Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 7063–7068, July 1996
Cell Biology

Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1

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Communicated by Peter K. Vogt, The Scripps Research Institute, La Jolla, CA, March 20, 1996 (received for review January 9, 1996)

ABSTRACT **The Bcl-2 protein blocks programmed cell death (apoptosis) through an unknown mechanism. Previously we identified a Bcl-2 interacting protein BAG-1 that enhances the anti-apoptotic effects of Bcl-2. Like BAG-1, the serine/threonine protein kinase Raf-1 also can functionally cooperate with Bcl-2 in suppressing apoptosis. Here we show that Raf-1 and BAG-1 specifically interact *in vitro* and in yeast two-hybrid assays. Raf-1 and BAG-1 can also be coimmunoprecipitated from mammalian cells and from insect cells infected with recombinant baculoviruses encoding these proteins. Furthermore, bacterially-produced BAG-1 protein can increase the kinase activity of Raf-1 *in vitro*. BAG-1 also activates this mammalian kinase in yeast. These observations suggest that the Bcl-2 binding protein BAG-1 joins Ras and 14–3-3 proteins as potential activators of the kinase Raf-1.**

The anti-apoptotic protein Bcl-2 regulates a distal step in an evolutionarily conserved pathway for cell death (1–4). Overproduction of Bcl-2 occurs frequently in human cancers and contributes to tumor radio- and chemoresistance by blocking apoptosis induced by genotoxic injury and other types of damage (5). Conversely, reduced levels of Bcl-2 have been associated with higher rates of spontaneous and inducible apoptosis in circulating lymphocytes of persons infected with HIV and some other viruses (6, 7).

The Bcl-2 protein shares no significant amino acid sequence homology with other proteins for which a biochemical mechanism is known. To gain insights into the function of the Bcl-2 protein therefore we recently attempted to identify proteins with which Bcl-2 physically interacts, thus leading to the discovery of a protein BAG-1 that binds to Bcl-2 *in vitro* and that enhances the anti-apoptotic activity of Bcl-2 in cotransfection assays (8). Like BAG-1, the serine/threonine protein kinase Raf-1 can cooperate with Bcl-2 in suppressing apoptosis, based on cotransfection assays using Bcl-2 and a transforming version of Raf-1 consisting only of the catalytic domain devoid of its N-terminal negative-regulatory domain and Ras-binding site (9). Furthermore, full-length Raf-1 protein, as well as Raf-1 deletion mutants containing only the catalytic domain, can be coimmunoprecipitated with Bcl-2 from mammalian cells and from Sf9 insect cells when infected with recombinant Bcl-2 and Raf-1 baculoviruses. However, Raf-1 may not directly bind to Bcl-2 and indeed does not induce phosphorylation of the Bcl-2 protein *in vitro* or in cells (9).

An N-terminal domain in Bcl-2 that is conserved among the anti-apoptotic members of the Bcl-2 protein family (termed A-box or BH4 domain) is required for its association with BAG-1 and Raf-1 *in vitro* (unpublished data). The pro-apoptotic Bcl-2 family protein Bax lacks this domain and fails to interact with either BAG-1 or Raf-1 *in vitro*. We therefore asked whether BAG-1 might bind to Raf-1. Our findings indicate that BAG-1 not only binds to Raf-1 but can also activate this kinase, suggesting that BAG-1 represents a novel type of Raf-1 activating protein.

MATERIALS AND METHODS

Coimmunoprecipitation Assays. Sf9 cells (6×10^6) were coinfected with BAG-1 and either Raf-1 or β-galactosidase (β-gal) recombinant baculoviruses (multiplicity of infection ≈10). Cells were lysed after 60 hr in 0.65 ml of Nonidet P-40 (NP-40) lysis buffer (10 mM Hepes, pH 7.5/142.5 mM KCl/5 mM $MgCl_2$/1 mM EGTA/0.2% NP-40) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, and 5 μg/ml aprotinin. After preclearing with normal rabbit antiserum (50 μl/ml) and 50 μl protein A-Sepharose at 4°C for 1 hr, immunoprecipitations were performed by incubating 0.2 ml of lysate with 20 μl of protein A-Sepharose preadsorbed with 10 μl of anti-Raf-1 antiserum (10), anti-BAG-1 antiserum (8), or normal rabbit antiserum as a negative control at 4°C for 3 hr. After extensive washing in NP-40 lysis buffer, beads were boiled in 60 μl of Laemmli buffer and 20 μl of the eluted proteins were subjected to SDS/12% PAGE immunoblot analysis using 0.2% (vol/vol) anti-Raf-1 monoclonal antibody ascites (URP30) (lanes 1–3) or anti-β-gal monoclonal antibody (Santa Cruz Biotechnology), followed by 0.3 μg/ml HRPase-goat anti-mouse (Bio-Rad), and detected using an enhanced chemiluminescence system (Amersham).

Cos-7 cells (1×10^6) in 10 ml of DMEM containing 10% fetal calf serum were transiently transfected with 20 μg of pcDNA3-HA-BAG-1 or pcDNA3 parental vector with 20 μg of pKRSPA-BXB(Raf-1) or pKRSPA parental plasmid DNA by a calcium-phosphate precipitation method. Cells were lysed 60 hr later in 0.35 ml of NP-40 lysis buffer and incubated at 4°C for 3 hr with 20 μl of protein G-Sepharose preadsorbed with 3 μg of anti-HA (hemagglutinin) mouse monoclonal antibody (12CA5, Boehringer Mannheim). After washing 3 times in 1.5 ml of NP-40 lysis buffer, immune complexes were subjected to SDS/4–20% PAGE immunoblot analysis using anti-Raf-1 rabbit antiserum or anti-BAG-1 rabbit antiserum, followed by HRPase-goat anti-rabbit (Bio-Rad) and detection by enhanced chemiluminescence.

***In Vitro* Binding Assays.** Glutathione *S*-transferase (GST) fusion proteins (≈10 μg) were immobilized on glutathione-Sepharose and incubated with 10 μl of reticulocyte lysates (TNT-lysates, Promega) containing *in vitro* translated [^{35}S]methionine-labeled Raf-1 or R-Ras. After extensive washing, beads were boiled in Laemmli buffer and eluted proteins were analyzed by SDS/12% PAGE and detected by fluorography.

Yeast Two-Hybrid Assays. EGY191 strain yeast were transformed with various combinations of the pEG202 expression plasmids producing LexA DNA-binding domain fusion proteins and pJG4–5 plasmids encoding B42 transactivation domain fusion proteins (11). Growth on leucine-deficient me-

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Abbreviations: NP-40, Nonidet P-40; β-gal, β-galactosidase; GST, glutathione *S*-transferase; HA, hemagglutinin.

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dium that contained glucose or galactose for repression or activation, respectively, of the Gal1 promoter in pJG4–5 was scored 4 days later, as described (11, 12).

***In Vitro* Kinase Assays.** Sf9 cells (10^7) were infected with recombinant baculoviruses (multiplicity of infection ≈ 10) encoding Raf-1. After ≈60 hr, cells were lysed in 1 ml RIPA buffer (25 mM Tris, pH 8.0/150 mM NaCl/0.1% SDS/0.5% sodium deoxycholate/1% NP-40/10% glycerol/2 mM EDTA) containing 1 mM sodium orthovanadate, 5 mM NaF, 1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. After preclearing with 200 μl of protein A-Sepharose, Raf-1 was immunoprecipitated using 0.2 ml of protein A-Sepharose preadsorbed with 0.1 ml anti-Raf antiserum and the resulting immune complexes were washed twice in Triton X-100 buffer (20 mM Tris, pH 7.4/150 mM NaCl/1% Triton X-100/10% glycerol/2 mM EDTA) containing 1 mM sodium orthovanadate, 5 mM NaF, 1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin and then washed in kinase buffer (25 mM Hepes, pH 7.4/150 mM NaCl/25 mM glycerol phosphate/1 mM DTT/5 mM $MgCl_2$/5 mM $MnCl_2$). One-tenth (20 μl) of the resulting immune complexes were incubated with various amounts of purified GST or GST-BAG-1 in 20 μl of PBS at 4°C for 15 min. Kinase buffer (30 μl) containing 1 μg purified GST-MEK protein, 10 μM ATP, and 20 μCi [γ-^{32}P]ATP was then added for 30 min at 25°C. The samples were centrifuged at 15,000 × *g* for 1 min, and the supernatant containing GST-MEK was analyzed by SDS/PAGE and the results quantified using a β-scanner (Bio-Rad; GS-525 Molecular Imager System).

The 293 cells were transfected with 20 μg of pcDNA3-HA-BAG-1, pcDNA3-HA-BAG-1(ΔN), or pcDNA3 parental plasmid DNA by a $CaPO_4$ precipitation method and selected in 800 μg/ml G418. Clones expressing high levels of HA-BAG-1 proteins were isolated and 10^7 transfected cells were lysed in NP-40 lysis buffer or RIPA buffer containing 1 mM sodium orthovanadate, 5 mM NaF, 1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. Endogenous Raf-1 protein was immunoprecipitated with protein A-Sepharose preadsorbed with anti-Raf-1 rabbit antiserum and *in vitro* kinase assays were performed using 1 μg of GST-MEK as a substrate (13).

Assay for Raf-1 Activation in Yeast. *Saccharomyces cerevisiae* strain SY1984-RP was used to detect effects of BAG-1 on Raf-1 activity, essentially as described (14). SY1984-RP cells were transformed with pAAH5-BAG-1, pAD4-Bcl-2, pAD4-SOD, YEplac181-Mas70P, pAAH5 empty vector, YEp13 parental vector, or YEp13-RAS1 plasmid DNA. Activation of Raf-1 was detected by growth on histidine-deficient SC-plates for 3 days, indicating activation of the *FUS1*::*HIS3* reporter gene.

RESULTS

For initial experiments, Raf-1 and BAG-1 were coexpressed in Sf9 insect cells using recombinant baculoviruses. Immunoprecipitations were performed using antisera specific for Raf-1 and BAG-1, or with normal rabbit serum as a control, followed by SDS/PAGE immunoblot assay using anti-Raf-1 antibodies. Under these conditions ≈5% of the total Raf-1 coimmunoprecipitated with BAG-1 (Fig. 1*A*) and vice versa (not shown). The association of Raf-1 with the BAG-1 protein was specific, since in Sf9 cells coinfected with a β-gal- and a BAG-1-producing virus, β-gal protein failed to coimmunoprecipitate with BAG-1 (Fig. 1*A*).

The domain within the Raf-1 protein required for its association with BAG-1 was mapped using recombinant baculoviruses encoding GST-fusion proteins that contained: (*i*) full-length Raf-1, (*ii*) a mutant consisting essentially only of the catalytic domain [Raf-BXB], and (*iii*) a mutant lacking the catalytic domain [Raf-GRS] (Fig. 1*B*). Sf9 cells were coinfected with a BAG-1 virus and one of these GST-Raf-1-producing viruses. GST-fusion proteins were then recovered on glutathione-Sepharose and associated BAG-1 was detected by SDS/PAGE immunoblot assay, revealing that BAG-1 specifically associated with full-length Raf-1 and the Raf-1(BXB) protein, which consists essentially only of the catalytic domain, but not with the Raf-1(GRS) mutant that lacks the catalytic domain of Raf-1 (Fig. 1*B*). Incubating the blot with an anti-GST monoclonal antibody however confirmed production of the GST-Raf-1(GRS) protein, excluding problems with expression of the GST-Raf-1(GRS) protein as an explanation for its failure to associate with BAG-1 in Sf9 cells. The catalytic domain of Raf-1 therefore appears to be sufficient for interactions with BAG-1, similar to our previous studies where the domains in Raf-1 required for coimmunoprecipitation with Bcl-2 were mapped (9).

To confirm that association of BAG-1 and Raf-1 can occur in mammalian cells, Cos-7 cells were transiently cotransfected with expression plasmids producing an HA-tagged BAG-1 protein and the catalytic domain of Raf-1(BXB). Immunoprecipitates were prepared using anti-HA antibody and subjected to SDS/PAGE immunoblot assays using anti-Raf-1 or anti-BAG-1 antibodies, revealing that Raf(BXB) protein can coimmunoprecipitate with HA-Bag-1 (Fig. 1*C*). Endogenous full-length p72–74 Raf-1 could also be coimmunoprecipitated with HA-BAG-1 from 293 cells, though the signals were more difficult to see because of the relative low levels of Raf-1 in these cells (not shown). The proportion of Raf-1(BXB) associated with BAG-1 represented ≈1% of the total cellular amount of this protein (Fig. 1*A*).

The only region in the BAG-1 protein that shares significant amino acid homology to other known proteins is a ubiquitin-like domain located between residues 43 and 89. To explore whether binding of BAG-1 to Raf-1 requires this domain, an N-terminal deletion mutant of BAG-1 that lacks the first 89 amino acids was expressed in bacteria as a GST-fusion protein and compared with full-length GST-BAG-1 for ability to interact *in vitro* with *in vitro* translated ^{35}S-Raf-1 protein. The full-length BAG-1 and BAG-1(ΔN) GST-fusion proteins bound to Raf-1 with comparable efficiencies (Fig. 1*D*), indicating that the ubiquitin-like domain of BAG-1 is not required for its interaction with Raf-1 and demonstrating that residues 90 → C terminus of BAG-1 are sufficient for binding to Raf-1. This experiment also complements the above studies where Raf-1 and BAG-1 were expressed in mammalian or insect cells, showing that bacterially-produced BAG-1 can bind *in vitro* to Raf-1 produced in reticulocyte lysates. The failure of GST-BAG-1 to bind to *in vitro* translated R-Ras protein, as well as the lack of binding of *in vitro* translated Raf-1 with GST-CD40 and GST-TNFRI confirmed the specificity of these protein interactions (Fig. 1*D*).

Evidence has been obtained that the enzymatic activity of Raf-1 can be increased through interactions with other proteins, including some members of the Ras and 14-3-3 protein families, though these protein–protein interactions are probably insufficient by themselves for fully activating the kinase (14–20). Like BAG-1, 14-3-3 family proteins can interact with Raf-1 at least in part through binding to its catalytic domain (CR3), whereas Ras proteins bind by means of a domain (CR1) located in the N-terminal portion of Raf-1 (14–20). We therefore tested whether bacterially-produced GST-BAG-1 protein could influence the enzymatic activity of Raf-1. For these experiments, full-length Raf-1 was immunoprecipitated from Sf9 cells that had been infected with a Raf-1 baculovirus and the resulting immune complexes were incubated *in vitro* with 1 μg of a physiological Raf-1 substrate (bacterially-produced, affinity-purified GST-MEK) and various amounts of purified GST-BAG-1 or control GST proteins. GST-BAG-1, but not control GST, increased the specific activity of Raf-1 as measured by phosphorylation *in vitro* of GST-MEK (Fig. 2*A*). The BAG-1-mediated increase in the kinase activity of Raf-1 was linear up to ≈5 μg of GST-BAG-1 protein, after which addition of more GST-BAG-1 protein had either no

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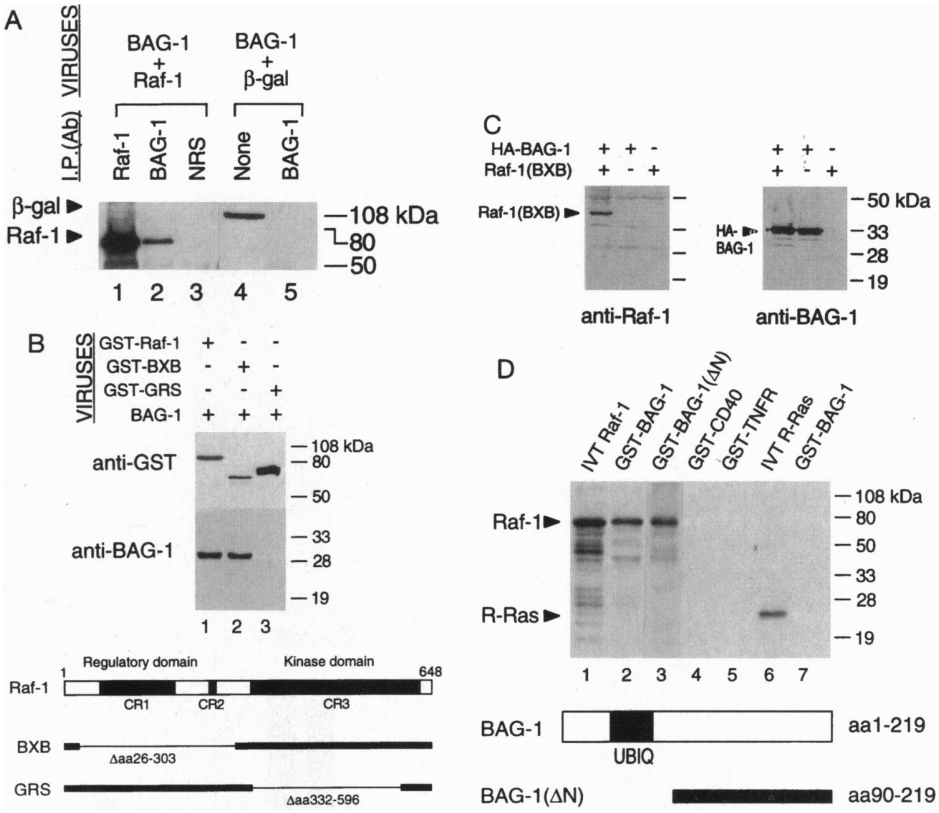

FIG. 1. Raf-1 binds to BAG-1. (*A*) Coimmunoprecipitation experiment using lysates prepared from Sf9 cells coinfected with either BAG-1 and Raf-1 or BAG-1 and β-gal-producing recombinant baculoviruses. Immunoprecipitations were performed using either normal rabbit serum as a negative control or polyclonal rabbit antisera specific for Raf-1 (10) or BAG-1 (8). Western blots were developed with anti-Raf-1 antibody (lanes 1–3) or anti-β-gal antibody (Santa Cruz Biotechnology) (lanes 4 and 5). Sf9 lysate (5 μl) was run directly in gel for lane 4, without immunoprecipitation. (*B*) Sf9 cells (6×10^6) were coinfected with BAG-1 baculovirus or various GST-Raf-1 viruses as indicated. Cell lysates were prepared using 0.5 ml of NP-40 lysis buffer and incubated with 25 μl of glutathione-Sepharose. After washing 4 times with 1 ml of NP-40 buffer, the recovered proteins were analyzed by Western blot analysis using anti-BAG-1 and anti-GST monoclonal antibodies (S.T., Kristine Kochel, and J.C.R., unpublished data) and an enhanced chemiluminescence detection system. The structures of the GST-Raf-1 proteins are depicted at bottom. (*C*) Coimmunoprecipitation of HA-BAG-1 and Raf-1(BXB) from Cos-7 cells. Cos-7 cells were transiently transfected with expression plasmids producing HA-BAG-1 or Raf-1(BXB) proteins as indicated (+), or the same parental vectors lacking BAG-1 or Raf-1 cDNAs (−). Cell lysates were prepared and immunoprecipitation were done using anti-HA monoclonal antibody followed by Western blot analysis of the resulting immune complexes using either anti-Raf-1 (*left*) or anti-BAG-1 (*right*) antisera. The position of the Raf-1(BXB) protein is noted. Similar results were obtained when 293 cells were employed (not shown). (*D*) Bacterially produced BAG-1 binds to *in vitro* translated Raf-1 *in vitro*. Various GST-fusion proteins were immobilized on glutathione-Sepharose and then incubated with *in vitro* translated ^{35}S-Raf-1 (lanes 2–5) or ^{35}S-R-Ras (lane 7), washed, and the recovered proteins were analyzed by SDS/PAGE. In some cases, an equivalent amount of *in vitro* translated Raf-1 or R-Ras protein was run directly in the gel (lanes 1 and 6). The structures of the mouse BAG-1 and BAG-1(ΔN) proteins are indicated below.

effect or began to exert inhibitory effects, possibly because of interference with Raf-1 access to GST-MEK substrate. An ≈5-fold elevation in the specific activity of Raf-1 was induced by 5 μg GST-BAG-1, after correcting for any nonspecific effects of the GST control protein. BAG-1 did not directly phosphorylate the GST-MEK substrate, based on experiments where GST-BAG-1 was incubated with GST-MEK in the presence of [γ-^{32}P]ATP or where GST-BAG-1 was added to immune complexes that had been prepared from Sf9 cells infected with a β-gal baculovirus instead of Raf-1 virus (Fig. 2*B* and not shown). An His$_6$-tagged, affinity-purified BAG-1 protein activated Raf-1 *in vitro* to a similar extent as GST-BAG-1 (≈3.5-fold increase), implying that the GST moiety is unimportant for this effect (Fig. 2*B*, lane 1). The GST-BAG-1(ΔN) protein also activated Raf-1 *in vitro*, almost as efficiently as full-length GST-BAG-1 protein (≈3-fold) (Fig. 2*B*, lane 3). Various GST control proteins, including GST, GST-Bcl-2, and GST-Bax, did not induce elevations in Raf-1 activity above their baseline levels (Fig. 2*B*, lanes 4 and 5, and not shown).

Though Raf-1 bound to BAG-1 *in vitro*, it did not induce phosphorylation of BAG-1 protein. For example, experiments were performed in which GST-BAG-1 protein or GST-MEK as a positive control were incubated with Raf-1(259D), a transforming constitutively active form of Raf-1. The Raf-1(259D) protein induced no detectable phosphorylation of BAG-1, whereas GST-MEK was heavily phosphorylated (Fig. 2*C*, lanes 2 and 4). Use of another mutant of Raf-1 that lacks enzymatic activity, Raf-1 (YY340, 341FF), served as a negative control (lanes 1 and 3) (21, 22). Raf-1 also did not induce detectable phosphorylation of BAG-1 in ^{32}PO$_4$-labeled Sf9 cells (not shown). Thus, Raf-1 fails to phosphorylate both BAG-1 and Bcl-2 (Fig. 2*C*) (9), though it can be coimmunoprecipitated with these proteins.

To gain insights into whether BAG-1 can activate Raf-1 in mammalian cells, expression plasmids producing BAG-1 or BAG-1(ΔN) protein were stably transfected into 293 cells and Raf-1 was immunoprecipitated under conditions of gentle detergent (0.2% NP-40) designed to preserve protein–protein interactions or using harsh detergent (RIPA buffer) conditions that disrupt most protein–protein interactions. When using gentle conditions, Raf-1 immunoprecipitated from BAG-1 and BAG-1(ΔN) expressing cells had 2–2.5 higher specific activity than when immunoprecipitated from control transfected cells under the same the conditions. In contrast, when Raf-1

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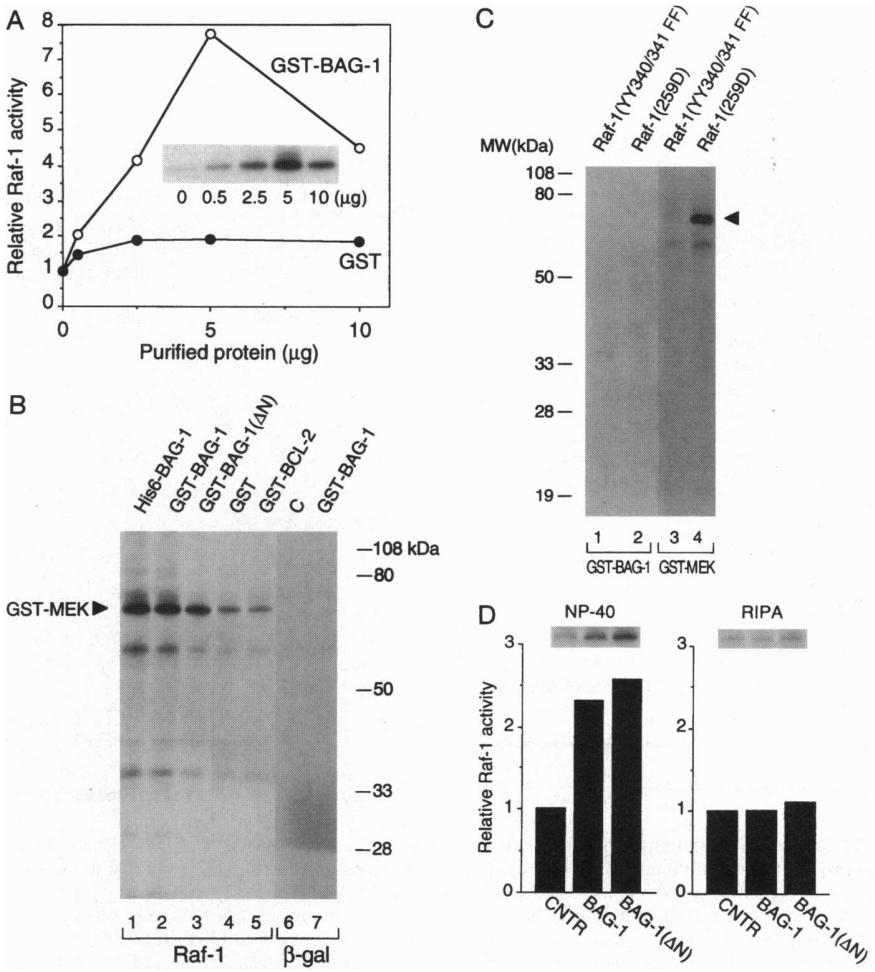

FIG. 2. BAG-1 stimulates increases in kinase activity of Raf-1. (*A*) Human Raf-1 protein was expressed in Sf9 cells and immunoprecipitated using anti-Raf-1 antiserum. Raf-1-containing immune complexes bound to protein A-Sepharose were aliquoted into equal portions and incubated with various concentrations of purified GST-BAG-1 or GST control proteins in a kinase buffer containing 1 μg of purified GST-MEK substrate and 20 uCi [γ-^{32}P]ATP. The soluble fraction was analyzed by SDS/PAGE autoradiography and the results quantified using a β-imager (Bio-Rad; GS-525). Representative of two of two experiments. (*B*) Recombinant purified His$_6$- or GST-fusion proteins (5 μg) were mixed with Raf-1 immune complexes that were prepared from Sf9 cells infected with either Raf-1 (lanes 1–5) or β-gal (lanes 6 and 7) baculoviruses. *In vitro* kinase assays were then performed using GST-MEK substrate and [γ-^{32}P]ATP as described above. C, control in which no GST- or His$_6$-recombinant protein was added. (*C*) BAG-1 is not a substrate of Raf-1 *in vitro*. Constitutively active Raf-1 (259D) or inactive Raf-1 (YY340, 341FF) protein was expressed in Sf9 cells (9, 21, 22), immunoprecipitated with anti-Raf-1 antibody, and incubated with either 3 μg GST-BAG-1 (lanes 1 and 2) or 1.5 μg GST-MEK (lanes 3 and 4) in a kinase buffer containing [γ-^{32}P]ATP. Phosphorylated proteins were analyzed by SDS/PAGE autoradiography. Arrow indicates position of GST-MEK phosphoprotein. (*D*) 293 cells that had been stably transfected with control (CNTR) plasmid or expression plasmids producing BAG-1 or BAG-1 (ΔN) proteins were lysed using a buffer containing 0.2% NP-40 (*left*) or in RIPA buffer (*right*). Raf-1-containing immune complexes recovered on protein A-Sepharose were subjected to *in vitro* kinase assays using GST-MEK substrate and [γ-^{32}P]ATP, and the soluble fraction was subjected to SDS/PAGE analysis. Autoradiograms show phosphorylated GST-MEK protein. Data were quantified using a β-imager and normalized relative to Raf-1 derived from control-transfected 293 cells. Western blot analysis of the pellets (Raf-1 immune complexes on protein A-Sepharose) confirmed the presence of equivalent amounts of Raf-1 protein in all lanes (not shown). Data are representative of two of two experiments.

immunoprecipitates were prepared using harsh conditions, no differences were noted in the activity of Raf-1 from the control-transfected and the BAG-1- or BAG-1(ΔN)-transfected 293 cells. Immunoblot analysis of immune complexes prepared under the same conditions revealed similar amounts of Raf-1 protein for all samples and demonstrated that BAG-1/Raf-1 interactions were preserved in 0.2% NP-40 but not in RIPA buffer (not shown). These findings therefore suggest that for BAG-1 to stimulate increases in Raf-1 activity, it or an associated protein must be bound to Raf-1.

Because the experiments described above did not involve use of purified Raf-1 protein, we cannot determine whether BAG-1 directly binds to and activates this kinase. However, Raf-1 also displayed specific interactions with Bag-1 in yeast two-hybrid experiments (Fig. 3*A*), implying either that these two proteins directly bind to each other or that other required proteins are conserved even in budding yeast. As expected, BAG-1 also interacted with Bcl-2 in these two-hybrid assays, but not with Ha-Ras (V12), Bax, or Fas (Fig. 3*A* and data not shown). Moreover, BAG-1 also activated mammalian Raf-1 in budding yeast (Fig. 3*B*), based on experiments using the same reporter gene system that has been employed previously to document activation of Raf-1 by Ras and 14–3-3 proteins (14).

DISCUSSION

Taken together, the observations described here indicate that BAG-1 represents a novel Raf-1 activating protein. The BAG-1 protein shares no obvious amino acid sequence homology with the other known Raf-1 activating proteins, Ras

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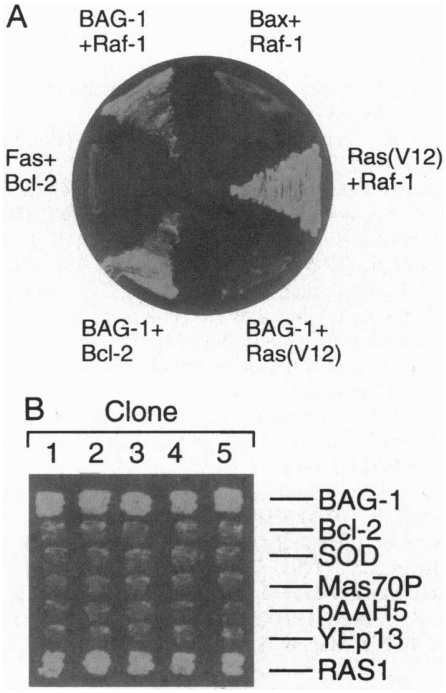

FIG. 3. BAG-1 binds to and activates Raf-1 in budding yeast. (*A*) Two-hybrid analysis of BAG-1/Raf-1 interactions. Pairs of fusion proteins containing either a N-terminal LexA DNA-binding domain (*top*) or a B42 transactivation domain (*bottom*) were expressed in EGY191 strain *S. cerevisiae* (*MATa trp1, his3, ura3 LEU2::(lexAop)2-LEU2*). Cells were streaked on leucine-deficient medium containing galactose and growth was monitored 4 days later (11, 12). (*B*) Yeast strain SY1984 cells (*ste11Δ his3Δ FUS1::HIS3*) that express mammalian Raf-1 and STE7^{P368} proteins were transformed with plasmids encoding BAG-1 and various negative control proteins (Bcl-2, superoxide dismutase, Mas-70p), yeast Ras-1 as a positive control, or the empty vectors pAAH5 and YEp13. Raf-1-dependent activation of the *FUS1/HIS3* reporter gene was monitored by growth of five independent transformants on histidine-deficient medium for 3 days (14).

and 14–3-3. The region within BAG-1 that is sufficient for binding to and activating Raf-1 (amino acid 90 → C terminus), however, is predicted to contain several α-helical regions, and to that extent has some similarity with 14–3-3 proteins (23, 24). Because BAG-1 specifically binds to the catalytic domain of Raf-1, presumably the mechanism by which it induces activation of this kinase is more similar to 14–3-3 proteins that can also bind with at least weak affinity to the catalytic domain of Raf-1, than Ras proteins that interact with a domain in the N-terminal regulatory region of this protein kinase (14–20). Although we can only speculate as to how the interaction of BAG-1 with Raf-1 promotes activation of the kinase, conceivably BAG-1 may dislodge the negative regulatory domain of Raf-1 from its catalytic domain, stabilize the kinase in an active conformation once activated by other mechanisms, or protect Raf-1 from inactivation by phosphatases (25–27). Alternatively, BAG-1 may recruit other proteins to Raf-1 that are directly responsible for activating the kinase. Given that BAG-1 also activated Raf-1 in yeast, however, its seems more likely that BAG-1 directly activates Raf-1, but we cannot exclude the possibility that the cellular machinery required for BAG-1-mediated activation of Raf-1 is well conserved throughout evolution or that Raf-1 activation is a (at least) two-step process with BAG-1 fulfilling one of the necessary requirements for activation of Raf-1 in yeast and other evolutionarily conserved proteins playing an essential role as well. In this regard, our previous demonstration that Bcl-2 and Raf-1 can be coimmunoprecipitated from Sf9 cells coinfected with Raf-1 and Bcl-2 baculoviruses implies that either Sf9 cells or baculovirus may encode a homolog of BAG-1 that facilitates interactions of Raf-1 with Bcl-2 (9). Finally, by analogy to Ras that may activate Raf-1 at least in part by targeting it to membranes, it is conceivable that BAG-1 may promote Raf-1 activation by pulling Raf-1 into the vicinity of intracellular membranes through its interactions with integral membrane proteins such as Bcl-2 (28, 29).

The finding that BAG-1 can bind to and activate Raf-1 raises the possibility that Raf-1 may become activated locally in the vicinity of Bcl-2 though a protein–protein interaction mechanism, thus potentially targeting Raf-1 to unique substrates presumably involved in the regulation of apoptosis as opposed to the mitogen-activated protein kinase-signaling pathway in which Raf-1 has traditionally been implicated. In this regard, we have obtained evidence that BAG-1, Raf-1, and Bcl-2 can form trimolecular complexes, but their stoichiometry appears to be low (≈1%), suggesting that only a small proportion of the total cellular Raf-1 may be found in such complexes (unpublished observations). Consistent with this idea, however, targeting of the kinase domain of Raf-1[Raf-BXB] to mitochondrial membranes using the transmembrane domain of the yeast outer mitochondrial membrane protein Mas-p70 markedly increases its anti-apoptotic effects in a hemopoietic cell line in which withdrawal of lymphokines results in programmed cell death (data not shown). Given that Bcl-2 is located primarily in the membranes of the mitochondria, nuclear envelope and parts of the endoplasmic reticulum (30), presumably whatever substrates to which Bcl-2/BAG-1 complexes might target Raf-1 would be different at least in part from those associated with the plasma membrane where Raf-1 participates in growth factor receptor signal transduction. Candidates for such substrates that have been hypothesized as potentially explaining the anti-apoptotic actions of Bcl-2 include antioxidant enzymes, Ca^{2+} transporters, and members of the ced-3 family of proteases or their regulators (reviewed in ref. 1).

The kinase inhibitor staurosporine has been reported to induce apoptosis in a wide variety of types of cells at concentrations typically of ≤1–10 μM, implying that inhibition of certain kinases is a stimulus for apoptosis. Overexpression of Bcl-2 however protects cells from staurosporine-induced apoptosis (8, 31), implying that Bcl-2 does not require a staurosporine-sensitive kinase for its death-suppressing function. Though staurosporine has been reported to completely inhibit the activity of purified protein kinase C *in vitro* at 10 nM (32), we observed that the activity of Raf-1 was entirely unaffected by ≤5 μM of staurosporine and only 20% inhibition was produced by 20 μM (data not shown). Raf-1 therefore is a staurosporine-resistant kinase, consistent with the possibility that anti-apoptotic function of Bcl-2 may be at least in part dependent on Raf-1. It remains to be determined whether the interaction of BAG-1 with Raf-1 is essential for suppression of apoptosis.

We thank K. Matsumoto for the yeast Raf-1 reporter system, E. Golemis for the two-hybrid system, W. Kolch for GST-Raf-1 baculoviruses, D. Bredesen for Bcl-2 and superoxide dismutase yeast expression plasmids, G. Schatzl for the Mas70-P plasmid, C. Marshall for the GST-MEK plasmid, and C. Stephens for manuscript preparation. This work was supported by National Cancer Institute Garnt CA-67329. J.C.R. is a Scholar of the Leukemia Society of America.

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