing the same blot with anti-Raf antibody confirmed production of comparable amounts of the M-Raf-1, M-Raf-1(K375W), and Raf-1–CAAX proteins (data not shown).

(D) 293 cells were transiently cotransfected with 20 μg of pFLAG-CMV2-BAD and 20 μg M-Raf-1, M-Raf-1(K375W), or Raf-1-CAAX-producing plasmids and labeled with $^{32}PO_4$ 2 days later. FLAG–BAD protein was immunoprecipitated with anti-FLAG antibody, subjected to SDS–PAGE, and transferred to nitrocellulose filters, followed by exposure to X-ray film to detect ^{32}P-labeled FLAG–BAD (top). The blot was subsequently probed with anti-FLAG antibody and developed by ECL, demonstrating comparable amounts of FLAG–BAD protein in all samples (bottom).

be drawn between the mechanisms by which p21^{ras} and Bcl-2 target Raf-1 to membranes. For example, because the Bcl-2 protein resides in the outer mitochondrial membrane, with its C-terminal TM domain integrated into membranes and the bulk of the protein oriented toward the cytosol (Krajewski et al., 1993; Lithgow et al., 1994), we presume that the Bcl-2–Raf-1 interaction brings Raf-1 to the surface of the outer mitochondrial membrane. Similarly, p21^{ras} family proteins orient their C-termini toward the plasma membrane because of covalent attachment of lipids at the CAAX box motif and can bring Raf-1 from the cytosol to the plasma membrane through Ras–Raf-1 protein–protein interactions (Hall, 1994). Furthermore, just as targeting of Raf-1 to plasma membranes by fusing the CAAX box motif onto Raf-1 has been shown to allow for Ras-independent activation of Raf-1 and phosphorylation of the Raf-1 substrate MEK (Stokoe et al., 1994; Leevers et al., 1994), fusing activated Raf-1 with the TM domain Mas70p allowed for Bcl-2-independent inhibition of apoptosis by Raf-1. These similarities between Ras and Bcl-2 with regards to interactions with Raf-1 emphasize the importance of protein–protein interactions for the intracellular targeting of kinases to sites of biological importance (Faux and Scott, 1996). Presumably, it is through these protein–protein interactions that specificity is achieved in terms of targeting kinases to appropriate substrates, a concept supported by the differential phosphorylation of ERK1, ERK2, and BAD shown here in 32D.3 cells expressing mitochondrial (M-Raf-1) and plasma (Raf-1–CAAX) membrane–targeted versions of active Raf-1.

An interesting difference in the ways that p21^{ras} proteins and Bcl-2 interact with Raf-1 is that while Ras binds to the N-terminal regulatory domain of Raf-1 (Zhang et al., 1993), Bcl-2 interacts with the C-terminal catalytic domain (Wang et al., 1994). Indeed, it was this difference in the locations on the Raf-1 protein where Bcl-2 and Ras bind that allowed us to explore the effects of constitutively active and kinase-inactive Raf-1 mutants (which lacked the Ras-binding domain) on the Bcl-2 pathway for suppression of apoptosis, in the absence of interfering effects on p21^{ras} function.

Overexpression of Bcl-2 can inhibit cell proliferation in some situations, perhaps by slowing the rate of progression through G_1–S phase (Pietenpol et al., 1994; Borner, 1996; Mazel et al., 1996). Given the ability of p21^{ras} and Bcl-2 to target Raf-1 to different membrane compartments, with Raf-1 presumably participating in pathways involved in mitogenesis at the plasma membrane and other pathways related to cell survival control at mitochondrial membranes, it is tempting to speculate that this phenomenon of growth suppression by Bcl-2 may reflect a competition between Ras and Bcl-2 family proteins for limiting amounts of Raf-1.

Though Ras can target Raf-1 to plasma membranes, resulting in activation of the kinase, it remains controversial whether this protein–protein interaction is sufficient to activate Raf-1. Indeed, the bulk of evidence supports the idea that other proteins, including other protein kinases, are required for Ras-mediated activation of Raf-1 (Jelinek et al., 1996). In the experiments presented here, we have bypassed the need for kinase activation by deleting the negative regulatory domain of Raf-1. The question then is how is the kinase activity of Raf-1 normally turned on when brought into the vicinity of the mitochondrial or other intracellular membranes where

Bcl-2 resides? A potential answer has come recently from the finding that another Bcl-2-associated protein, BAG-1, can bind to and activate Raf-1 through an unknown mechanism (Wang et al., 1996). Like Bcl-2, the catalytic domain of Raf-1 is sufficient for the interaction with BAG-1. Also, binding of BAG-1 to Bcl-2 is dependent on the BH4 domain, similar to the interaction of Raf-1 with Bcl-2. Thus, a preformed BAG-1–Raf-1 complex may bind to the BH4 domain of Bcl-2, thus bringing Raf-1 to mitochondrial membranes associated with an activating protein, BAG-1. Alternatively, since Bcl-2 can form dimers or larger oligomers (Oltvai et al., 1993; Sato et al., 1994), it is possible that Raf-1–BAG-1 complexes assemble on Bcl-2 dimers/oligomers via interactions with BH4 domains on different Bcl-2 protein molecules. Clarifying the structural details of how these protein complexes form and the precise mechanisms responsible for activation of Raf-1 in the vicinity of Bcl-2 represents important issues for future investigations.

Though it remains to be clarified what the substrates of Raf-1 are within its context as a Bcl-2-binding protein, the findings first that activated Raf-1 efficiently phosphorylates the BAD protein in vitro and second that BAD becomes phosphorylated in cells expressing mitochondria-targeted but not plasma membrane–targeted Raf-1 suggest (but do not prove) that BAD represents at least one (but perhaps not the only) such substrate. At present we do not know the functional significance of Raf-mediated phosphorylation of the BAD protein. However, it might hypothetically prevent BAD from binding to Bcl-2 and Bcl-X_L, thus relieving repression of these antiapoptotic proteins by allowing them to homodimerize with themselves, interact with other proteins, or form pores in membranes.

Bcl-X_S is the only known proapoptotic member of the Bcl-2 family that contains a BH4 domain. Since Bcl-X_s can interact wtih Raf-1, conceivably one mechanism by which Bcl-X_S may antagonize the function of Bcl-2 is by competing with Bcl-2 for binding to Raf-1. However, since Bcl-X_S probably resides in the same membrane compartments with Bcl-2 (González-Garcia et al., 1994), there must be other reasons why Bcl-X_S cannot substitute for Bcl-2 in either targeting Raf-1 to appropriate substrates or providing the means for activation of Raf-1 within the vicinity of mitochondrial or other internal membranes. One likely explanation for the differential outcome of these interactions of Raf-1 with Bcl-2 versus Bcl-X_S can be attributed to the capacity of the former to dimerize with BAD and the inability of latter to do so (Sedlak et al., 1995), though other explanations are also possible.

While the Bcl-2–Raf-1 interaction defines a novel regulatory event in the control of apoptosis, it is unclear whether it is essential for the function of Bcl-2 as a suppressor of cell death. The observation that Raf-1 is a staurosporine-resistant kinase that can collaborate with Bcl-2 in the inhibition of staurosporine-induced apoptosis is at least consistent with the idea that the Bcl-2–Raf-1 interaction may be critical, inasmuch as staurosporine is a universal inducer of apoptosis (Weil et al., 1996). Genetic analysis of Raf-1 homologs in the fly Drosophilia melanogaster (D-Raf) and the nematode Caenorhabditis elegans (LIN-45), however, fail to suggest an essential role for Raf in preventing programmed cell death (Han et al., 1993; Lu et al., 1994; Tsuda et al., 1993). Moreover, the recently reported structure of the Bcl-X_L protein suggests that Bcl-2 family proteins may have intrinsic functions as pore-forming proteins (Muchmore et al., 1996), irrespective of their interactions with other proteins. Thus, it seems unlikely that the Raf-1–Bcl-2 interaction is absolutely required for suppression of apoptotic cell death. Nevertheless, the finding that a dominant-inhibitory mutant of Raf-1 lacking kinase activity completely abrogated the survival-promoting effects of Bcl-2 in 32D.3 cells argues that Raf-1 can at least be a potent modulator of Bcl-2 function in some types of cells. The interaction of Raf-1 with Bcl-2, therefore, may provide a target for future attempts at modulating Bcl-2 protein activity for therapeutic benefit.

Experimental Procedures

Plasmids

pcDNA3-Raf-1/CAAX chimeric plasmid encoding Raf-1(Δ26–303) joined with the 20 C-terminal amino acids of K-Ras at the C-terminus of Raf-1 was generated by polymerase chain reaction (PCR) mutagenesis using the plasmids pRSV-BXB (Bruder et al., 1992) and pZIP-raf/CAAX (gift of Channing Der) as templates and the primers 5′-CGGGCTCCTGGCTCCCTCAGGTTTAAGAATTG-3′ (forward 1), 5′-AATTGCTCTGGGGTTGGGTCGACAAC-3′ (reverse 1), 5′-TGCAG TAAAGATCCTAAAGGTTGTC-3′ (forward 2), and 5′-CGGGATCCTT ACATAATTACACACTTTGTC-3′ (reverse 2). pcDNA3-Mas70/Raf-1 (Cat) and pcDNA3-Mas70/Raf-1(Cat)(K375W) containing the Mas-70p TM domain (residues 1–29) fused to the N-terminus of Raf-1 (Δ26–303) or Raf-1(Δ26–303)(K375W) were generated by using the plasmids YEplac181-Mas70 (gift of Gottfried Schatz) (Hase et al., 1984), pKSII-BXB, and pRSV-BXB(K375W) (Bruder et al., 1992) as templates and the primers 5′-GGAATTCGCCACCATGAAGAGCTTC ATTACAAGG-3′ (forward 1), 5′-CCTGTATGTGCTCCATGTAATAATA GTAGGCACCGATGGC-3′ (reverse 1), 5′-TGCCTACTATTATTACATG GAGCACATACAGGGAGCTTG-3′ (forward 2), and 5′-CAGTCTAGAA GGTCCTTAGCAGAGC-3′ (reverse 2). The pGFP-Raf-1(Δ26–303) plasmid was generated by subcloning the Raf-1(Δ26–303) cDNA from pKSII-BXB into the pGFP-C1 (Clontech, Incorporated) by using EcoRI–XbaI sites. PCR-generated cDNAs encoding the human Bcl-2 (ΔTM) (i.e., without C-terminal TM domain), Bcl-X_L(ΔTM), Bcl-X_s(ΔTM), Mcl-1(ΔTM), Bax(ΔTM), Bak (ΔTM), BAD, and BAG-1 proteins were subcloned into the EcoRI and XhoI sites of pGEX-4T-1, in-frame with GST, as described (Hanada et al., 1995). The human *BAD* cDNA was PCR-amplified from a HepG2 cDNA library by using forward (5′-GGAATTCATGTTCCAGATCCCAGAGTTTG AGC-3′) and reverse (5′GCTCTAGATCACTGGGAGGGGGCGGAGC TTCC-3′) primers based on sequences of EST database clones 239688 and 301984. After digestion with EcoRI and XbaI, the *BAD* cDNA was subcloned into the HA tag vector pShin-HA (Wang et al., 1996) and a modified version of the FLAG-tag vector pFLAG-CMV2. All other plasmids have been described (Wang et al., 1994; Hanada et al., 1995).

Cell Transfections

The 32D.3 cells were maintained in IL-3-containing medium, and 20 μg of plasmid DNAs was introduced by electroporation as described (Wang et al., 1994, 1995). The 293 cells were transfected by a calcium phosphate method (Wang et al., 1996).

Coimmunoprecipitation Assay

Infections of Sf9 cells with recombinant baculoviruses and coimmunoprecipitation assays were performed as described (Wang et al., 1994, 1995, 1996) with antisera specific for Raf-1, Bcl-2, Bcl-X, or Bax, or normal rabbit serum (control) for immunoprecipitations and an anti-Raf-1 monoclonal antibody (URP30) for blotting.

In Vitro Protein Binding Assay

Sf9 cells (8×10^7) were infected with GST–Raf-1 or GST–Raf-1(ΔCR3) recombinant baculoviruses (MOI ~10), and lysates were prepared

in 4 ml of Triton X-100 lysis buffer (150 mM NaCl, 10 mM Tris [pH 7.4], 5 mM EDTA [pH 8.0], 1% Triton X-100) containing 1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. Lysates were mixed with 100 μl of glutathione–Sepharose for 3 hr at 4°C. The beads were washed three times with 1 ml of NP-40 lysis buffer supplemented with protease inhibitors and resuspended in 0.5 ml of the same solution. GST-loaded beads (5 μg protein on ~20 μl) were incubated with 10 μl of reticulocyte lysates (TNT-lysates; Promega, Incorporated) containing in vitro translated [^{35}S]methionine-labeled Bcl-2, Bcl-2(ΔBH4), Bcl-X_S, or Bax for 3 hr at 4°C. After extensive washing in NP-40 lysis buffer, beads were boiled in Laemmli buffer and eluted proteins analyzed by SDS–PAGE (12% gels) and detected by fluorography.

Yeast Two-Hybrid Assay

The cDNA fragments encoding Raf-1, Bcl-2(1–218) without its TM domain, Bcl-2(1–218)(ΔBH4), Bcl-2(1–81), Bcl-2(1–81)(ΔBH4), Bcl-2(83–218), and Bax(1–171) without its TM were subcloned from pEG202 (Hanada et al., 1995) into the two-hybrid vector pGilda (gift of E. Golemis and D. Buckholtz), which produces fusion proteins with a LexA DNA-binding domain at the N-terminus under the control of a Gal1 promoter (Estojak et al., 1995). EGY191 strain yeast was cotransformed with various pGilda expression plasmids in combination with various pJG4–5 plasmids, which produce B42 *trans*-activation domain fusion proteins as described (Sato et al., 1994; Hanada et al., 1995). Filter assays were performed for β-galactosidase measurements, by using cells plated on either galactose- or glucose-containing MM medium supplemented with 20 μg/ml leucine. Colorimetric results were photographed after 2.5 hr.

GFP Studies

The 293 cells were transfected with 20 μg of pGFP–Raf-1(Δ26–303) or pGFP parental plasmid DNA and selected in 0.8 mg/ml G418. The stably transfected cells were then transiently transfected with 20 μg of pcI-Neo-Bcl-2 or pcI-Neo-Bcl-2(ΔBH4) plasmid DNA. After ~48 hr, cells were seeded into 24-well plates containing coverslips pretreated with fibronectin and were returned to culture overnight. The medium was replaced with fresh medium containing 25 nM MitoTracker (Molecular Probes, Incorporated). After 0.5 hr, the cells were then washed with PBS and fixed in 3.7% paraformaldehyde for 15 min, washed three times in PBS, and mounted in Vectashield mounting medium (Vector Labs, Incorporated) for confocal analysis and photographing with an Axiophot (Zeiss, Incorporated).

Cell Viability and Apoptosis Assays

Relative numbers of viable 32D.3 cells were estimated by MTT assay, and flow cytometric DNA content analysis was performed by propidium iodide staining (Wang et al., 1994, 1995).

Preparation of Subcellular Fractions

32D.3-M–Raf-1 cells or 32D.3-M–Raf-1(K375W) cells (6×10^7) were washed twice in ice-cold PBS and resuspended in 2 ml of hypotonic buffer (5 mM Tris [pH 7.4], 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA [pH 8.0], 1 mM DTT) containing 0.2 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 0.7 μg/ml pepstatin A, and incubated on ice for 30 min. After homogenization for 20–30 strokes with a Dounce homogenizer, samples were transferred to Eppendorf centrifuge tubes and centrifuged at 500 × g for 5 min at 4°C to discard nuclei. The resulting supernatant was centrifuged at 10,000 × g for 0.5 hr at 4°C to obtain the HM fraction (pellet), and this supernatant was then centrifuged for 1.5 hr at 150,000 × g to obtain the LM (pellet) and cytosolic (supernatant) fractions. The HM and LM material was resuspended in 0.1 ml of Triton X-100 lysis buffer.

In Vitro Kinase Assay

Raf-1(259D) protein was immunoprecipitated from lysates prepared from 2×10^7 infected Sf9 cells by using 50 μl of anti-Raf-1 peptide antibody (Reed et al., 1991b) and 200 μl of BSA-preadsorbed protein A–Sepharose. Immune complexes were divided into 10 tubes and incubated for 30 min at 25°C in 30 μl of kinase buffer (Wang et al., 1994, 1995) containing 20 μCi of [^{32}P]-γATP, 1 μg of GST–MEK, and 1 μl of DMSO containing 0, 10 nM, 50 nM, 100 nM, 1 μM, 5 μM, 10 μM, or 20 μM staurosporine. Phosphorylation of GST–MEK was analyzed by SDS–PAGE/autoradiography, and the results were quantified with a β-scanner (Bio-Rad, Incorporated). Alternatively, 1 μg of GST–BAD or other GST fusion proteins was added, instead of GST–MEK.

Immunoblot and Immunoprecipitation Assays

32D.3 cells (5×10^6) were washed three times with Iscove's modified Dulbecco's medium (IMDM) and cultured in 10 ml IMDM containing 10% FCS without IL-3 for 6 hr. Cells were lysed in Triton X-100 lysis buffer containing protease inhibitors and phosphatase inhibitors (10 mM sodium β-glycerophosphate [pH 7.4], 1 mM Na_3VO_4, 5 mM NaF, 2 mM $Na_4P_2O_7$, 50 mM 4-nitrophenyl phosphate, and 1 μM microcystin). The resulting lysates (25 μg per lane) were subjected to 10% SDS–PAGE immunoblot analysis (10% gels with 2% SDS) using 0.1 μg/ml anti-ERK rabbit antiserum (Santa Cruz Biotech, Incorporated), followed by 0.3 μg/ml horseradish peroxidase–goat anti-rabbit (Bio-Rad) and detection by an enhanced chemiluminescence (ECL) system (Amersham).

After transfection (2 days), 293 cells were cultured in medium with 0.5% serum for 6 hr, and cell lysates (50 μg of total protein) were incubated with 1 U of alkaline phosphatase at 37°C for 4 hr in the presence or absence of phosphatase inhibitors prior to SDS–PAGE immunoblot analysis using anti-HA monoclonal antibody and ECL-based detection. Alternatively, the transfected cells were labeled with $^{32}PO_4$ for 10 hr in phosphate-free Dulbecco's modified Eagle's medium containing 0.5% dialyzed serum and 0.5 mCi/ml $^{32}PO_4$. FLAG–BAD protein was immunoprecipitated with the M2 antibody (Kodak, Incorporated).

Acknowledgments

Correspondence should be addressed to J. C. R. This work was supported by National Institutes of Health grant CA-67329.

Received July 10, 1996; revised September 20, 1996.

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