R-Ras Promotes Apoptosis Caused by Growth Factor Deprivation Via a Bcl-2 Suppressible Mechanism

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Abstract. The Bcl-2 protein is an important regulator of programmed cell death, but the biochemical mechanism by which this protein prevents apoptosis remains enigmatic. Recently, Bcl-2 has been reported to physically interact with a member of the Ras superfamily of small GTPases, p23-R-Ras. To examine the functional significance of R-Ras for regulation of cell death pathways, the IL-3–dependent cells 32D.3 and FL5.12 were stably transfected with expression plasmids encoding an activated form (38 Glycine → Valine) of R-Ras protein. R-Ras(38V)–producing 32D.3 and FL5.12 cells experienced increased rates of apoptotic cell death relative to control transfected cells when deprived of IL-3. Analysis of several independent clones of transfected 32D.3 cells revealed a correlation between higher levels of R-Ras protein and faster rates of cell death upon withdrawal of IL-3 from cultures. 32D.3 cells cotransfected with R-Ras(38V) and Bcl-2 exhibited prolonged cell survival in the absence of IL-3, equivalent to 32D.3 cells transfected with Bcl-2 expression plasmids alone. R-Ras(38V) also increased rates of cell death in serum-deprived NIH-3T3 cells, and Bcl-2 again abrogated most of this effect. The ratio of GTP and GDP bound to R-Ras(38V) was not significantly different in control 32D.3 cells vs those that overexpressed Bcl-2, indicating that Bcl-2 does not abrogate R-Ras–mediated effects on cell death by altering R-Ras GDP/GTP regulation. Moreover, purified Bcl-2 protein had no effect on the GTPase activity of recombinant wild-type R-Ras in vitro. When expressed in Sf9 cells using recombinant baculoviruses, R-Ras(38V) bound to and induced activation of Raf-1 kinase irrespective of whether Bcl-2 was coproduced in these cells, suggesting that Bcl-2 does not nullify R-Ras effects by interfering with R-Ras–mediated activation of Raf-1 kinase. Taken together, these findings suggest that R-Ras enhances the activity of a cell death pathway in growth factor–deprived cells and imply that Bcl-2 acts downstream of R-Ras to promote cell survival.

SEVERAL genes have been identified that participate as either inducers or repressors of programmed cell death and apoptosis. Among these is *bcl-2* (B-cell lymphoma-2)[1], a blocker of cell death which was first discovered by virtue of its involvement in the t(14;18) chromosomal translocations found in the majority of non-Hodgkin's B cell lymphomas (Tsujimoto et al., 1985; Tsujimoto and Croce, 1986). Dysregulation of *bcl-2* expression contributes to neoplastic cell expansion by preventing normal cell turnover due to programmed cell death, rather than by accelerating rates of cellular proliferation (Vaux et al., 1988; McDonnell et al., 1989; Katsumata et al., 1992). Gene transfer studies have shown that elevations in Bcl-2 protein levels can provide protection against cell death induced by a broad spectrum of stimuli, suggesting that this protein regulates a distal step in a final common pathway for apoptotic cell death (for review see Reed, 1994). Furthermore, elements of this pathway appear to be well conserved throughout evolution in that the human Bcl-2 protein can also provide cell death protection to insect cells, worms, and yeast under at least some circumstances (Alnemri et al., 1992; Vaux et al., 1992; Kane et al., 1993; Sato et al., 1994; Hengartner and Horvitz, 1994).

The biochemical mechanism of action of the Bcl-2 protein remains enigmatic, principally because its predicted amino acid sequence shares no significant homology with other proteins whose functions are known. In mammalian species, the *bcl-2* gene encodes a 25–26-kD intracellular protein that

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1. *Abbreviations used in this paper*: *bcl-2*, B-cell lymphoma-2; GM-CSF, Granulocyte/Macrophage-Colony Stimulating Factor; IL-3, Interleukin-3; PI, propidium iodide.

contains a hydrophobic stretch of amino acids near its carboxy terminus, accounting for its posttranslational insertion into membranes (Chen-Levy and Cleary, 1990). The intracellular locations where the Bcl-2 protein resides include predominantly the outer mitochondrial membrane, nuclear envelope, and parts of the ER (Krajewski et al., 1993; Jacobson et al., 1993; de Jong et al., 1994; Lithgow et al., 1994). Electron microscopic data suggest that Bcl-2 may be associated at least in part with nuclear pore complexes and mitochondrial junctional complexes, where the inner and outer membranes of these DNA-containing organelles come into contact and where various transport phenomena occur (Krajewski et al., 1993; de Jong et al., 1994). Consistent with this localization, evidence has recently been obtained suggesting that Bcl-2 may impair the translocation of the p53 tumor suppressor as well as the Cdc-2 and Cdk-2 kinases into nuclei (Ryan et al., 1994; Meikrantz et al., 1994). However, Bcl-2 can also provide protection against apoptotic-like phenomena that occur in the cytosol in the absence of a nucleus, based on studies involving enucleated cytoplasts and a cell-free apoptosis assay (Jacobson et al., 1994; Newmeyer et al., 1994). Furthermore, evidence has been obtained suggesting that Bcl-2 may regulate intracellular Ca^{2+} homeostasis or an antioxidant pathway (Baffy et al., 1993; Lam et al., 1994; Kane et al., 1993; Hockenbery et al., 1993). Nevertheless, at this point, no direct cause and effect data exist that conclusively link Bcl-2 to protein transport, Ca^{2+} regulation, antioxidant enzymes, or other biochemical events.

Recently, Bcl-2 has been reported to physically associate with p72 Raf-1, a serine/threonine-specific protein kinase (Wang et al., 1994), and p23-R-Ras (Fernandez-Sarabia and Bischoff, 1993), a GTPase member of the Ras family (Lowe et al., 1987), suggesting that this apoptosis-blocking protein may control a signal transduction pathway located in the vicinity of mitochondrial, nuclear, and perhaps other intracellular membranes, instead of the plasma membrane. In cotransfection studies, the combination of elevations in Bcl-2 protein levels and Raf-1 kinase activity were shown to provide synergistic protection from cell death induced by growth factor withdrawal from a diploid hematopoietic cell line (Wang et al., 1994). The function of R-Ras and the significance of its interaction with Bcl-2 however have not been explored where regulation of cell death is concerned.

In Rat-1 fibroblasts, R-Ras and mutant versions of this protein that presumably remain mostly in an active GTP-bound state due to impaired GTPase activity were reported to lack transforming activity, unlike oncogenic mutants of their p21-Ha-Ras counterparts (Lowe and Goeddel, 1987). In NIH-3T3 fibroblasts, however, versions of R-Ras with mutations at condons 38 or 87, analogous to the oncogenic position 12 and 61 mutants of Ha-Ras, confer ability to proliferate in low serum, form colonies in soft agar, and form tumors in nude mice, as well as inducing morphological transformation to variable extents (Cox et al., 1994; Saez et al., 1994). Similar to Ha-Ras proteins, R-Ras has also been reported to bind to Raf-1 kinase in vitro in a GTP-dependent fashion, stimulate activation of the mitogen-activated protein kinases p44-ERK-1 and p42-ERK-2, and induce transcription of *ras*-responsive reporters in a Raf-dependent manner in NIH-3T3 cells (Cox et al., 1994; Rey et al., 1994; Spaargaren et al., 1994). These similarities to Ha-Ras notwithstanding, R-Ras does not induce maturation of *Xenopus* oocytes or differentiation of PC12 cells (Rey et al., 1994). Taken together, therefore, the data available to date indicate that while some overlap in the biochemical and cellular functions of Ha-Ras and R-Ras exist, these proteins appear to play different biological roles. Here we present evidence that one of the roles played by R-Ras can be to promote apoptotic cell death in the setting of growth factor withdrawal.

Materials and Methods

Expression Plasmids

The plasmids pEXV-R-Ras(WT) and pEXV-R-Ras(38V) contain R-Ras and R-Ras(38V) cDNAs under the control of a SV40 promoter/enhancer (gift of Alan Hall) (Rey et al., 1994). R-Ras cDNA sequences were liberated from these pEXV plasmids by digestion with EcoRI and subcloned into the EcoRI site of pGEX-3X (Pharmacia LKB Biotechnology, Piscataway, NJ) for production of GST-R-Ras and GST-R-Ras(38V) proteins in *E. coli*. These cDNAs were also subcloned into the EcoRI site of the baculovirus transfer vector pVL1393 and recombinant baculoviruses were prepared using the BaculoGold system (PharMingen, Inc., San Diego, CA).

Cell Transfections

32D.3 cells (Mavillo et al., 1989) were maintained essentially as described (Baffy et al., 1993; Tanaka et al., 1993) at 0.05–1 × 10^6 cells per ml in Iscove's modified Dulbecco medium (IMDM) (Irvine Scientific, Inc., Irvine, CA) supplemented with 1 mM L-glutamine, 100 U/ml penicillin-G, 100 μg/ml streptomycin, 10% (vol/vol) heat-inactivated FCS and 20% (vol/vol) of conditioned medium from the IL-3-producing cell line WEHI-3B. FL5.12 cells were similarly maintained except that RPMI-1640 medium was used and 25 mm Hepes (pH 7.4) and 50 μM β-mercaptoethanol were added. A combination of 20 μg of PvuI-linearized pEXV-R-Ras(WT) or pEXV-R-Ras(38V) or pEXV parental vector with 5 μg of NdeI-linearized pZip-Neo or pZip-Bcl-2 plasmid DNA (Miyashita and Reed, 1992) was mixed with 5 × 10^6 of 32D.3 or FL5.12 cells in 0.8 ml of Dulbecco's PBS (pH 7.4) on ice. For 32D.3 cells, plasmid DNAs were then introduced by electroporation using a double pulse consisting of 2,000 V/cm; 40 μF; 74 ohms followed by 250 V/cm; 1,500 μF; 74 ohms, using an apparatus from EquiBio, Inc. (Angleur, Belgium) (CellJect), and 2 d later cells were selected in medium containing IL-3 and 800 μg/ml G418 (GIBCO BRL, Gaithersburg, MD). FL5.12 cells similarly received a single pulse of 500 V/cm at 900 μF, and were selected in medium containing 1 mg/ml G418. For subcloning, G418-resistant cells were seeded at an average of 0.5 cells per well in 0.1 ml of IL-3-containing medium in 96-well plates.

NIH-3T3 cells were grown in 100-mm dishes in DMEM (MediaTech, Inc., Herndon, VA) supplemented with antibiotics, glutamine, and 10% (vol/vol) calf serum. Cells were transfected by a calcium-phosphate precipitation method and selected in 400 μg/ml G418, essentially as described (Cox et al., 1994).

Cell Viability and Apoptosis Assays

32D.3 and FL5.12 cells (2 × 10^6) were washed three times with IMDM or RPMI and cultured in 10 ml IMDM or RPMI containing 10% FCS without IL-3 for various times. Cells in culture supernatants were recovered by centrifugation and pooled with cells that were detached from flasks using HBSS containing 0.02% EDTA. The percentage of viable cells was then determined by trypan blue dye exclusion, counting a minimum of 200 cells each for triplicate samples. In some cases, after ~1 d of culture without IL-3, 32D.3 cells were recovered from flasks as above, washed in PBS (pH 7.4), and fixed in 3.7% formaldehyde in PBS for 10 min at room temperature. After three washes in PBS, cells were resuspended in PBS containing 0.1 μg/ml of 4',6-diamindino-2-phenylinodole (DAPI) for 15 min, washed several times in PBS, and examined using a Nikon fluorescence microscope. Alternatively, 10^6 cells were fixed on ice in PBS containing 30% ethanol, and the DNA of RNAse-treated cells was subsequently stained with propidium iodide (PI) as described previously (Wang et al., 1994). DNA content analysis was accomplished by flow cytometry using a FACStar-Plus (Becton-Dickinson Immunocytometry Sys., Mountain View, CA).

NIH-3T3 cells were seeded at 5 × 10^4 cells per well of 24-well plates in 0.5 ml DMEM complete medium. The next day, cells were washed in DMEM without serum and cultured for 48 h without serum. Floating apoptotic cells were gently washed off using PBS and the remaining viable at-

tached cells were photographed under phase contrast using a Nikon light-microscope. Alternatively, 5×10^4 cells were seed into 24-well plates and, after culturing overnight, the cells were washed once in DMEM without serum and cultured without serum for 48 h. The plates were centrifuged at 400 *g* for 5 min to pellet floating apoptotic cells, and after washing in PBS and fixing in 3.7% formaldehyde in PBS, the cells were stained with 0.1 μg/ml DAPI and the numbers of normal and apoptotic cells were counted using a UV-microscope.

Preparation of R-Ras Antibodies

A polyclonal antiserum was produced in rabbits using as a immunogen a synthetic peptide (NH_2-Cys-Arg-Gly-Arg-Pro-Arg-Gly-Gly-Gly-Pro-Gly-Pro-Arg-Asp-Pro-Pro-Pro-Gly-Glu-Thr-His-Tyr-amide) corresponding to amino acids 11-31 of the mouse R-Ras protein with a NH_2-terminal cysteine appended to facilitate conjugation to maleimide-activated Keyhole Limpet Hemocyanin (KLH) (Pierce, Rockford, IL), using methods essentially as described previously (Reed et al., 1991).

GTPase Assays and Nucleotide-binding Studies

To produce the GST-R-Ras and GST-R-Ras(38V) proteins, DH5-α strain *E. coli* containing pGEX-3X-R-Ras or pGEX-3X-R-Ras(38V) were cultured at 37°C in 1 liter of LB medium containing 50 μg/ml ampicillin until the $O.D._{590nm}$ reached 0.6 to 1.0. IPTG was then added to 0.2 mM and the cultures were continued for 5 h at 25°C. Bacteria were recovered by centrifugation, washed in ice-cold PBS, and resuspended in 10 ml of PBS containing 1% Triton X-100, 20 μg/ml aprotinin, 1 mM PMSF, 1 mg/ml lysozyme. After 10 min at 37°C, the cells were sonicated on ice, using 3 pulses of 30 s each from a 5-mm microtip sonicator (model X L2020; Heat Systems, Inc., Farmingdale, NY). Cell lysates were centrifuged at 15,000 *g* for 15 min, and the resulting supernatant was applied to a 2-ml column of glutathione-Sepharose (Pharmacia LKB Biotechnology). The column was washed with 10-bed volumes of ice-cold PBS containing 1 mM PMSF and 20 μg/ml aprotinin. GST-fusion proteins were then eluted in 2 bed volumes of 50 mM Tris (pH 8.0) containing 5 mM glutathione. After dialysis in 20 mM Tris (pH 7.5), 1 mM DTT, 0.1 mM PMSF, 10% glycerol, ~10 μg of affinity-purified proteins were incubated for 10 min at 30°C in 25 μl of 50 mM Tris (pH 7.5), 50 mM NaCl, 0.1 mM DTT, 10 μM [^{32}P]γ-GTP. The samples were then placed on ice and $MgCl_2$ was added to achieve a final concentration of 5 mM. GTPase assays (Garrett et al., 1989) were then initiated by placing at 30°C 1 μg of [^{32}P]GTP-loaded proteins in 50 mM Tris (pH 7.5), 0.1 mM DTT, 5 mM $MgCl_2$, 1 mg/ml BSA, 1 mM GTP, with or without 0.5 μg of purified Bcl-2 protein. At various times, 5 μl aliquots were diluted in 1 ml of 50 mM Tris (pH 7.5), 0.1 mM DTT, 5 mM $MgCl_2$ and filtered through nitrocellulose (BA85 filters; Schleicher & Schuell, Inc., Keene, NH). Filters were washed with 10 ml of the same solution, dried, and subjected to scintillation counting. Bcl-2 protein was immunoaffinity purified by the method of Haldar et al. (1994) from Sf9 cells infected with a recombinant *bcl-2* baculovirus using the monoclonal antibody 4D7 (Reed et al., 1992).

To measure relative amounts of GTP and GDP bound to R-Ras, 32D-R-Ras(38V) or 32D-R-Ras(38V)/Bcl-2 cells were cultured for 5 h in phosphate-free RPMI-1640 medium containing 10% dialyzed FCS and 0.2 mCi/ml $^{32}PO_4$ (ICN Radiochemicals, Inc., Costa Mesa, CA). After washing three times in ice-cold PBS, cells were lysed for 10 min on ice in 0.5 ml of 50 mM Hepes (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.1% BSA, 1% aprotinin, 100 μg/ml leupeptin, 10 μg/ml pepstatin, 10 mM benzamidine, 10 μg/ml soybean trypsin inhibitor. After centrifugation at 16,000 *g* for 3 min, the resulting supernatants were brought to 500 mM NaCl, 0.5% deoxycholate, 0.05% SDS, and precleared using 25 μl (packed volume) of preimmune serum adsorbed protein A-Sepharose. R-Ras was then immunoprecipitated at 4°C for 0.5–1 h using 10 μl of anti-R-Ras antiserum preadsorbed protein A-Sepharose. Immune complexes were washed eight times in 1 ml of 50 mM Hepes (pH 7.5), 500 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.005% SDS. Bound nucleotides were eluted in 2 mM EDTA, 2 mM DTT, 0.2% SDS at 68°C for 20 min, and chromatographed on PEI-cellulose plates in 0.75 M KH_2PO_4 (pH 3.5) along with GDP and GTP standards (Sigma, Inc., St. Louis, MO). Data were analyzed by autoradiography and quantifications were performed using a radioisotope detector system (Ambis-100).

Raf-1 Kinase Assays

Sf9 cells were infected with recombinant baculoviruses (Reed et al., 1992; Xian-Feng et al., 1993) encoding Raf-1, Raf-1(259D), Raf-1 (YY340, 341FF), R-Ras, R-Ras(38V), Bcl-2, or various combinations of these viruses, essentially as described (Wang et al., 1994). After ~60 h, cells were lysed in 25 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% Na-deoxycholate, 1% NP-40, 10% glycerol, 2 mM EDTA, 1 mM Na-ortho-vanadate, 1 mM PMSF, 20 μM leupeptin, 5 μg/ml aprotinin. Raf-1 was then immunoprecipitated using protein A-Sepharose preadsorbed with an anti-Raf COOH-terminal peptide antiserum (Reed et al., 1991*b*) and the resulting immune complexes washed twice with 20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 1 mM Na-ortho-vanadate and protease inhibitors, before performing a final wash in kinase buffer (25 mM Hepes [pH 7.4], 150 mM NaCl, 25 mM glycerol phosphate, 1 mM DTT, 5 mM $MgCl_2$), followed by incubation in 30 μl of kinase buffer containing 0.1–1 μg purified recombinant MEK protein, 10 μM ATP, and 20 μCi [^{32}P]γ-ATP for 30 min at ~25°C. The samples were then centrifuged at 16,000 *g* for 1 min, and the supernatant containing MEK and the pellet containing Raf-1 immune complexes were separately size fractionated by SDS-PAGE. Phosphorylated MEK was detected by autoradiography in the dried gel, whereas Raf-1 was transferred to nitrocellulose and detected by immunoblotting using anti-Raf-1 antibody, essentially as described (Wang et al., 1994).

Coimmunoprecipitation Assays

Coimmunoprecipitation experiments were preformed as described previously (Wang et al., 1994) using polyclonal antisera and either transfected 32D.3 cells or baculovirus-infected Sf9 cells. In addition, for some experiments, lysates were prepared from 2×10^6 Sf9 cells in 0.25 ml of 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, 0.2 mM PMSF, 5 μg/ml aprotinin, 0.7 μg/ml pepstatin, 5 μg/ml leupeptin) on ice for 0.5 h, and nuclei and cell debris were removed by centrifugation at 15,000 *g* for 15 min. After preclearing with 25 μl of protein G-Sepharose (Zymed Labs, S. San Francisco, CA), immunoprecipitations were performed using 2 μg of the 4D7 monoclonal antibody (Reed et al., 1992) or a mouse IgG_1 control antibody (Dako, Inc., Carpinteria, CA) for 3 h at 4°C. Immune complexes were recovered using 25 μl Protein G-Sepharose, washed three times in lysis buffer, and subjected to SDS-PAGE/immunoblot assay using 0.1% vol/vol polyclonal anti-Raf-1 or anti-R-Ras antisera followed by HRPase-donkey anti-rabbit (Amersham Corp., Buckinghamshire, England) and either colorimetric detection using 3,3′-diaminobenzidine (DAB) or detection using an ECL system (Amersham Corp.).

Results

32D.3 cells are a murine myeloid progenitor cell clone that is absolutely dependent on either Interleukin-3 (IL-3) or Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF) for proliferation and survival in vitro. These cells have a normal diploid karyotype and, like freshly isolated bone marrow progenitors, retain the ability to undergo differentiation to mature granulocytes upon stimulation with appropriate lymphokines (Mavilio et al., 1989). Removal of IL-3 and GM-CSF from cultures of 32D.3 cells results in G_0–G_1-arrest followed by apoptosis. Because these cells have been used extensively as a model for investigations of mechanisms of programmed cell death in the setting of growth factor withdrawal (for examples, see references by Askew et al., 1991; Nunez et al., 1990; Baffy et al., 1993; Wang et al., 1994; Cleveland et al., 1994; Ando et al., 1993), we employed them for gene transfer studies of R-Ras.

R-Ras Increases Rates of Apoptotic Cell Death in IL-3–deprived 32D.3 Cells

To study the effects of R-Ras alone and in combination with Bcl-2, 32D.3 cells were cotransfected with expression plasmids encoding human Bcl-2 and either normal human p23-R-Ras or a version of R-Ras (gly→val 38) that contains a mutation analogous to the gly→val 12 mutation of activated Ha-Ras oncoproteins (Lowe and Goeddel, 1987). As a control, cells were also transfected with the parental vectors lacking *R-ras* or *bcl-2* cDNA inserts ("32D-NEO"). In addi-

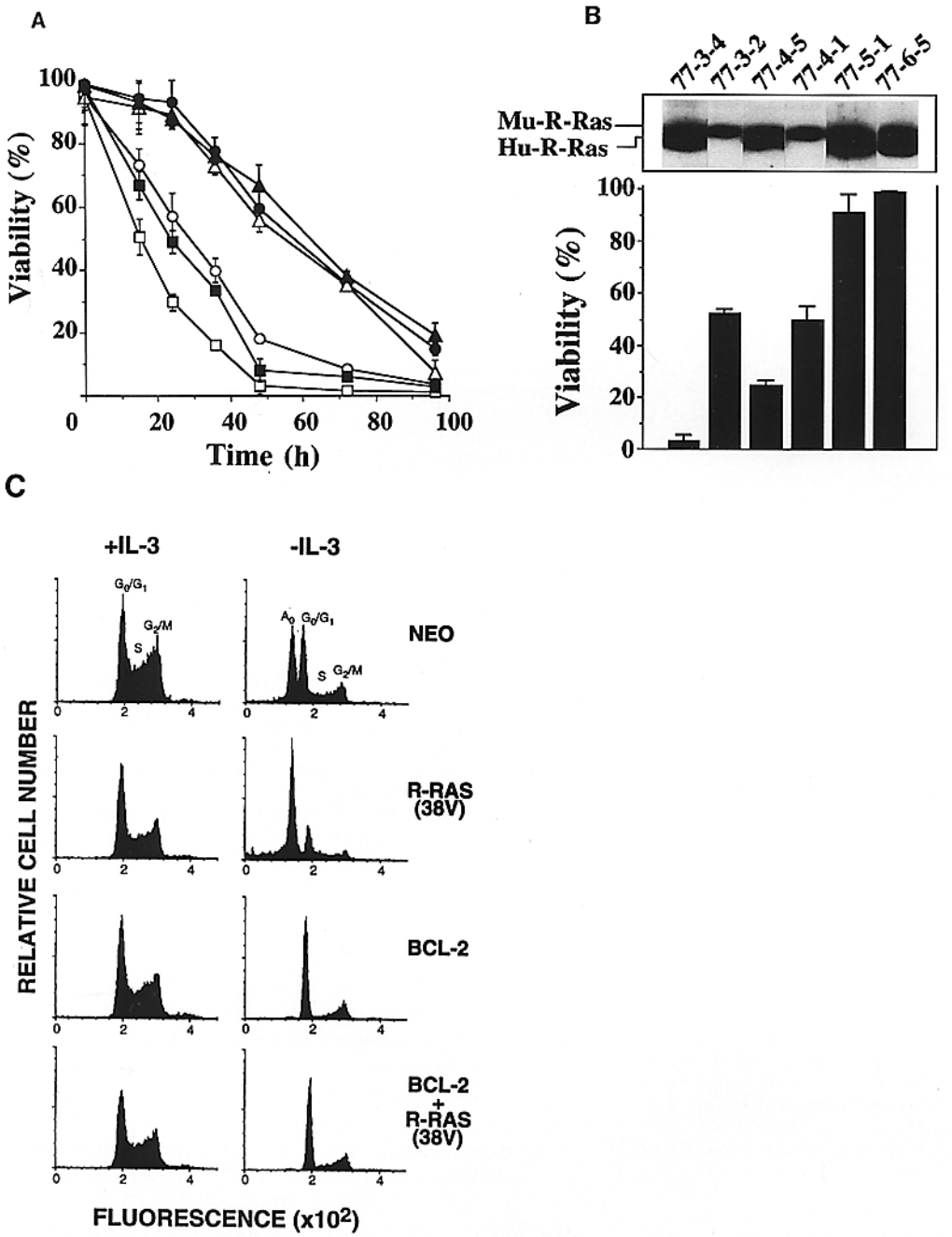

Figure 1. Effects of R-Ras and R-Ras(38V) on 32D.3 cells survival and growth. In *A*, transfected 32D.3 cells were cultured at 10^6 per ml in medium without 10% WEHI-3B conditioned medium as a source of IL-3. At various times thereafter, the percentage of viable cells was determined by counting cells in the presence of trypan blue dye. Data represent mean ± SD (n= 3). Symbols represent 32D-Neo cells (*open circles*), 32D-Bcl-2 (*closed circles*), 32D-R-Ras(38V) (*open squares*), 32D-R-Ras (*closed squares*), 32D-R-Ras(38V) +Bcl-2 (*open triangles*), 32D-R-Ras + Bcl-2 (*closed triangles*). In *B*, transfected 32D.3 cells were subcloned by limiting dilution and detergent lysates were prepared for immunoblot analysis (50 μg per lane) using anti-R-Ras antiserum (*top panel*). The positions of the endogenous mouse and transgene-derived human R-Ras proteins are shown. The same subclones were cultured in the absence of IL-3 for 24 h and the percentage of viable cells was determined by counting cells in the presence of trypan blue dye (mean ± SD; n = 3) (*bottom panel*). Clones represent 32D.3 cells transfected with the following plasmids: pEXV-R-Ras(38V) + pZip-Neo (77-3-4 and 77-3-2); pEXV-R-Ras + pZip-Neo (77-4-5 and 77-4-1); pEXV-R-Ras(38V) + pZip-Bcl-2 (77-5-1) and pEXV-R-Ras + pZip-Bcl-2 (77-6-5). In *C*, the subclones 77-1-1 (32D-Neo), 77-3-4 (32D-R-Ras[38V]), 77-2-1 (32D-Bcl-2), and 77-5-1 (32D-Bcl-2/R-Ras[38V]) were cultured at 10^5 cells per ml in medium with (+) or without (−) 20% WEHI-3B conditioned medium for ~15 h. Cells were then fixed, permeabilized, and treated with RNAse before incubation in 50 μg/ml propidium iodide (PI) and analysis by flow cytometry. Data represent fluorescence intensity (abscissa) vs relative cell number (ordinant). DNA contents indicative of apoptotic cells (A_0) and viable cells in G_0/G_1, S, or G_2/M-phases of the cell cycle are indicated.

tion, 32D.3 cells were stably cotransfected with various combinations of parental ("empty") vectors and plasmids encoding Bcl-2, R-Ras, or R-Ras(38V) to express Bcl-2 or R-Ras proteins individually rather than in combination. After selection in G418, immunoblot analysis was performed on the resulting polyclonal bulk transfected cell lines, confirming elevations in the relative levels of R-Ras (38V), R-Ras, and Bcl-2 proteins in the relevant transfectants but not in the 32D-NEO control cells (not shown).

Transfected 32D.3 cells were washed to remove IL-3, and then cultured for various times in medium without lymphokines. Cell viability was assessed based on ability to exclude trypan blue dye. As shown in Fig. 1 *A*, 32D.3 cells expressing the R-Ras(38V) protein died with accelerated kinetics relative to control-transfected 32D-NEO cells. In the experiment shown, for example, only ~30% of 32D-R-Ras(38V) cells remained viable at 24 h after factor withdrawal, compared to nearly 60% of 32D-NEO cells. At 36 h

post-IL-3 withdrawal, ~15% of 32D-R-Ras(38V) cells were alive compared to ~40% of 32D-NEO cells. 32D.3 cells expressing the normal R-Ras protein tended to die somewhat faster than control 32D-NEO cells, but the difference was not significant. Microscopic examination of the dead cells under UV-illumination after staining with the DNA-binding fluorochrome DAPI demonstrated typical apoptotic morphology, including condensed chromatin, fragmented nuclei, and shrunken cell size (not shown). Consistent with previous reports (Baffy et al., 1993; Tanaka et al., 1993), expression of the Bcl-2 protein markedly prolonged the survival of 32D.3 cells in the absence of lymphokines, relative to control 32D-NEO cells (Fig. 1 *A*). Coexpression of Bcl-2 also completely abrogated the effects of R-Ras(38V), such that the cell death kinetics of 32D-Bcl-2/R-Ras(38V) cells were essentially superimposable with 32D-Bcl-2 cells. Similar results were obtained in multiple experiments and by use of cells derived from two independent transfections.

Analysis of Independent Clones of Transfected 32D.3 Cells Demonstrates Correlation of Rates of Apoptosis with Levels of R-Ras(38V) Protein

To further examine the association between expression of the R-Ras(38V) protein and promotion of apoptosis, several independent clones of 32D.3 transfectants were obtained by limiting dilution and their relative levels of R-Ras proteins were determined by immunoblotting using a R-Ras-specific antiserum. Clones of 32D-R-Ras(38V) and 32D-R-Ras transfectants with a wide range of R-Ras protein levels were found, presumably reflecting clonal variability in the genomic integration sites of the R-Ras and R-Ras(38V) expression plasmids. Functional assessments of these clones with regard to cell death rates in the absence of lymphokines demonstrated a clear correlation between higher levels of p23-R-Ras(38V) protein and faster cell death kinetics. Similarily, 32D-cell clones expressing higher levels of the normal p23-R-Ras protein died at accelerated rates relative to clones that contained lower levels R-Ras, but the difference between high and low expressors was less striking than for the clones expressing the mutant activated R-Ras(38V) protein. Fig. 1 *B* shows representative results for a pair of high and low expressing R-Ras(38V) and a pair of R-Ras-producing 32D.3 cells, but similar results were obtained for multiple independent clones. Also shown are typical results obtained for clones that coexpressed Bcl-2 and either R-Ras(38V) or R-Ras, demonstrating that these cells enjoyed markedly prolonged survival relative to 32D.3 cells that expressed R-Ras(38V) or R-Ras in the absence of gene transfer-mediated elevations in the levels of Bcl-2 protein.

Next, flow cytometric analysis of propidium iodide (PI)-stained cells was performed to measure the DNA content of various clones of transfected 32D.3 cells when cultured in the presence or absence of IL-3. When supplied with exogenous IL-3, clones of transfected 32D.3 cells were distributed in all phases of the cell cycle. No significant differences in the proportions of cells in G_0/G_1, S, or G_2/M-phases was observed among these transfectants, regardless of their relative levels of R-Ras, R-Ras(38V), and Bcl-2 proteins. Fig. 1 *C*, for example, presents results for some of the same subclones shown in Fig. 1 *B* that contained high levels of either p23-R-Ras(38V), p26-Bcl-2, or both of these proteins. The relative portion of 32D-R-Ras(38V) cells in G_0/G_1, S, and G_2/M-phases was 47%, 20%, and 27%, respectively, compared to ranges of 40–43% (G_0/G_1), 21–23% (S), and 30–33% (G_2/M) among 32D-Bcl-2, 32D-Bcl-2/R-Ras(38V), and 32D-NEO cells. Thus, R-Ras did not significantly alter the cell cycle distribution of 32D.3 cells. When cultured for ~1 d in the absence of IL-3, however, a large proportion of 32D-R-Ras(38V) cells (~71% in the experiment shown) contained hypodiploid DNA contents, indicative of the genomic digestion that occurs during apoptosis. The remaining presumably viable cells with normal DNA contents were distributed throughout the cell cycle, with 47% in either S or G_2/M-phases. Similarly, 32D-NEO cells experienced DNA degradation, though in smaller proportions (~41%), with 44% of the remaining viable cells in either S or G_2/M-phases. In contrast, 32D.3 cells expressing high levels of Bcl-2 retained mostly normal DNA contents >95%, regardless of whether R-Ras(38V) protein was also present in the cells at high levels. Interestingly, fewer 32D-Bcl-2 and 32D-Bcl-2/R-Ras(38V) cells were found in S- or G2/M-phases at ~1 d after IL-3 withdrawal (32–34%).

Based on these observations, we conclude that R-Ras(38V) increases the rate of cell death when 32D.3 cells are deprived of lymphokines through a mechanism consistent with apoptosis as opposed to necrosis. R-Ras(38V), however, did not alter the cell cycle distribution of 32D.3 cells when cells were cultured with saturating amounts of IL-3. Moreover, in additional experiments not presented, the rate of growth of 32D-R-Ras(38V) clones and the 32D-R-Ras(38V) bulk transfectants in IL-3–containing medium was not appreciably different from the other transfectants during the first 2 d after initiation of cultures, based on determination of viable cell numbers by trypan blue dye exclusion. After 2 d, however, when IL-3 became limiting in the cultures, numbers of viable 32D-R-Ras(38V) cells began to fall precipitously, compared to other transfectants. Moreover, rates of [^{3}H]thymidine incorporation into DNA were also not different for 32D-R-Ras(38V) cells compared to other transfectants, at least when measured at various times within the first day of initiation of cultures with optimal amounts of IL-3. These findings however do not exclude a potential role for R-Ras in promoting cell proliferation under conditions where suboptimal amounts of lymphokine are supplied, analogous to the previously reported results for R-Ras(38V) in NIH-3T3 cells cultured in low serum (Cox et al., 1994). Finally, expression of R-Ras or R-Ras(38V) in 32D.3 cells did not alter their state of differentiation, based on examination of cell morphology by Giemsa-Wright staining, histostaining for the presence of nonspecific esterase, and immunophenotyping for Mac-1 and other cell surface markers (not shown).

R-Ras(38V) Also Increases Rates of Apoptotic Cell Death in Cultures of IL-3–deprived FL5.12 Cells and Serum-deprived NIH-3T3 Fibroblasts

To determine whether the ability of R-Ras(38V) to increase rates of cell death in the absence of growth factors was unique to 32D.3 cells or more broadly applicable, we evalutated the effects of R-Ras(38V) on apoptosis induced by IL-3 deprivation in FL5.12 cells and by serum withdrawal in NIH-3T3 cells. For these experiments, FL5.12 and NIH-3T3 cells were transfected with expression plasmids encoding R-Ras (38V) or the same parental vector lacking R-Ras as a control (Neo). Some cells were also transfected with a Bcl-2 expres-

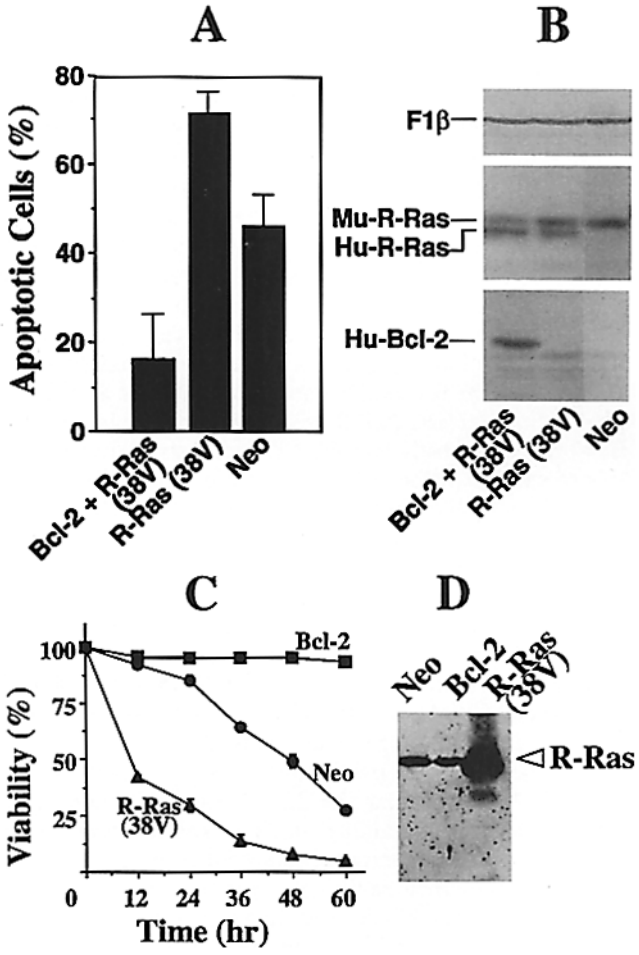

Figure 2. Immunoblot and functional analysis of transfected NIH-3T3 and FL5.12 cells. In *A*, transfected 3T3 cells were seeded at 5×10^4 cells per well of 24-well plates. The following day, cells were cultured for ~48 h without serum and the cells were stained with DAPI. The percentage apoptotic cells was then determined, counting ~800 cells per sample (mean ± SD [$n = 3$]). In *B*, stably transfected NIH-3T3 cells were analyzed by immunoblotting (50 μg total protein per lane) using antibodies specific for R-Ras, Bcl-2, or F_1-β-ATPase. The positions of the endogenous mouse R-Ras and F_1-β-ATPase proteins are indicated, as well as the transgene-derived human R-Ras and Bcl-2. In *C*, stably transfected FL5.12 cells were cultured at 10^6 cells/ml in medium without IL-3. The percentage of viable cells was determined at various times thereafter by trypan blue dye exclusion (mean ± SD; $n = 3$). In *D*, detergent-lysates were prepared from G418-resistant FL5.12 cells that had been stably transfected with Neo-control, R-Ras(38V), or Bcl-2 expression plasmids. Proteins (50 μg/lane) were subjected to SDS-PAGE/immunoblot assay using anti-R-Ras antiserum.

sion vector or were cotransfected with R-Ras(38V) and Bcl-2 expression plasmids. Immunoblot analysis confirmed the production of the human R-Ras(38V) protein, in addition to the endogenous mouse R-Ras protein, in those NIH-3T3 cells that received the R-Ras(38V) expression plasmid but not in the control (Neo) transfected cells (Fig. 2 *B*). Levels of p23-R-Ras protein were also markedly elevated in R-Ras(38V)-transfected FL5.12 cells, compared to FL5.12 cells that received either the Neo-control plasmid or a Bcl-2 expression plasmid (Fig. 2 *D*). Human Bcl-2 protein was also detected by immunoblotting in the cells that received the Bcl-2 expression plasmid but not in other transfected NIH-3T3 and FL5.12 cells (Fig. 2 *B* and data not shown). Evaluation of the relative levels of the mitochondrial protein F_1-β-ATPase served as a control, verifying loading of approximately equal amounts of total protein in all lanes (Fig. 2 *B* and data not shown).

These NIH-3T3 cells were then cultured for 2 d in the absence of serum, and cell viability was assessed by DAPI staining to distinguish apoptotic cells from cells that retained normal nuclear morphology. As shown in Fig. 2 *A*, ~70–80% of R-Ras(38V)-producing NIH-3T3 cells had undergone apoptosis after 2 d without serum growth factors, compared to only ~40–50% of Neo control cells. In contrast, relatively few of the NIH-3T3 cells that coexpressed R-Ras(38V) and Bcl-2 had become apoptotic (<20%). Similarly, the kinetics of cell death were markedly accelerated in cultures of IL-3–deprived R-Ras(38V)–expressing FL5.12 cells compared to 32D-NEO control cells. Conversely, FL5.12 cells that contained high levels of Bcl-2 protein survived for prolonged periods in the absence of IL-3 (Fig. 2 *C*). These findings indicate that R-Ras(38V) can also increase the rate of cell death in serum-deprived NIH-3T3 cells and lymphokine-starved FL5.12 cells.

Bcl-2 Abrogates R-Ras–promoted Cytotoxicity without Altering GTP Binding or GTPase Activity of R-Ras Proteins

The ability of Bcl-2 to completely abrogate the effects of R-Ras(38V) and R-Ras on cell death induced by factor withdrawal raised the possibility that Bcl-2 nullifies some biochemical function of the R-Ras protein, particularly since Bcl-2 and R-Ras have been reported to physically interact based on yeast two hybrid experiments and coimmunoprecipitation assays (Ferendez-Sarabia and Bischoff, 1993). One of the mechanisms by which Bcl-2 could potentially interfere with R-Ras function is by either accelerating the rate of hydrolysis of bound GTP, analogous to GTPase-activating proteins, or by inhibiting GDP/GTP-exchange and thus leaving R-Ras in an inactive GDP-bound state.

To begin to examine these possibilities, R-Ras and R-Ras (38V) were expressed in *E. coli* as GST-fusion proteins, affinity purified, and loaded with [^{32}P]γ-GTP. The GST-R-Ras protein hydrolyzed GTP to GDP in vitro such that ~35–40% of the total GTP was converted to GDP within 30 min. As expected, but never before confirmed in the literature, the GST-R-Ras(38V) protein exhibited no GTPase activity under these conditions. Addition of ~0.5 μg of purified baculovirus-produced Bcl-2 protein to these reactions had no influence on R-Ras or R-Ras(38V) GTPase activity (Fig. 3 *A*). Similar results were obtained using two independent preparations of immunoaffinity-purified Bcl-2 protein (not shown). Bcl-2 protein prepared in this same manner has been shown to be biologically active, based on experiments using a cell-free assay for apoptosis (Newmeyer et al., 1994) and on electroporation of Bcl-2 protein into human B-cell lines (Haldar et al., 1994). Thus, at least when tested in vitro using purified proteins, Bcl-2 did not directly modulate the GTPase activity of the R-Ras or R-Ras(38V) proteins. In preliminary experiments, recombinant Bcl-2 protein also appeared to have no GDP/GTP-exchange activity on GDP-

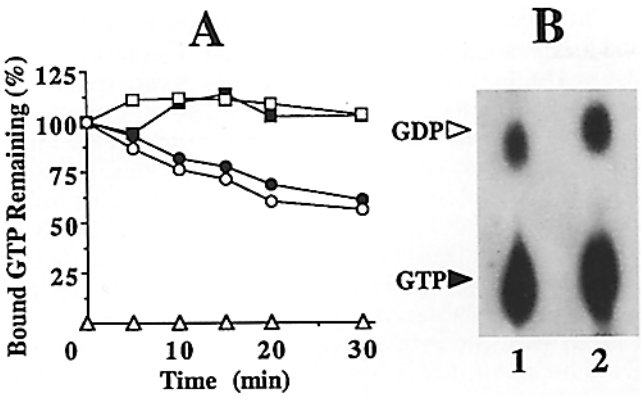

Figure 3. Effects of Bcl-2 on R-Ras GTPase activity and GTP binding. In *A*, GST-R-Ras (*circles*) and GST-R-Ras(38V) proteins (*squares*) were loaded with [^{32}P]γ-GTP and incubated with (*dark symbols*) or without (*open symbols*) 0.5 μg of affinity-purified Bcl-2 protein. Reactions were initiated by placing samples at 30°C. At various times thereafter, aliquots of samples were filtered through nitrocellulose, washed, subjected to scintillation counting, and the percentage of [^{32}P]γ-GTP remaining was calculated relative to T_o. Results for GST nonfusion protein are also shown as a negative control, where the data are calculated relative to GST-R-Ras(38V) at T_o. Data are representative of two experiments. In *B*, 32D-R-Ras(38V) cells (lane *1*) and 32D-R-Ras(V38)+Bcl-2 cells (lane *2*) were metabolically labeled with $^{32}PO_4$. Detergent lysates were prepared from 10^7 cells, and R-Ras was immunoprecipitated. Associated nucleotides were eluted from immune complexes and chromatographed on PEI-cellulose along with GTP and GDP standards. Filters were subjected to autoradiography and the positions of [^{32}P]GTP and [^{32}P]GDP indicated. Data are representative of two experiments.

loaded GST-R-Ras and GST-R-Ras(38V) proteins in vitro (not shown). However, several exchange proteins, including Sos (Buday and Downward, 1993), cdc25, and Vav also failed to catalyze GDP/GTP-exchange on GST-R-Ras and GST-R-Ras(38V), while some of these (Sos and cdc25) were effective on GST-Ha-Ras. Thus, we cannot completely discount the possibility that Bcl-2 might have exhibited exchange activity had alternative conditions for these in vitro assays been tried, but nevertheless consider it unlikely.

Though Bcl-2 had no GAP or GDP/GTP-exchange activity on R-Ras in vitro, these results do not exclude the possibility that Bcl-2 could modulate the ratio of GTP/GDP bound to R-Ras protein in cells through indirect effects on other proteins that control R-Ras GTPase activity or nucleotide exchange. Since Bcl-2 can completely abrogate the cell death promoting activity even of the mutant R-Ras(38V) which lacks detectable GTPase activity, we compared the ratio of GTP/GDP bound to R-Ras(38V) in 32D.3 cells that contained normal levels of Bcl-2 protein vs those with gene transfer-mediated elevations in the relative amounts of Bcl-2 protein. For these experiments, 32D-R-Ras(38V) and 32D-Bcl-2/R-Ras(38V) cells were metabolically labeled with $^{32}PO_4$. R-Ras proteins were then immunoprecipitated from these cells and the relative amounts of GTP and GDP associated with immune complexes were determined by thin layer chromatography. Both the total amount of guanine nucleotides and the relative proportions of GTP and GDP associated with R-Ras proteins were not significantly different for 32D-R-Ras(38V) and 32D-Bcl-2/R-Ras(38V) cells. In the experiment presented in Fig. 3 *B*, for example, the percentage of bound GTP relative to total associated guanine nucleotides (GDP + GTP) was 82% and 78% for 32D-R-Ras(38V) and 32D-Bcl-2/R-Ras(38V) cells, respectively. Immune complexes prepared from these cells also contained essentially equivalent amounts of R-Ras proteins (not shown). We conclude therefore that Bcl-2 does not block R-Ras(38V)- mediated effects on apoptotic cell death by altering the relative amounts or proportions of GTP-bound to R-Ras proteins.

Investigations of Bcl-2 Effects on R-Ras Interactions with Raf-1 Kinase

Recently, R-Ras has been shown to bind to Raf-1 kinase in vitro based on experiments employing purified recombinant proteins and in yeast two hybrid experiments (Rey et al., 1994; Spaargaren et al., 1994). Presumably the region in R-Ras required for interaction with Raf-1 corresponds to the presumptive effector domain of this GTPase (residues 51 to 72), which shares significant amino acid sequence homology with the effector domain of Ha-Ras where Raf-1 binds, including 9 of 9 residue identities in the core of this domain (amino acids 58-66) of R-Ras (32-40 of Ha-Ras) (Lowe and Goeddel, 1987; Self et al., 1993; Zhang et al., 1993). Like Ha-Ras, the interaction of R-Ras with Raf-1 is dependent on R-Ras being in the GTP-bound state (Rey et al., 1994; Spaargaren et al., 1994). Using recombinant baculoviruses, Raf-1 was coexpressed in Sf9 cells with either R-Ras or R-Ras (38V). Coimmunoprecipitation experiments showed that Raf-1 associated with R-Ras(38V) and to a lesser extent with the normal R-Ras protein (not shown), consistent with the GTP dependence of R-Ras/Raf-1 interactions. Coexpression of R-Ras(38V) but not R-Ras also resulted in elevations in the specific activity of the Raf-1 kinase in Sf9 cells, based on immune complex kinase assays where Raf-1 was immunoprecipitated and assayed for ability to phosphorylate recombinant purified MEK protein in vitro (Fig. 4). These data thus provide the first direct demonstration that R-Ras(38V) can promote Raf-1 kinase activation. Since these Raf-1 immune complexes were prepared under conditions that do not preserve in vitro interactions with R-Ras (not shown), presumably R-Ras(38V) induced activation of Raf-1 in Sf9 cells by bringing it into contact with Raf-1 kinases or other Raf-activating proteins that are associated with membranes in Sf9 cells (Leevers et al., 1994; Stokoe et al., 1994). Next, the effects of Bcl-2 on R-Ras-mediated activation of Raf-1 kinase were explored in the Sf9 cells to determine whether Bcl-2 is capable of blocking this R-Ras effector mechanism. As shown in Fig. 4, expression of Bcl-2 in Sf9 cells did not interfere with R-Ras(38V)-induced elevations in Raf-1 kinase activity. Approximately equivalent amounts of Raf-1 protein were produced in all Sf9 samples, excluding variations in the levels of Raf-1 protein as an explanation for the higher levels of Raf-1 kinase activity seen in R-Ras(38V)-containing Sf9 cells (*bottom panel*; Fig. 4).

Analysis of R-Ras Interactions with Bcl-2 by Coimmunoprecipitation Assays

To begin to examine the physical interactions among R-Ras, Raf-1, and Bcl-2 proteins, coimmunoprecipitation experi-

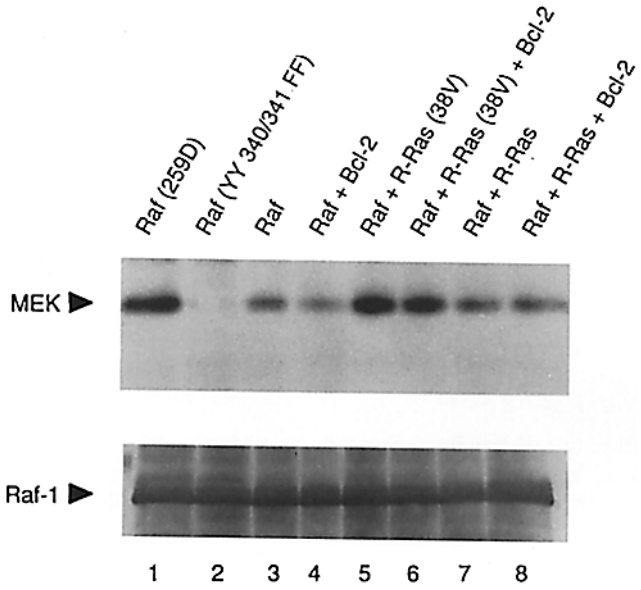

Figure 4. R-Ras(38V) induces elevations in Raf-1 kinase activity in Sf9 cells. Sf9 cells were infected with various recombinant baculoviruses, including: (lane *1*) Raf(259D), a constitutively active form of Raf-1 kinase used as a positive control; (lane *2*) Raf(Y340F, Y341F), an inactive form of Raf-1 kinase used as a negative control; (lane *3*) normal Raf-1 kinase; (lane *4*) Raf-1 and Bcl-2; (lane *5*) Raf-1 and R-Ras(38V); (lane *6*) Raf-1, R-Ras(38V), and Bcl-2; (lane *7*) Raf-1 and R-Ras; and (lane *8*) Raf-1, R-Ras, and Bcl-2. 2 d later, cells were lysed in RIPA buffer and Raf-1 kinase was immunoprecipitated. The resulting immune complexes were employed for in vitro kinase assays using MEK as an exogenous substrate and [^{32}P]γ-ATP. Reactions were centrifuged to separate MEK protein (supernatant; *top panel*) and Raf-1 immune complexes (pellet; *bottom panel*). Proteins were size fractioned by SDS-PAGE and the resulting gels either dried for autoradiographic detection of phosphorylated MEK (*top*) or transferred to nitrocellulose and incubated with anti-Raf-1 antiserum to compare relative levels of Raf-1 protein (*bottom*). The production of high levels of Bcl-2 and R-Ras proteins was also verified by immunoblot analysis of a portion of the cell lysates (not shown).

ments were performed using recombinant baculoviruses so that high levels of these proteins could be produced in Sf9 cells, thus facilitating detection of protein–protein associations. When Sf9 cells were coinfected with Bcl-2 and either R-Ras or R-Ras(38V), and then cell lysates were subjected to immunoprecipitation using anti–R-Ras antibody, little or no associated Bcl-2 protein was detected by subsequent immunoblotting using anti–Bcl-2 antibodies (see Fig. 5 *A*; lane *5*; and data not shown). Under these same experimental conditions where essentially no association of Bcl-2 with either R-Ras or R-Ras(38V) was seen, Raf-1 kinase was coimmunoprecipitated with R-Ras or R-Ras(38V) at easily detectable levels (representing ~10–20% of total Raf) from either Sf9 cells coinfected with Raf-1 and R-Ras alone or cells triply infected with Bcl-2, Raf-1, and R-Ras (Fig. 5 *A*; lanes *1* and *2*; and data not shown). Similar results were obtained when anti–Bcl-2 antibodies were employed for immunoprecipitations and immunoblotting was performed with anti–R-Ras antibodies. As shown in Fig. 5 *B*, for example, very little R-Ras was coimmunoprecipitated with Bcl-2 from Sf9 cells that had been infected with the combination of either Bcl-2 and R-Ras(38V) baculoviruses (lanes *1* and *2*). Direct immunoblot analysis of an aliquot of these same lysates confirmed the presence of high levels of R-Ras(38V) protein, excluding failure to produce R-Ras(38V) as an explanation of the minimal amounts of this protein that could be recovered with Bcl-2 immune complexes (Fig. 5 *B*, lane *3*). Performing triple infections with Bcl-2, Raf-1, and either R-Ras or R-Ras(38V) baculoviruses did not change the results, though in some experiments slightly more Bcl-2 could be coimmunoprecipitated with R-Ras when Raf-1 was added (not shown). In addition, in yeast two hybrid assays, no significant interaction above background of R-Ras or R-Ras(38V) with Bcl-2 was detected, when using fusion proteins that lacked the CAAX boxes of R-Ras and R-Ras(38V) and the transmembrane domain of Bcl-2 so as not to interfere with nuclear targeting. Under these same conditions, however, R-Ras and R-Ras (38V) did strongly interact with Raf-1 (unpublished observations).

R-Ras(38V) and R-Ras Do Not Coimmunoprecipitate with Bax

The finding that R-Ras promotes apoptotic death of 32D.3 cells in the setting of factor deprivation prompted us to explore the possibility of an association between R-Ras and Bax, a protein that shares 21% amino acid sequence homology with Bcl-2, forms heterodimers with Bcl-2, and accelerates rates of cell death in IL-3–dependent hematolymphoid cells when these cells are cultured in the absence of lymphokines (Oltvai et al., 1993). For these experiments, 32D.3 cell transfectants were lysed using gentle detergent conditions (0.2% NP-40) and immune complexes were prepared using antibodies specific for either Bax or R-Ras. The resulting immune complexes were then assessed for associated R-Ras, Bax, and Bcl-2 proteins by immunoblot assay. As shown in Fig. 6, no Bax was detected in anti–R-Ras immunoprecipitates. Similarly no detectable R-Ras was observed anti-Bax immune complexes (not shown). Conversely, Bcl-2 was coimmunoprecipitated with Bax. Thus, if R-Ras does interact with Bax, it presumably does so with lower affinity or with lower stoichiometry than Bcl-2/Bax interactions. Moreover, in yeast two hybrid assays, no interaction of R-Ras or R-Ras (38V) (expressed without CAAX boxes) with Bax (without its transmembrane domain) was detected above background, whereas Bcl-2 and Bax did interact strongly in these experiments (unpublished observations).

We also could not detect Bcl-2 associated with immune complexes prepared with anti–R-Ras antibodies from 32D.3 cell lysates (Fig. 6), consistent with the data presented above for Sf9 cells. R-Ras(38V) and R-Ras also did not detectably coimmunoprecipitate with Bcl-2 when anti–Bcl-2 antibodies were used for immunoprecipitation (not shown).

Discussion

R-Ras has been reported to interact with the Bcl-2 protein, thus suggesting a role for this GTPase in the regulation of physiological cell death pathways (Ferendez-Sarabia and Bischoff, 1993). Here we show for the first time that an activated version of R-Ras can increase the rate of apoptotic cell death in the setting of growth factor withdrawal and that Bcl-2 completely abrogates this effect of R-Ras. The mechanism

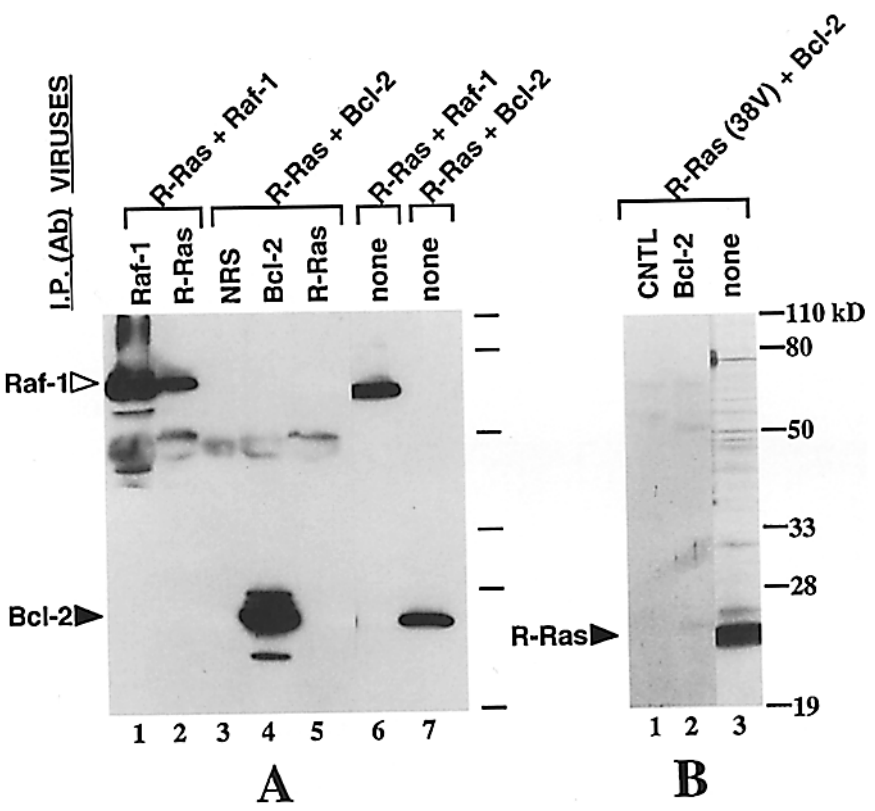

Figure 5. Coimmunoprecipitation analysis of R-Ras interactions with Bcl-2 and Raf-1 in Sf9 cells. In *A*, Sf9 cells were coinfected with either Raf-1 and R-Ras recombinant baculoviruses (lanes *1*, *2*, and *6*) or Bcl-2 and R-Ras (lanes *3–5*, *7*). Approximately 60 h later, detergent lysates (0.2% NP-40) were prepared and immunoprecipitations were performed using 200 μl of lysate and 5–10 μl of anti–R-Ras antiserum (lanes *2* and *5*), anti–Bcl-2 antiserum (lane *4*), anti–Raf-1 antibody (lane *1*) or normal rabbit antiserum as a negative control (lane *3*). In addition to immune complexes, 15 μl of the Sf9 cell lysates were applied directly to the gel without immunoprecipitation (lanes *6* and *7*). Proteins were subjected to SDS-PAGE and transferred to nitrocellulose filters which were incubated with antisera specific for Raf-1 (*top half*) or Bcl-2 (*bottom half*) and detected by ECL method. The bands seen at ~50 kD in lanes *1–5* represent rabbit immunoglobulin heavy chain proteins derived from the immunoprecipitations. In *B*, Sf9 cells were coinfected with Bcl-2 and R-Ras(38V) recombinant baculoviruses, and immunoprecipitations were performed using either the anti–Bcl-2 monoclonal antibody 4D7 (Reed et al., 1992) or IgG1 control antibody (*CNTL*) (lanes *1* and *2*). Immune complexes were subjected to SDS-PAGE immunoblot analysis using anti–R-Ras antiserum. An aliquot of the Sf9 cell lysates equivalent to 8% of that used for coimmunoprecipitation experiments was run directly in the gel, to verify production of R-Ras protein (lane *3*). Antibody detection was by a diaminobenzidine colorimetric method. Similar results were obtained using ECL for detection (not shown).

by which Bcl-2 nullifies R-Ras effects on cell death was examined with regards to R-Ras GTP-binding and GTPase activity. Bcl-2 did not affect R-Ras GTPase activity nor did it act as an exchange protein for R-Ras in vitro. Though we cannot exclude the possibility that Bcl-2 indirectly regulates the activity of R-Ras through effects on R-Ras GAPs or GDP exchange proteins in cells, this does not appear to provide an explanation for how Bcl-2 abrogates R-Ras effects on cell death. In 32D.3 cells, for example, R-Ras(38V) bound GTP to the same extent regardless of whether Bcl-2 was overproduced. Thus, Bcl-2 did not prevent R-Ras(38V) from assuming an active GTP-bound state. The implication of this finding is that Bcl-2 lies downstream of R-Ras in a cell death pathway, and is able to nullify the effects of R-Ras on growth factor withdrawal-induced apoptosis even when R-Ras assumes an active GTP-bound conformation.

How then does Bcl-2 interfere with R-Ras(38V)–mediated effects on apoptotic cell death? One possibility is that Bcl-2 somehow interrupts the interaction of activated GTP-bound R-Ras with a downstream effector protein. Based on the data presented here showing that coexpression of R-Ras(38V) with Raf-1 in Sf9 cells leads to elevations in Raf-1 kinase activity (Fig. 4), Raf-1 appears to be one potential downstream effectors of R-Ras. In this regard, Cox et al. (1994) have shown that expression of an activated form of R-Ras in NIH-3T3 cells leads to increased phosphorylation of p44-ERK-1 and p42-ERK-2 and that a dominant inhibitory mutant of Raf-1 that lacks kinase activity inhibits R-Ras-induced increases in activity of a *ras*-responsive promoter. Moreover, Raf-1 kinase has been shown to bind to R-Ras in vitro and in yeast two hybrid assays in a GTP-dependent manner (Rey et al., 1994; Spaargaren et al., 1994), but not to a mutant form of R-Ras that contains a serine 43 to asparagine amino acid substitution which is analogous to a Asn17 dominant-inhibitory form of Ha-Ras (Spaargaren et al., 1994). The region within Raf required for interaction with R-Ras corresponds to a segment within the NH_2-regulatory domain of Raf-1 (amino acids 51-131) that also is necessary for interactions with Ha-Ras (Vojtek et al., 1993). Conversely, the region in Raf that is sufficient for binding to Bcl-2 resides in the carboxyl-half of this protein, where the kinase domain is located (Wang et al., 1994). Consistent with the mapping of the Ras-binding and Bcl-2-association domains within Raf-1 to distinctly different domains, coproduction of Bcl-2 protein in Sf9 cells with R-Ras(38V) and Raf-1 did not prevent R-Ras(38V) from inducing increases in Raf-1 kinase activity (Fig. 4). Bcl-2 also did not detectably diminish coimmunoprecipitation of R-Ras(38V) and Raf-1 when the three proteins were simultaneously produced in Sf9 cells (unpublished observations). Thus, it seems unlikely that Bcl-2 thwarts the effects of R-Ras on cell death by blocking R-Ras–mediated activation of Raf-1 kinase, but additional work must be done before this possibility can be completely excluded. It should be noted, however, that Raf-1 may be only one of several potential downstream effectors of R-Ras, given that Ha-Ras has been shown to interact with Raf-1, phosphatidylinositol-3′-kinase, NF1-GAP and p120 ras-GAP via its effector domain (Vojtek et al., 1993; Zhang et al.,

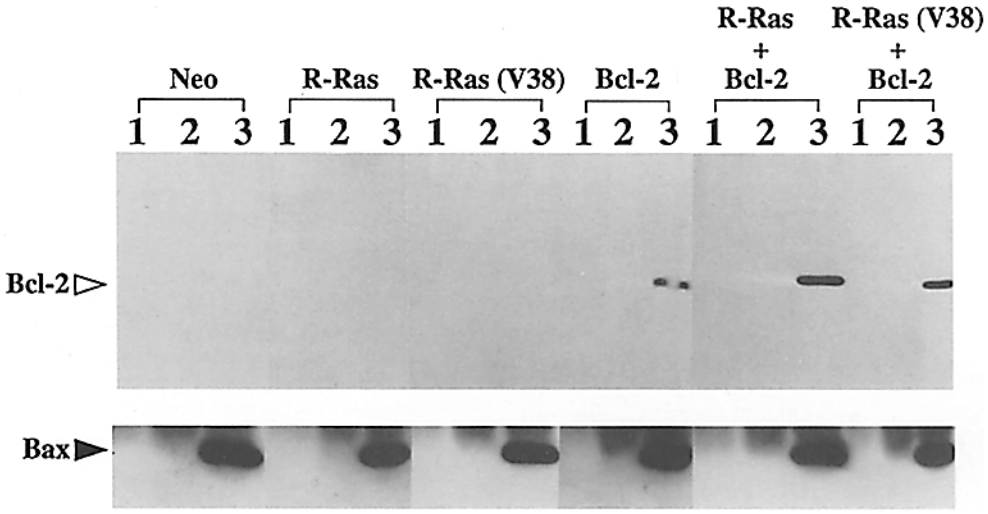

Figure 6. Coimmunoprecipitation analysis of R-Ras interactions with Bax and Bcl-2 in 32D.3 cells. Detergent lysates (0.2% NP-40) were prepared from various transfected 32D.3 cells (10^7 cells) and immunoprecipitations were performed using preimmune rabbit serum as a negative control (lane *1*), anti-R-Ras antiserum (lane *2*), or anti-Bax antiserum (lane *3*). Immune complexes were subjected to SDS-PAGE and transferred to nitrocellulose. The resulting blots were incubated sequentially with antibodies specific for Bcl-2 (*top panel*), followed by Bax (*bottom panel*). Antibodies were detected by an ECL method. Blots were stripped of the previous antibody using 2% SDS, 0.1 M 2-mercaeptoethanol, 62.5 mM Tris (pH 6.7) at 50°C for 0.5 h. The positions of the endogenous mouse Bax and transgene-derived human Bcl-2 proteins are shown.

1993; Rodriguez-Viciana et al., 1994; Frech et al., 1990) and that the effector domains of Ha-Ras and R-Ras have either identical or closely related amino acid sequences, depending on how this domain is defined (Lowe and Goeddel, 1987; Rey et al., 1994). Consequently, it remains possible that Bcl-2 directly interferes with the interaction of R-Ras with some other unknown downstream effector that regulates a signal transduction pathway involved in apoptosis.

Any model that attempts to position Bcl-2 as a direct inhibitor of R-Ras, however, must take into consideration the poor efficiency with which these proteins could be coimmunoprecipitated from transfected 32D.3 and baculovirus-infected Sf9 cells. Our virtual inability to coimmunoprecipitate Bcl-2 and R-Ras differs from a report by Fernandez-Sarabia and Bischoff (1993) where Bcl-2 and R-Ras were coimmunoprecipitated from HeLa cells in which overproduction of these proteins was achieved by gene transfection. Though a variety of technical issues may account for the differences in our results, we were able to readily coimmunoprecipitate R-Ras and Raf-1 from Sf9 cells and we also could easily coimmunoprecipitate Bax and Bcl-2 from 32D.3 cells, under the same conditions where Bcl-2 and R-Ras were coimmunoprecipitated minimally, if at all. These findings therefore argue that the binding of R-Ras and Bcl-2 may occur with low affinity or low stoichiometry, compared to interactions of R-Ras with Raf-1 and of Bcl-2 with Bax. Alternatively, it is possible that an additional unknown third protein is required to facilitate interactions of R-Ras with Bcl-2 or that this protein–protein interaction is modulated by post-translational modifications that failed to occur in our 32D.3 or Sf9 cells. Finally, we cannot exclude the possibility that transient interactions of Bcl-2 with R-Ras can occur, which are somehow dependent on whether or not guanine nucleotides are bound to R-Ras.

In contrast to activated R-Ras which accelerated the rate of 32D.3 cell death when cultured without IL-3, expression of *v-raf* in 32D.3 cells has been reported to delay programmed cell death caused by lymphokine withdrawal (Cleveland et al., 1994). Moreover, whereas 32D.3 cells cotransfected with R-Ras(38V) and Bcl-2 exhibit cell survival kinetics comparable to cells transfected with Bcl-2 alone, 32D.3 cells coexpressing Bcl-2 and an activated version of *c-raf-1* enjoyed markedly prolonged survival relative to cells expressing Bcl-2 alone (Wang et al., 1994). Thus, Raf-1 can cooperate with Bcl-2 to provide enhanced protection from apoptosis caused by growth factor deprivation. Since R-Ras can induce Raf-1 activation, these observations suggest a potential paradoxical situation where activated Raf-1 kinase can prolong cell survival in one scenario, but then possibly accelerate cell death in another. However, we do not know whether the mechanism that R-Ras uses to increase rates of cell death involves Raf-1 vs some other downstream effector protein. Also, it is possible that Raf-1 can have different effects on apoptosis depending on the substrates to which it is targeted by its interactions with various proteins such as R-Ras, Ha-Ras, and Bcl-2.

The increased rates of cell death seen in IL-3–deprived 32D.3 cells that contain R-Ras(38V) is reminiscent of previous results obtained for Myc oncoproteins, which have been shown to promote apoptosis in 32D.3 and other cells when deprived of growth factors (Askew et al., 1991; Evan et al., 1992). Under circumstances where growth factors are absent but c-Myc remains elevated, a clash in cell cycle signals occurs, resulting in the triggering of apoptosis via a mechanism that is suppressible by Bcl-2 (Bissonnette et al., 1992; Fanidi et al., 1994). In this regard, activated R-Ras has been reported to promote growth of NIH-3T3 cells in reduced serum, suggesting an effect on cell cycle (Cox et al., 1994). Despite some similarities, however, R-Ras and Myc clearly perform distinct biological functions, since coexpression of R-Ras(38V) and Bcl-2 in IL-3–dependent cells did not result in factor-independent growth, unlike coexpression of Myc and Bcl-2 which does (Vaux et al., 1988). Also of interest with regards to the proapoptotic effects of R-Ras on factor-deprived cells are studies showing that oncogenic Ha-Ras can accelerate rates of apoptosis in murine fibroblasts when

cultured in growth factor–deficient medium or exposed to γ radiation or chemotherapeutic drugs (Tanaka et al., 1994). Thus, R-Ras may share some characteristics in common with Ha-Ras with regards to sensitizing cells to apoptosis. Further work is required therefore to delineate the mechanisms by which R-Ras accelerates apoptotic cell death in growth factor–deprived cells and yet can also promote anchorage-independent growth in vitro and tumor formation in vivo, at least for some types of cells such as NIH-3T3 fibroblasts (Cox et al., 1994; Saez et al., 1994). Regardless of these mechanisms, the data shown here argue that this R-Ras–mediated pathway for apoptosis is ultimately governed in a dominant fashion by Bcl-2.

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