Grb10 and Active Raf-1 Kinase Promote Bad-dependent Cell Survival*

The proapoptotic protein Bad is a key player in cell survival decisions, and is regulated post-translationally by several signaling networks. We expressed Bad in mouse embryonic fibroblasts to sensitize them to apoptosis, and tested cell lines derived from knock-out mice to establish the significance of the interaction between the adaptor protein Grb10 and the Raf-1 protein kinase in anti-apoptotic signaling pathways targeting Bad. When compared with wild-type cells, both Grb10 and Raf-1-deficient cells exhibit greatly enhanced sensitivity to apoptosis in response to Bad expression. Structure-function analysis demonstrates that, in this cellular model, the SH2, proline-rich, and pleckstrin homology domains of Grb10, as well as its Akt phosphorylation site and consequent binding by 14-3-3, are all necessary for its anti-apoptotic functions. As for Raf-1, its kinase activity, its ability to be phosphorylated by Src on Tyr-340/341 and the binding of its Ras-associated domain to the Grb10 SH2 domain are all necessary to promote cell survival. Silencing the expression of either Grb10 or Raf-1 by small interfering RNAs as well as mutagenesis of specific serine residues on Bad, coupled with signaling inhibitor studies, all indicate that Raf-1 and Grb10 are required for the ability of both the phosphatidylinositol 3-kinase/Akt and MAP kinase pathways to modulate the phosphorylation and inactivation of Bad. Because total Raf-1, ERK, and Akt kinase activities are not impaired in the absence of Grb10, we propose that this adapter protein creates a subpopulation of Raf-1 with specific anti-apoptotic activity.

The best known models for receptor-mediated anti-apoptotic cascades include interleukin-3 signaling in hematopoietic cells and insulin-like growth factor 1 (IGF-1) signaling in fibroblasts. To date, several distinct anti-apoptotic signaling pathways have been characterized (1, 2) including activation of the Akt kinase following the production of phosphatidylinositol 3,4-biphosphate by phosphoinositol 3-kinase (PI3K) (3, 4). A second anti-apoptotic pathway is the mitogenic MAPK cascade that is characterized by the sequential activation of the Raf, MEK, ERK, and p90RSK kinases (5, 6). Finally, Peruzzi et al. (6) have identified an additional IGF-1-dependent pathway that requires the mitochondrial relocation of a complex containing a 14-3-3 protein, the E3 ubiquitin ligase Nedd4, and the Raf-1 kinase (6). One of the end points of these signaling pathways is the serine phosphorylation and inactivation of the Bcl-2 protein family member Bad (7). This phosphorylation event is followed by the binding of Bad to 14-3-3 proteins and its sequestration in the cytoplasm, thus preventing it from inactivating the anti-apoptotic proteins Bcl-2 and Bcl-xL on the mitochondrial surface (8). Several serine residues have been identified as sites for Bad-inactivating phosphorylation; Akt promotes cell survival by phosphorylating serine 136 (9, 10), serine 112 is the substrate for the MAPK downstream signaling effector p90RSK (5, 11) as well as PAK-phosphorylated Raf-1 (12), whereas the cAMP-dependent protein kinase A phosphorylates serine 155. Phosphorylation on any of these serine residues appears sufficient to permit binding to 14-3-3 isoforms (13, 14).

Grb10 is a member of a superfamily of adaptor proteins that includes Grb7 and Grb14 (15, 16); these share a common overall structure, including an N-terminal region harboring a conserved proline-rich motif, a central pleckstrin homology (PH) domain, a C-terminal Src homology 2 (SH2) domain, and a conserved region located between the PH and SH2 domains (the BPS) that has been shown to act as a pseudosubstrate for the kinase domain of receptor-tyrosine kinases (17). Extensive experimentation directed at understanding the possible roles of Grb10 in regulating the metabolic and mitogenic responses to insulin and IGF-1 has indicated a multitude of functions both positive and negative (18–20). In particular, several lines of evidence implicate Grb10 in apoptosis including an extensive list of cell survival-related binding partners such as the IGF-1 receptor, Raf-1, MEK1, and Akt kinases, the p85 subunit of the fetal bovine serum; HEK, human embryonic kidney; HA, hemagglutinin; GST, glutathione S-transferase; GFP, green fluorescent protein; siRNA, small interfering RNA; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.
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PI3K, the 14-3-3 adaptor protein, and the Nedd4 ubiquitin ligase (20, 21). In addition, overexpression of a Grb10 protein containing an inactivating mutation in its SH2 domain promotes apoptosis in transfected cells (22). Immunofluorescence microscopy and subcellular fractionation clearly demonstrate that most of the endogenous Grb10 protein is peripherally associated with the mitochondria, the organelle that is at the center of the apoptosis control machinery, and that these molecules transiently relocalize to the plasma membrane following receptor activation. Finally, the endogenous Grb10 and Raf-1 proteins can be co-immunoprecipitated from mitochondrial extracts, an interaction that is enhanced following the activation of Raf-1 by ultraviolet radiation (23). Overall, an important component of Grb10 function in cell survival appears to be dependent on its relationship with the Raf-1 kinase.

The Raf proteins are highly conserved serine-threonine protein kinases that transmit signals between a wide range of cell surface receptors and the MAPK cascade that contains the extracellular signal-regulated kinases (ERK). Activated Ras binds to Raf with high affinity and mediates its translocation from the cytosol to the plasma membrane, where kinase activation occurs via phosphorylation (24). Recent studies have revealed a new role for the Raf-1 kinase that is independent of the activation of the MAPK cascade and whose effect is to increase resistance to apoptosis (25–29). The importance of Raf-1 as an apoptotic modulator was greatly reinforced by the phenotypic analysis of mouse raf-1 knock-outs. Raf-1-deficient embryos die in gestation and show increased apoptosis in several tissues, whereas a cardiac-specific deletion ultimately leads to heart failure also linked to excess apoptosis. Mouse embryonic fibroblasts (MEFs) isolated from Raf-1−/− embryos are more sensitive to apoptosis induced by Fas, etoposide, and staurosporine. Activated Ras binds to Grb10 with high affinity and mediates its translocation from the cytosol to the plasma membrane, where kinase activation occurs via phosphorylation (24). Recent studies have revealed a new role for the Raf-1 kinase that is independent of the activation of the MAPK cascade and whose effect is to increase resistance to apoptosis (25–29). The importance of Raf-1 as an apoptotic modulator was greatly reinforced by the phenotypic analysis of mouse raf-1 knock-outs. Raf-1-deficient embryos die in gestation and show increased apoptosis in several tissues, whereas a cardiac-specific deletion ultimately leads to heart failure also linked to excess apoptosis. Mouse embryonic fibroblasts (MEFs) isolated from Raf-1−/− embryos are more sensitive to apoptosis induced by Fas, etoposide, and staurosporine. Surprisingly, activation of the MAPK pathway in Raf-1 knock-outs remained unchanged, possibly due to redundancy provided by the isoforms A-Raf and B-Raf (26, 27, 29). In this report, we use a novel in vivo assay to measure the effectiveness of anti-apoptotic signaling pathways and demonstrate that the interaction between the adaptor Grb10 and kinase Raf-1 is important for cell survival.

EXPERIMENTAL PROCEDURES

Generation and Analysis of Grb10−/− Mice and Cell Lines—Generation of Grb10/LacZ knock-in mice expressing the bacterial β-galactosidase cDNA under the transcriptional control of the endogenous Grb10 promoter has been performed by conventional gene targeting methodology. Briefly, a XbaI–Xhol genomic fragment of mGrb10 was isolated (positions 11808–5675 relative to AL663087) to generate the required homology arms for successful targeting. At the unique SmaI site at position 10,155 (and position 340 relative to the mGrb10 cDNA BC016111), a cassette containing (from 5′ to 3′) stop codons in all three reading frames, an IRES–LacZ–pa element, and finally a floxed PGK–NeoR–bpA element was inserted. Correctly targeted ES clones were verified by Southern analysis with external 5′ and 3′ probes and used to generate mutant mice by standard techniques. The resulting mice phenocopy two other mutants that are functionally null for the imprinted gene Grb10, a retroviral insertion mutant of Grb10 (30), and a mouse that inherits a paternal disomy for proximal chromosome 11 (31).

Primary mouse epithelial fibroblasts were produced from day 13 mouse embryos (day of plug = day 1) from appropriate crosses. After dissection, embryos were washed twice in sterile phosphate-buffered saline solution. Embryos were decapitated, eviscerated, and then passed several times through an 18-gauge needle. Each embryo was resuspended in 1 ml of 10% heat inactivated fetal bovine serum (FBS) in Dulbecco’s modified Eagle’s medium supplemented with 100 μg/ml DNase and 500 μg/ml collagenase. Subsequent to trituration, embryos were incubated at 37 °C for 30 min and centrifuged at 1,200 × g for 5 min. Ultimately, each disassociated embryo was plated in a 100-mm dish in 10% FBS in Dulbecco’s modified Eagle’s medium supplemented with penicillin and streptomycin.

Six independent pools of Grb10+/+ and Grb10−/− MEF cell lines were produced from two primary mouse epithelial fibroblasts lines from each genotype by spontaneous immortalization over a period of 6 weeks. All showed similar growth rates and activation profiles of the ERK and Akt kinases following serum treatment. Transcriptional profiles, as determined with DNA microarrays, were similar within the same genotypes and, in a comparison of the Grb10+/+ with Grb10−/− cells, did not show any significant changes in the levels of transcripts encoding known regulators of mitochondrial apoptosis (data not shown).

Cell Culture—Human embryonic kidney 293A (HEK293A) cells and the human breast adenocarcinoma cell line MCF-7 were grown in Dulbecco’s modified Eagle’s medium (Hyclone) containing 10% FBS (Hyclone), whereas MEF were grown in Dulbecco’s modified Eagle’s medium high glucose (Hyclone) containing 10% FBS. A human breast adenocarcinoma cell line MDA-MB231, purchased from ATCC, was grown in RPMI 1640 (Hyclone) medium supplemented with 10% FBS. Raf-1 knock-out, and the wild-type control MEF cell lines were kindly provided by Dr. C. Pritchard (University of Leicester, UK) (26). Grb14 knock-out with their wild-type control MEFs were a kind gift from Dr. R. J. Daly (Garvan Institute of Medical Research, St Vincent’s Hospital, Sydney, Australia) (32).

Expression Constructs and Transfection Methods—Mouse Grb106 (mGrb106) and Raf-1 kinase (mRaf-1) were cloned from cDNA prepared from MEF cells mRNA, using the Expand Long Template PCR System® (Roche), subcloned into pcDNA 3.1myc/his plasmid (Invitrogen), and sequenced. Mouse Bad cDNA, kindly provided by Dr. G. Shore (McGill Cancer Center, McGill University, Montreal, Canada), was subcloned into the bicistronic vector pIRES-hrGFP-1a (Stratagene). Point mutations into mGrb106, mRaf-1, and Bad were performed using the QuikChange® multisite-directed mutagenesis kit (Stratagene). The mammalian GST-Bad construct, pEBGmBad was purchased from Cell Signaling Technology. Deleted constructs were generated by overlapping PCR (33, 34), subcloned into pcDNA 3.1myc/his plasmid (Invitrogen), and sequenced. The HA-tagged human Grb10 construct subcloned into pcDNA3 was provided by Dr. S. Giorgetti-Peraldi (INSERM U145, Institut Federatif de Recherche 50, Faculté de Médecine, Nice, France). Transient transfections of these constructs in HEK293 and MEF cells were performed according to the manufacturer’s...
recommendations, using Superfect® transfection reagent (Qiagen). In Fig. 4, following transfection MEFs were treated with 150 nM Src Inhibitor I (Calbiochem), 10 nM of the PI3K inhibitor wortmannin, or 5 μM of the MEK1 inhibitor PD98059 (Cell Signaling Technology) and respective cell lysates were processed for Western blotting.

Fluorescence Microscopy—Phase-contrast and epifluorescence images were taken using a Leica DMI2500 inverted microscope (Leica Microsystems Canada) equipped with a Hamamatsu cooled CCD camera at ×200 or 400 magnification, using the appropriate filters. Openlab software (Improvision, MA) was used for image acquisition. For each field, 2 separate pictures were taken: phase-contrast and green fluorescence (for imaging the cells expressing green fluorescent protein (GFP)).

Apoptotic Sensitivity Assay—MEF were seeded in 6-well plates and transiently transfected with 0.5 μg of either of the empty reporter vectors (pIRES-hrGFP-1a or vectors) containing the various pIRES-Bad-hrGFP constructs. Alternatively, we also cotransfected pIRES-Bad-hrGFP with 0.3 μg of expression plasmids containing the various tagged Raf-1 or Grb10 genes. 16–18 h post-transfection, GFP-positive cells were visualized microscopically and apoptotic cells were distinguished from live cells based on their morphology; small, round, blebbing, and/or floating cells were scored as apoptotic, whereas flat adherent cells were scored as normal. Hoechst staining confirmed that these rounded/blebbing cells also contained condensed and fragmented nuclei, another hallmark of apoptosis (not shown). Unless otherwise indicated the data presented are the mean ± S.D. of four independent experiments.

Immunoblotting, Immunoprecipitation, and Pull-down Experiments—Transfected HEK293A and MEF were washed in ice-cold phosphate-buffered saline, and lysed in lysis buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, supplemented with protease inhibitors (Roche) and Phosphatase Mixtures I and II (Sigma). All lysates were cleared by centrifugation at 14,000 × g, Raf-1 (E10), Grb10 (K20), c-myc (9E10), and actin antibodies were purchased from Santa Cruz Biotech. Bad Ser-112, Bad Ser-136, Bad Ser-155, and Bad antibodies were from Cell Signaling Technology. Monoclonal antibody to HA was produced in our monoclonal antibody facility and was a kind gift of Anne Marcil (BRI-NRC, Montreal, Canada). For immunoblotting, total cell lysates (50 μg of protein) were resolved by SDS-PAGE and transferred onto either nitrocellulose or polyvinylidene difluoride membranes. After incubation with appropriate primary antibodies, signals were revealed by chemiluminescence (Roche) using corresponding horseradish peroxidase-conjugated secondary antibodies. For co-immunoprecipitations, 1 mg of clarified lysates were incubated overnight at 4 °C with 3 μg of anti-c-myc monoclonal antibody. Immune complexes were collected on protein L-agarose (Sigma), washed five times in lysis buffer, and processed for immunoblotting. Cell lysate from MEFs overexpressing mammalian GST-Bad alone or with either mycRaf-1 or mycGrb106 were subjected to an overnight GST pull down at 4 °C. Following several washes with lysis buffer, proteins were eluted from beads and processed for Western blotting using phosphoserine-specific Bad antibodies.

Small Interfering RNA—MCF-7 and MDA-MB231 cells plated at a density of 0.5 to 1 × 10^6 cells per well in six-well plates were transfected with 2 μM small interfering RNA (siRNA) oligoduplexes using Oligofectamine reagent (Invitrogen) according to the manufacturer’s protocols. The sequences of each oligoduplex targeting specifically human Grb10 (hGrb10) and human Raf-1 (hRaf-1) genes were as following: hGrb10, 5’-CAGUCUGAGAAUUUGGdTdT-3’, duplexed with AUCAGGUCAGAAUUUGGdTdT-3’, and hRaf-1, 5’- AGCAAAAGACUGUGGUAaTdTdT-3’, duplexed with 5’- UUGACACUGUUCUUUGCUaTdTdT-3’. Silencing was assessed 48 h post-transfection by Western blotting using specific Grb10 and Raf-1 antibodies. Alternatively, Grb10 or Raf-1 knocked down cells were rescued with either mouse Grb106 (mGrb106) or mouse Raf-1 (mRaf-1) cDNA, respectively, 24 h following siRNA transfection. Transfection of both human Grb10-specific oligos with its respective human cDNA targets, i.e. MycGrb10, lowered the expression of the transfected human mycGrb10 constructs, whereas the same oligos transfected with the mouse mycGrb10 did not alter the expression of the mouse Grb10 construct. The same experiments were performed with Raf-1 oligos (data not shown).

RESULTS

Bad-dependent Apoptotic Sensitivity in MEFs—To directly measure the effectiveness of signaling pathways leading to cell survival in cell lines lacking the Grb10 adaptor protein, we developed an assay to evaluate apoptotic sensitivity based on the expression of the pro-apoptotic modulator Bad. We have generated transgenic mice lacking functional Grb10 due to a LacZ insertion in the first coding exon of the Grb10 locus. Pups and newborn mice with an inactivated maternal Grb10 allele exhibited the same enhanced growth phenotype previously described by others (30). MEF originating from both wild-type and Grb10 knock-in mice express low levels of endogenous Bad (Fig. 1A). Transfection of small amounts of bicistronic Bad vector in which the expression of Bad is driven by the cytomegalovirus promoter and the expression of the GFP cistron is directed by an internal ribosomal entry site sequence, allowed us to study its regulation in these knock-out-derived cell lines. Microscopic examination of transfected MEFs was used to evaluate the percentage of apoptotic cells in the GFP-positive population 16–18 h post-transfection (Fig. 1A). Hoescht staining for the nucleus integrity revealed that apoptotic cells are round and detached, whereas healthy cells are flat and attached to the cell plate (data not shown). Sensitivity to apoptosis is presented as the ratio of scored apoptotic cells over total number of transfected cells.

Grb10−/− MEFs are more sensitive than wild-type to Bad expression and this pro-apoptotic phenotype can be reverted by expressing the wild-type gene (Fig. 1, A and B). Protective effect of Grb106 overexpression was achieved with 0.3–0.5 μg of cDNA transfected. As an additional control, Bad-mediated cell death assays were performed in MEFs lacking Grb14, an adaptor protein structurally related to Grb10 and belonging to the Grb7 superfamily (32, 35, 36). These showed no change in Bad-dependent apoptotic sensitivity when compared with wild-type cells, thus confirming the specific role of Grb10 in cell survival.
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(data not shown). Moreover, activation of the anti-apoptotic Akt and ERK kinases upon serum, IGF-I, or epidermal growth factor stimulation remained the same in these MEFs (data not shown).

To determine which domain modules of Grb10 were required for its anti-apoptotic activity, several mutants, illustrated in Fig. 1C, were tested in the Bad-mediated cell death assay (Fig. 1D). Constructs carrying deletions in the proline-rich (PR) and pleckstrin homology (PH) domains as well as a point mutation in a conserved arginine residue that is necessary for the SH2 domain to recognize its ligands (22, 33) are all unable to protect Grb10/H11002/H11002 MEFs from Bad-mediated apoptosis. Finally, Fig. 1E shows that mutagenesis of serine 428 to alanine, an Akt phosphorylation site known to promote the interaction of Grb10 to 14-3-3 (21), also prevented the Grb10 anti-apoptotic activity. Overall these results demonstrate that Grb10 promotes cell survival, and that its binding to other molecules through the SH2 domain, the PR region, as well as the PH domain, is essential for proper anti-apoptotic signaling.

Mapping the Requirements for Grb10-Raf-1 Interaction—We have previously demonstrated that the adaptor protein Grb10 interacts with Raf-1 (22, 23), a kinase known to be of great importance in promoting cell survival (25–29). Using a yeast two-hybrid mating strategy, we determined that the Grb10 SH2 domain recognizes the Ras-associated domain on the N-terminal half of Raf-1 (22, 37). Two Raf-1 point mutations, G46C and D117V, resulted in impaired binding to Grb10, whereas another point mutation, S120F, partially impaired binding to Grb10 in this two-hybrid assay (Fig. 2A).

FIGURE 1. Functional Grb10 promotes cell survival. A, MEF wild-type (+/+) and lacking functional Grb10 (−/−) were transfected with 0.5 μg of either an empty bicistronic GFP reporter vector (Mock) or the bicistronic GFP reporter vector expressing Bad (Bad). Right panel, graph of the relative percentage of apoptosis based on the morphology of GFP-positive cells. Experiments were performed five times on independent pools of immortalized cell lines and the results represent the mean ± S.D. The asterisks indicate significant differences as determined with a Student’s t test (p < 0.01). Lower panel, immunoblotting of total cell lysates (50 μg of protein) probed as indicated, with either Bad or Grb10 antibody. B, Bad-mediated cell death assay performed in MEF lacking functional Grb10 and cotransfected with 0.5 μg of bicistronic GFP vector expressing Bad along with increasing amounts of a mycGrb10-expressing plasmid. Experiments were performed three times and the results represent the mean ± S.D. with the asterisks signifying p < 0.001. Bottom panels show the immunoblotting of total cell lysates probed with either anti-Bad (upper panel) or Grb10 (lower panel) antibodies. C, schematic representation of various mGrb10 constructs used in domain characterization. Grb10SH2M and Grb10(5428A) carry mutations in the SH2 domain or Akt phosphorylation site, whereas Grb10PR and Grb10PH carry deletions in the proline-rich region and PH domain, respectively. D and E, Bad-mediated cell death assay, as performed above, in MEF lacking functional Grb10 and transfected with 0.3 μg of the different myc-Grb10 constructs shown in C. Mock denotes cells transfected with 0.5 μg of the empty GFP bicistronic reporter vector. Bottom panels show an immunoblot of whole cell lysates probed with either anti-Bad (upper panel) or myc antibody (lower panel). F, co-immunoprecipitation of 14-3-3 and Raf-1 with myc-tagged Grb10 or myc-tagged Grb10(5428A). WB, Western blot.
To verify that the interaction specificity is the same in mammalian cells, we co-transfected HEK293A cells with vectors expressing each of the myc-tagged Raf-1 mutants along with HA-tagged Grb10. Fig. 2C, upper panel, shows that the Raf-1 mutants G46C and D117V (fourth and fifth lanes) failed to immunoprecipitate HA-Grb10, whereas wild-type Raf-1, and Raf-1 S120F, can be found in a complex with HA-Grb10 (sixth and seventh lanes), thus supporting most of the two-hybrid experiments. However, the weaker binding of Grb10 to Raf-1 S120F observed in the yeast model was not reproduced in mammalian cells. In addition, the role of the Raf-1 kinase activity in these interactions has also been demonstrated; a Raf-1 kinase-dead mutant (K375W) (Raf-1KD) (38) is still capable of binding to Grb10 (Fig. 2C, last lane 7). Raf-1 kinase activity requires the phosphorylation of one of two pairs of residues, either serines 338 and 339 by PAK, or tyrosines 340 and 341 by Src. The non-phosphorylatable mutants Raf-1(S338A/S339A) and Raf-1(Y340F/Y341F) are still capable of binding Grb10 (Fig. 2C, right panel). A reciprocal experiment was performed in HEK293A cells expressing Myc-tagged Grb10 mutants (shown in Fig. 2D). Binding to endogenous Raf-1 kinase was not shown with wild-type, Grb10Δ PR and Grb10Δ PH proteins (Fig. 2D, upper panel, third, fifth, and sixth lanes), whereas the Grb10SH2M failed to immunoprecipitate Raf-1 (Fig. 2D, upper panel, fourth lane). The interaction of Grb10 with Raf-1 seems to be stabilized by 14–3–3 proteins because the Grb10(S428A) mutant fails to co-immunoprecipitate Raf-1 (Fig. 1F). Finally, Raf-1 immunonokinase assays were performed on all the Raf-1 mutants expressed in Raf1−/− MEFS stimulated with serum and these showed similar levels of kinase activity, thus demonstrating that Raf-1 kinase activity is not significantly altered in mutants impaired in Grb10 binding (data not shown). Overall, these results confirm that, in HEK293A cells, Grb10 interacts in vivo with Raf-1 and that this interaction is mediated through the SH2 domain of Grb10 and the RA domain of Raf-1.

To demonstrate whether the interaction between Raf-1 and Grb10 is required for the anti-apoptotic function of Raf-1, we employed a Raf1−/− MEF deficient cell death assay in vivo. As shown in Fig. 3A, Raf-1−/− cells deficient in Raf-1 expression are more sensitive to Bad-mediated apoptosis. Full rescue of the Raf1−/− MEF from Bad-dependent cell death with exogenous Raf-1, comparable with the wild-type levels, was achieved at 0.3–0.5 μg of transfected plasmid (Fig. 3B, lanes 1 and 4). Higher or lower concentrations of Raf-1 CDNA were detrimental to the cells (Fig. 3B, lanes 3, 5, and 6). As previously shown by others, no significant changes were observed in the activation of the Akt and ERK kinases in the Raf-1 knock-out cells (Refs. 26 and 27, and results not shown).

We have used the Bad-mediated cell death assay and the Raf-1 binding-specific mutants to confirm the role of the Raf-1-Grb10 molecular complex in anti-apoptotic signaling. MEFS lacking the Raf-1 gene were co-transfected with Raf-1 mutants G46C, D117V, and S120F that partially or fully impair the binding to Grb10. As shown in Fig. 3C, the mutants Raf-1 G46C and D117V (lanes 4 and 5) failed to rescue Raf1−/− cells from Bad-induced apoptosis, whereas the Raf-1 S120F mutant prevented Bad-induced apoptosis to the same extent as the wild-type Raf-1 (Fig. 3C, lane 6). Finally, Raf1−/− cells cotransfected with increasing amounts of wild-type Grb10 and a constant concentration of the Bad b Specifies the Raf1−/− cells re-expressing wild-type Raf-1 (data not shown). These results suggest that each protein requires the other for its anti-apoptotic function.

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We then performed the Bad-mediated cell death assay in Raf-1-deficient MEFS cotransfected with increasing amounts of Myc-tagged mouse Raf-1 and a constant concentration of the Bad b Specifies the Raf1−/− cells re-expressing wild-type Raf-1 (data not shown). These results suggest that each protein requires the other for its anti-apoptotic function.

### Raf-1 Anti-apoptotic Activity Requires Src-dependent Phosphorylation

The Bad sensitivity assay also demonstrates that the kinase activity of Raf-1 is necessary for its anti-apopto-
tic function in MEFs because the kinase-dead K375W mutant failed to revert the enhanced apoptotic sensitivity of the Raf1\(^{-/-}\) cells (Fig. 3C, lane 7). Fig. 3C also shows that the non-phosphorylatable mutant Raf-1(S338A/S339A) retains its anti-apoptotic activity (ninth lane), whereas the Raf-1(Y340F/Y341F) and Raf-1(Y340F/Y341F + S338A/S339A) mutants cannot protect against Bad-mediated apoptosis (eight and tenth lanes). In addition, Fig. 4, A and B (fourth lane), shows that treatment of Grb10- and Raf-1-deficient MEFs with Src inhibitor I prevents the re-expressed genes from rescuing Bad-expressing cells. Taken together, these results demonstrate that, to prevent Bad-induced apoptosis in MEFs, Raf-1 requires: 1) its kinase activity, 2) its ability to be activated by the Src, but not the PAK, kinase, and 3) binding of its N-terminal domain to Grb10.

**Raf-1 and Grb10 Mediate Their Anti-apoptotic Effects through Both the MAPK and PI3K Signaling Pathways**—It is well established that the localization and binding activity of Bad is regulated through the phosphorylation of specific serine residues (8). For example, Ser-112 is phosphorylated by MAPK-activated p90\(^{RSK}\), Ser-136 by Akt (9, 10), and Ser-155 by protein kinase A (13, 14). We used phospho-specific antibodies to determine the phosphorylation profile of GST-Bad in Grb10 and Raf-1 knock-out cells. Purification on glutathione-Sepharose was necessary to obtain sufficient phosphorylated Bad for immunoblotting with phosphospecific antibodies. As shown in Fig. 5, A and B, second lane), phosphorylation of serines 112 and 136 on Bad were reduced in MEFs lacking either Grb10 or Raf-1, whereas phosphorylation of serine 155 remained unchanged in these cells. In addition, re-expression of Grb10 or Raf-1 in deficient MEFs restored the phosphorylation state of Bad to wild-type levels (Fig. 5, A and B, third lane, respectively). Furthermore, re-expression of Grb10 and Raf-1 mutants used in Figs. 1D and


3B, respectively, reduced Bad phosphorylation on serine 112 and 136 (Fig. 5, A and B). This suggests that there is a direct correlation between the ability of specific Grb10 and Raf-1 mutants to protect cells against Bad-mediated apoptosis and the resulting phosphorylation levels of serines 112 and 136. Concomitant re-expression of Raf-1 along with treatment with the MEK inhibitor PD98059 in the MEFs did not rescue the serine 112 phosphorylation, which suggests that, in MEFs, Raf-1 does not directly phosphorylate Bad on serine 112 as suggested in other cellular models (6). We have also generated non-phosphorylatable serine to alanine mutations at these positions in the Bad protein to determine whether they can prevent the rescue of knock-out cell lines by the re-expression of wild-type Raf-1 or Grb10. As shown in Fig. 4, C and D, second and third lanes, neither Grb10 nor Raf-1 can protect cells from the expression of Bad S112A or S136A in their respective knock-out cell lines. On the other hand, both wild-type proteins could revert the pro-apoptotic phenotype of Bad S155A (Fig. 4, C and D). Last, treatment of MEFs with inhibitors of either the PI3K or MAP kinase pathways prevented their rescue by Grb10 (Fig. 4A, fifth and sixth lanes) or Raf-1 (Fig. 4B, fifth and sixth lanes). Taken together, these results suggest that Grb10 and Raf-1 achieve their anti-apoptotic effect by modulating the ability of both the PI3K and MAP kinase pathways to phosphorylate Bad. These results suggest that, in MEF cells, a Grb10/Raf-1 molecular complex, as well as Src-dependent Raf-1 kinase activation are all required to modulate the phosphorylation state of serine residues on Bad that are targeted by both the PI3K/Akt and the mitogenic MAPK pathways.

Validation with Gene Knock-down Experiments—Whereas we expended significant efforts in assuring that concentrations of transfected proteins were close as possible to physiological levels, we wanted to confirm whether Grb10 and Raf-1 can modulate levels of endogenous Bad phosphorylation. To this end, we searched for established cell lines that 1) express both Grb10 and Raf-1, 2) express sufficient endogenous Bad protein to permit the use of phospho-specific antibodies in untransfected cells, and 3) have high transfection efficiencies. We settled on two human breast cancer cell lines, MCF-7 and MDA-MB231, that clearly exhibit serum-dependent Bad phosphorylation. As shown in Fig. 6A, serum starvation dramatically reduced serine 112 and 136 phosphorylation in both cell lines, whereas serine 155 phosphorylation remained unchanged. Down-regulation of either Grb10 or Raf-1 expression by transient transfections of human-specific Grb10 and Raf-1 siRNAs oligoduplexes in MCF-7 and MDA-MB231 cells effectively suppressed Grb10 and Raf-1 expression, respectively, whereas mock transfection (WT) had no effect (Fig. 6B and C). Importantly, phosphorylation of Bad on serines 112 and 136 was reduced in these cells, whereas concomitant re-expression of the mouse homologs of Grb10 or Raf-1 re-established the phosphorylation state of Bad to wild-type levels (Fig. 6, B and C).

FIGURE 3. Raf-1 anti-apoptotic function requires both its kinase activity and binding to Grb10. A, wild-type (+/+) and Raf-1 knock-out (−/−) MEFs expressing GFP with or without Bad. Cells were visualized 16–18 h post-transfection. Right panel, graph of the relative percentage of apoptosis based on the morphology of GFP-positive cells. Statistically significant differences are indicated with an asterisk (*p < 0.01). Lower panel, immunoblotting of total cell lysates (50 μg of protein) probed with anti-Raf-1 antibody. B, Bad-mediated cell death assay performed in Raf-1−/− MEFs cotransfected with 0.5 μg of bicistronic GFP vector expressing Bad and increasing amounts of myc-Raf-1 expression plasmids. Experiments were performed three times and the results represent the mean ± S.D. where an asterisk indicates a statistical significance, p < 0.001. Bottom panels show immunoblotting of total cell lysates with either anti-Bad (upper panel) or Myc (lower panel) antibodies. C, Bad-mediated cell death assay performed inraf-1−/− MEF expressing a variety of Myc-Raf-1 mutants. Experiments were performed four times. An asterisk indicates p < 0.001.

FIGURE 4. The anti-apoptotic effects of Raf-1 and Grb10 are mediated through the MAPK- and PI3K-dependent pathways. Effects of a 16–18 h treatment with 150 nM Src inhibitor I, 5 mM PD 98059, or 10 nM wortmannin on the ability of either MycGrb10 (A) or MycRaf-1 (B) to protect their respective knock-out MEF in the Bad-mediated cell death assay. In C and D, we tested the ability of MycGrb10(C) or MycRaf-1(D) to protect their respective knock-out cell lines following transfection with point mutants of Bad that are resistant to phosphorylation. Experiments were performed four times and the results represent the mean ± S.D., * at least p < 0.001.
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**DISCUSSION**

Great progress has been achieved in understanding the molecular mechanisms leading to apoptosis, including the identification of Bad, a pro-apoptotic member of the Bcl-2 family (8). Active (unphosphorylated) Bad induces apoptosis by inhibiting the activity of anti-apoptotic Bcl-2 family members, such as Bcl-x<sub>L</sub>, thereby promoting the aggregation of two other pro-apoptotic members, Bak and Bax, leading to the release of cytochrome c, caspase activation, and finally to apoptosis (39, 40). The activation of several major anti-apoptotic signaling pathways correlates with an increase in the phosphorylation of Bad on Ser-112, Ser-136, and Ser-155. These phosphorylation events result in Bad inactivation through sequestration by 14-3-3 proteins (9, 10). Although the phosphorylation of Bad provides an ideal mechanism for cell survival, it is less clear what regulates the coordination of the multitude of signaling events that can lead to its inactivation.

In this study, we established a practical assay to measure the effectiveness of anti-apoptotic pathways that specifically terminate in the phosphorylation and inactivation of Bad. Cell lines that originate from knock-in/knock-out mice embryos were transfected with a bicistronic vector in which Bad acts as an apoptotic promoter, whereas GFP is used as a transfection reporter. In our model, it appears that expression of Bad overrides signaling pathways leading to cell survival and therefore acts as an apoptotic stimulus. We used this assay in a structure-function study that demonstrated that the survival of mouse fibroblasts in response to the expression of Bad requires a functional Grb10 protein, a functional Raf-1 kinase, and the ability for both of these proteins to interact with each other, probably with assistance from 14-3-3 proteins. Grb10 and Raf-1 knock-out cells are more sensitive to Bad-mediated apoptosis than wild-type and this phenotype can be reversed by expression of appropriate amounts of the wild-type protein. This positive effect from the re-expression of Grb10 and Raf-1 disappeared when the proteins were overexpressed. Whereas this observation could be the consequence of non-physiological expression levels, it has also been suggested that signaling proteins, especially scaffolds like Grb10, can exhibit both positive and negative effects on signaling depending on the ratio between them and other components of the multiprotein complexes in which they are associated (41). Both proteins appear to be implicated in modulating the phosphorylation and inactivation of Bad by the MAPK and PI3K signaling pathways. We have also defined a new function for the Raf-1 RA domain. According to the three-dimensional structure of the Raf-1 and Ras molecular complex (42), Grb10 recognizes residues on the RA domain of Raf-1 on the opposite side of the Raf-1/Ras interacting interface, suggesting that Grb10 binding to Raf-1 would not interfere with Ras activation (Fig. 2B). Furthermore, the ability to interact with Grb10 and to protect cells against Bad-mediated apoptosis seems to be unique to the Raf-1 isoform. Immunoblotting has shown that both A-Raf and B-Raf are expressed in all of the cell lines used in this study. Furthermore, the Gly-46 and Asp-117 residues of Raf-1, which are indispensable for binding to Grb10, are not present in either of the two other isoforms.

There is growing evidence to suggest that the roles played by Grb10 and Raf-1 in anti-apoptotic signaling are more complex than the simple activation of the canonical anti-apoptotic kinases Akt or ERK1/2. This is obvious from the fact that these latter kinases are activated normally in all of the knock-out cell lines. As demonstrated previously for Raf-1 (43–46) and recently in PTEN-deficient tumor cells (47), both pathways act synergistically in cell survival. For instance, a study in the interleukin-3-dependent cell line 32D demonstrated that active Akt and MEK are necessary for Raf-1 to promote cell survival (45). Additionally, She et al. (47) showed that activation of the proapoptotic regulator Bad requires the inhibition of both EGFR/MAPK and PI3K/Akt pathways, which act through the respective phosphorylations of specific serine 112 and 136 residues on Bad. Interestingly, in epidermal growth factor receptor-dependent tumors lacking the tumor suppressor PTEN, a combined inhibition of epidermal growth factor receptor and PI3K signaling causes synergistic apoptosis (47), which suggests that killing these cancerous cells would require dual-therapy aimed at inhibiting both pathways. Although more studies
are needed to fully elucidate the mechanisms at hand. Fig. 7 illustrates that the Grb10-Raf-1 interface appears to be a site that can be used to regulate both pathways.

The mechanisms used by Raf-1 to affect cell survival seem to vary according to the cell line and the apoptotic inducer under study. Most notably, some mechanisms require Raf-1 to act as a kinase that would phosphorylate an effector other than MEK1/

MEK2. For example, in 293 cells, the PAK kinase is thought to phosphorylate Raf-1 at serines 338/339 allowing it to relocate to the mitochondria where it can bind to Bcl-2 and directly phosphorylate Bad on serine 112 (12). In other cases, Raf-1 seems to act as an adaptor protein because binding to a ligand is required and a kinase-dead mutant is just as effective as the wild-type protein. Kinase-independent mechanisms include the inhibition of the pro-apoptotic kinases ASK-1 and MTS-2 or the mitochondrial VDAC channel (29, 48, 49). Even the expression of the non-phosphorylatable (and theoretically kinase-dead) Raf-1-YY/FF mutant is still capable of reverting the embryonic lethality observed in Raf-1−/− mice (50). It was later shown that the embryonic lethality in Raf-1 knockout mice is caused by the lack of inhibition of the pro-apoptotic kinase ASK1 (29). This duality in Raf-1 function was best illustrated in a study of vascular cells where protection against intrinsic apoptotic signals (starvation, DNA damage) by basic fibroblast growth factor is mediated through the phosphorylation of Raf-1 at serines 338–339 by PAK kinases followed by its mitochondrial localization. In contrast, protection of these same vascular cells against extrinsic apoptotic signals (mediated by death receptors) by vascular endothelial growth factor requires the same vascular cells against extrinsic apoptotic signals (mediated by death receptors) by vascular endothelial growth factor requires the phosphorylation of proapoptotic protein Bad and Raf-1 binding complex.

**Figure 7.** Schematic model of the regulation of anti-apoptotic signaling leading to Bad inactivation by Grb10/Raf-1 binding complex. The Grb10 and Raf-1 binding complex favors both the PI3K/Akt and MEK/MAPK signaling pathways leading to phosphorylation of proapoptotic protein Bad and cell survival. This pathway clearly includes some sort of feedback mechanisms because the PI3K/Akt pathway has been shown to positively modulate Grb10 phosphorylation and 14-3-3 binding. The sites of action of the kinase inhibitors used in this study are also indicated.
we have not been successful in detecting changes in its intracellular localization (data not shown). MEF cells appear to use the “Raf-1 as a kinase” model and structure-function analysis clearly demonstrate that Grb10 participates in the formation of a multiprotein complex that allows the Raf-1 kinase to phosphorylate ligands that ultimately affect how the MAPK and PI3K pathways phosphorylate Bad.

This interpretation is buttressed by the fact that most of the conserved Grb10 domains are necessary to permit its cell survival effects, suggesting that its localization and/or its association with several anti-apoptotic effectors are required in this process. In support of this proposal, a recent proteomic screen using FLAG-tagged 14-3-3 3-y protein has revealed that Raf-1 and Grb10 exist in a multiple-molecular complex with 14-3-3y. Binding of Raf-1 to 14-3-3-y is mediated through multiple sites on the Ras-binding and kinase domains of the kinase, whereas binding to Grb10 is dependent on the PH domain, the Ras associating region, and the SH2 domain (55). In addition, Urschel et al. (21) showed that the Grb10/14-3-3 interaction requires PI3K-dependent phosphorylation of Grb10 on serine 428 (21). When tested in the Bad cell death assay, Grb10 mutated on serine 428 failed to rescue the Grb10 1/− cells, suggesting a role for 14-3-3 in Grb10-mediated cell survival (Fig. 1, E and F). Furthermore, immunoprecipitations of Grb10 have confirmed that binding to 14-3-3 requires intact SH2, PH, and PR domains on Grb10 (data not shown). Direct binding of 14-3-3 to Akt and PDK-1 has also been shown to promote the binding of Bad to 14-3-3 (56, 57). These observations may also explain why neither Grb10 nor Raf-1 can protect cells against apoptosis in the presence of PI3K inhibitors (Fig. 4, A and B). Finally, the Nedd4 ubiquitin ligase has been demonstrated to interact with both Grb10 and Raf-1 (6, 58). In the light of these findings, we can envision that Raf-1/Nedd4/Grb10/Akt and 14-3-3y could be part of a molecular complex that is required to modulate Bad phosphorylation. Furthermore, the role of Grb10 seems to be limited to the creation of a subpopulation of kinases with a specific role in anti-apoptotic signaling because the total kinase activities of Raf-1, ERK, and Akt do not appear to be significantly impaired in the absence of this adapter protein.

In summary, the findings presented in this study identified a molecular complex that recruits the adaptor Grb10 and a subpopulation of the kinase Raf-1 that contributes to cell survival by phospho-inactivating the proapoptotic protein Bad in a MEK/ERK and PI3K/Akt-dependent manner. Therefore, disruption of this complex may contribute in elaborating effective therapeutic strategies against cellular mechanisms underlying tumorogenesis.

Acknowledgments—We thank Dr. C. Pritchard (University of Leicester, UK) and Dr. R. J. Daly (Garvan Institute of Medical Research, St Vincent’s Hospital, Sydney, Australia) for the kind gift of the Raf-1 and Grb14 MEF knock-out lines, respectively. We are also grateful to Dr. G. Shore (McGill Cancer Center, McGill University, Montreal, Canada) for the Bad construct, Anne Marcel (BRI, NRC, Montreal, Canada) for the HA antibody, Dr. S. Giorgetti-Peraldi (INSERM, Nice, France) for HA-tagged human Grb10 constructs, Sylvie Berardi for siRNA synthesis, and Lucie Bourget for valuable help. We also thank Dr. C. M. Udell for critical reading.

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