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

HONG-GANG WANG*, SHINICHI TAKAYAMA*, ULF R. RAPP†, and JOHN C. REED**

The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037; and †Institute for Medical Cell Research, University of Würzburg, Versbacherstrasse 5, D-97078 Würzburg, Germany

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 S9 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. S9 cells (6 × 10⁶) were coinjected 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 MgCl₂/1 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 asctes (URP30) (lanes 1--3) or anti-β-gal monoclonal antibody (Santa Cruz Biotechnology), followed by 0.3 µg/ml HRP-antibody goat anti-mouse (Bio-Rad), and detected using an enhanced chemiluminescence system (Amersham).

Cos-7 cells (1 × 10⁶) 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-BXXB(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 HRP-antibody 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-lysatcs, Promega) containing in vitro translated [³⁵S]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-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

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

†To whom reprint requests should be addressed. e-mail: jreed@ijrf.edu.
dium that contained glucose or galactose for repression or activation, respectively, of the Gali promoter in pG4-J5 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 PMSE, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. After pre-clearing with 200 μl of protein A-Sepharose, Raf-1 was immunoprecipitated using 0.2 μl of protein A-Sepharose preadsorbed with 0.1 ml anti-Raf antisera 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 PMSE, 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 MgCl2/5 mM MnCl2). 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-32P]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 CaPO4 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 PMSE, 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 antisera 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, YEpl13 parental vector, or YEpl3-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 immunoprecipitated with BAG-1 (Fig. 1A) and vice versa (not shown).

The association of Raf-1 with the BAG-1 protein was specific, since SF9 cells coinfected with a β-gal- and a BAG-1-producing virus, β-gal protein failed to coimmunoprecipitate with BAG-1 (Fig. 1A).

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. 1B). 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. 1B).

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). Immunoprecipitations were performed using anti-HA antibody and subjected to SDS/PAGE immunoblot assays using anti-Raf-1 or anti-BAG-1 antibodies, revealing that Raf-1(BXB) protein can coimmunoprecipitate with HA-BAG-1 (Fig. 1C). 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. 1A).

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 35S-Raf-1 protein.

The full-length BAG-1 and BAG-1(ΔN) GST-fusion proteins bound to Raf-1 with comparable efficiencies (Fig. 1D), 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-TNFFR1 confirmed the specificity of these protein interactions (Fig. 1D).

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 specificity of activity of Raf-1 as measured by phosphorylation in vitro of GST-MEK (Fig. 2A). 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
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-32P]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. 2B and not shown). An His6-tagged, affinity-purified BAG-1 protein activated Raf-1 in vitro to a similar extent as GST-BAG-1 (5-fold increase), implying that the GST moiety is unimportant for this effect (Fig. 2B, 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. 2B, 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. 2B, 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. 2C, 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 32P-labeled Sf9 cells (not shown). Thus, Raf-1 fails to phosphorylate both BAG-1 and Bax (Fig. 2C, lane 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
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. 3A), 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. 3A and data not shown). Moreover, BAG-1 also activated mammalian Raf-1 in budding yeast (Fig. 3B), 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...
were binding medium for 3 proteins
with BAG-1 proteins, however, is monitored of this kinase because BAG-1 interacts with Ras-1, more than Raf-1-dependent Raf-1, has been reported to activate the kinase. Given that BAG-1 directly activated Raf-1 in yeast, however, it seems more likely that BAG-1 may directly activate 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 communoprecipitated 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, Ca²⁺ 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. Breiden 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 Grant CA-67329. J.C.R. is a Scholar of the Leukemia Society of America.