glomeruli with antibodies to IgG and the intense staining of tubular casts (Fig. 4D). Thus, the perturbed lymphocyte homeostasis produced by lack of Bim often culminates in a fatal systemic autoimmune disease.

The findings reported here indicate that, at least within the hematopoietic compartment, the BH3-only protein Bim is a key trigger of apoptosis and a critical physiological regulator of homeostasis. It influences T cell development and also may play a major role in terminating the normal immune response, because copious plasma cells accumulated in its absence, and renal failure often ensued due to a systemic autoimmune disease that has similarities to the human disorder systemic lupus erythematosus. Many of these effects, including the autoimmune disease (*19*), are highly reminiscent of those produced by overexpression of Bcl-2. These marked similarities argue that, at least for hematopoietic cells, Bim may well be the major physiological antagonist of the prosurvival proteins.

It might have been expected that a BH3-only protein would serve as the sentinel for damage to a single cellular component, but several disparate cytotoxic signals proved to be funneled through Bim. As well as microtubule perturbation (taxol) and Ca^{2+} flux, these included death upon cytokine deprivation. Apoptosis induced by growth factor withdrawal has been thought to proceed through Bad (*5*), but at least in B and T lymphocytes Bim must be the dominant transducer of this cytotoxic signal. In other responses that Bcl-2 can regulate, however, Bim apparently has a much smaller (or redundant) role, and those signals may well prove to be transduced mainly through other BH3-only proteins. In hepatocytes, but apparently not other cell types, Bid appears to play an important role in Fas-induced apoptosis (*9*), a response in which Bim appears to play no role. It thus appears likely that different BH3-only proteins are required to execute particular death responses in individual cell types.

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10. In the targeting vector (see Fig. 1A), prepared from 129SV/J DNA, the 110–base pair (bp) BH3-containing exon is replaced by the PGK*neo* expression cassette, flanked by loxP sites (*20*). Splicing of the remaining *bim* exons will produce a frameshift, which introduces a stop codon 12 bp downstream. Linearized targeting vector (30 mg) was electroporated into W9.5 ES cells. Hind III–digested DNA from 133 G418-resistant colonies was screened by Southern blotting with a 3-kb external probe (1.7 kb + 1.3 kb Bgl II fragments), for the 8-kb Hind III fragment. Two independent ES clones bearing a single targeted copy of the *bim* locus were microinjected into C57BL/6 blastocysts. Chimeric agouti-colored offspring were backcrossed to C57BL/6 mice or C57BL/6-Deleter mice (*11*).
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13. Some $bim^{-/-}$ males mated with $bim^{+/-}$ females routinely produced 50% $bim^{-/-}$ pups, but others sired hardly any $bim^{-/-}$ offspring. Because of the incomplete penetrance of the lethality, an inbred genetic background and large numbers of fetuses of different gestation ages will be required to identify the developmental defect imposed by Bim deficiency.
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22. Lysates precleared with an isotype-matched antibody (Ter119) were precipitated with rat anti-Bim monoclonal antibody 5E5 or 14A8 (*21*) and protein G–Sepharose. The washed immune complexes were fractionated electrophoretically on SDS–polyacrylamide gels and proteins transferred onto polyvinylidene difluoride membranes (Millipore). Bim protein was detected with 5E5 or 14A8, followed by a mouse anti-rat immunoglobulin reagent and ECL reaction (Amersham Pharmacia).
23. Peripheral blood erythrocytes and leukocytes were enumerated in a hemocytometer or with a ZM model Coulter counter and platelets were counted in a Sysmex NE8000 counter (TOA, Kobe, Japan). Suspensions of cells from thymus, spleen, lymph nodes, bone marrow, or blood were stained with cell type-specific monoclonal antibodies that had been purified on protein G–Sepharose and conjugated with fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), or biotin (Molecular Probes), the latter detected with PE-streptavidin (Caltag). Viable [excluding propidium iodide (PI)] $CD4^+8^+$ thymocytes and $B220^+$/surface (s)IgM^-/$sIgD^-$/$CD43^-$ pre-B cells were purified in a FACStar+ or a modified FACS II cell sorter (Becton-Dickinson).

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Differentiation Stage–Specific Inhibition of the Raf-MEK-ERK Pathway by Akt

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Extracellular signals often result in simultaneous activation of both the Raf-MEK-ERK and PI3K-Akt pathways (where ERK is extracellular-regulated kinase, MEK is mitogen-activated protein kinase or ERK kinase, and PI3K is phosphatidylinositol 3-kinase). However, these two signaling pathways were shown to exert opposing effects on muscle cell hypertrophy. Furthermore, the PI3K-Akt pathway was shown to inhibit the Raf-MEK-ERK pathway; this cross-regulation depended on the differentiation state of the cell: Akt activation inhibited the Raf-MEK-ERK pathway in differentiated myotubes, but not in their myoblast precursors. The stage-specific inhibitory action of Akt correlated with its stage-specific ability to form a complex with Raf, suggesting the existence of differentially expressed mediators of an inhibitory Akt-Raf complex.

The Raf-MEK-ERK and PI3K-Akt signaling pathways are often simultaneously activated in response to growth factors and hormones. In some systems, the small guanine nucleotide binding protein Ras acts as an upstream positive effector of both the Raf-MEK-ERK pathway and the PI3K-Akt pathway (*1, 2*). However, it has also been proposed that these two pathways

exert opposing effects. Manipulation of these pathways during muscle differentiation indicates that inhibition of the Ras-Raf-MEK-ERK pathway promotes differentiation, whereas inhibition of PI3K blocks differentiation (*3, 4*). However, the roles of these two pathways in the process of skeletal muscle hypertrophy has not previously been evaluated.

C2C12 myoblasts normally proliferate and are mononucleated (Fig. 1A). When deprived of serum at confluence, they fuse and differentiate into postmitotic, elongated, and multinucleated myotubes (Fig. 1B). The hypertrophic action of insulin-like growth factor–1 (IGF-1) on muscle cells in vivo is mimicked by the addition of IGF-1 during the differentiation of C2C12 myotubes in vitro, resulting in the generation of thicker myotubes (Fig. 1C). In addition to inducing hypertrophy of myotubes in vivo (*5*), IGF-1 has been shown to activate both the Raf-MEK-ERK pathway and the PI3K-Akt pathway (*6*).

The roles of these two pathways in the differentiation and hypertrophy of C2C12 myotubes were examined by genetic manipulation, which was accomplished by transfection of C2C12 cells with expression vectors encoding both the protein of interest and green fluorescent protein (GFP). This approach allowed the isolation of entire pools of transfected cells that express the test protein in sufficient amounts, with the use of a fluorescence-activated cell sorter (*7*). Expression of transgenes was confirmed by immunoblot analysis (Fig 1, E to G, inserts). Expression of GFP alone did not affect the differentiation of C2C12 cells (Fig. 1, B and D). Expression of a constitutively active form of Raf (c.a.-Raf) (*8*) resulted in the generation of smaller and thinner myotubes (Fig. 1E), whereas expression of a dominant negative form of Raf (d.n.-Raf) (*9*) resulted in markedly thicker myotubes (Fig. 1F). Thus, inhibition of the Raf-MEK-ERK pathway induced a hypertrophic phenotype similar to that elicited by IGF-1 treatment (Fig. 1, C and F). In contrast, activation of the Akt pathway by expression of a constitutively active form of Akt (c.a.-Akt) (*10, 11*) resulted in a hypertrophic phenotype more pronounced than that observed with d.n.-Raf and characterized by multinucleated myotubes that were both thickened and shortened (Fig. 1G). Thus, genetic manipulation of the Raf-MEK-ERK and PI3K-Akt pathways revealed opposing phenotypic effects of these pathways during muscle differentiation, with the Raf-MEK-ERK pathway inhibiting development of the hypertrophic phenotype and the PI3K-Akt pathway promoting it.

The similarity of the phenotypes induced by expression of d.n.-Raf and c.a.-Akt was confirmed by examining the abundance of mRNAs encoding myogenin and p21CIP, two markers of myoblast differentiation (*12*). Thus, whereas c.a.-Raf reduced the abundance of these RNAs, d.n.-Raf and c.a.-Akt each increased it (Fig. 2). Pharmacological manipulation of these two

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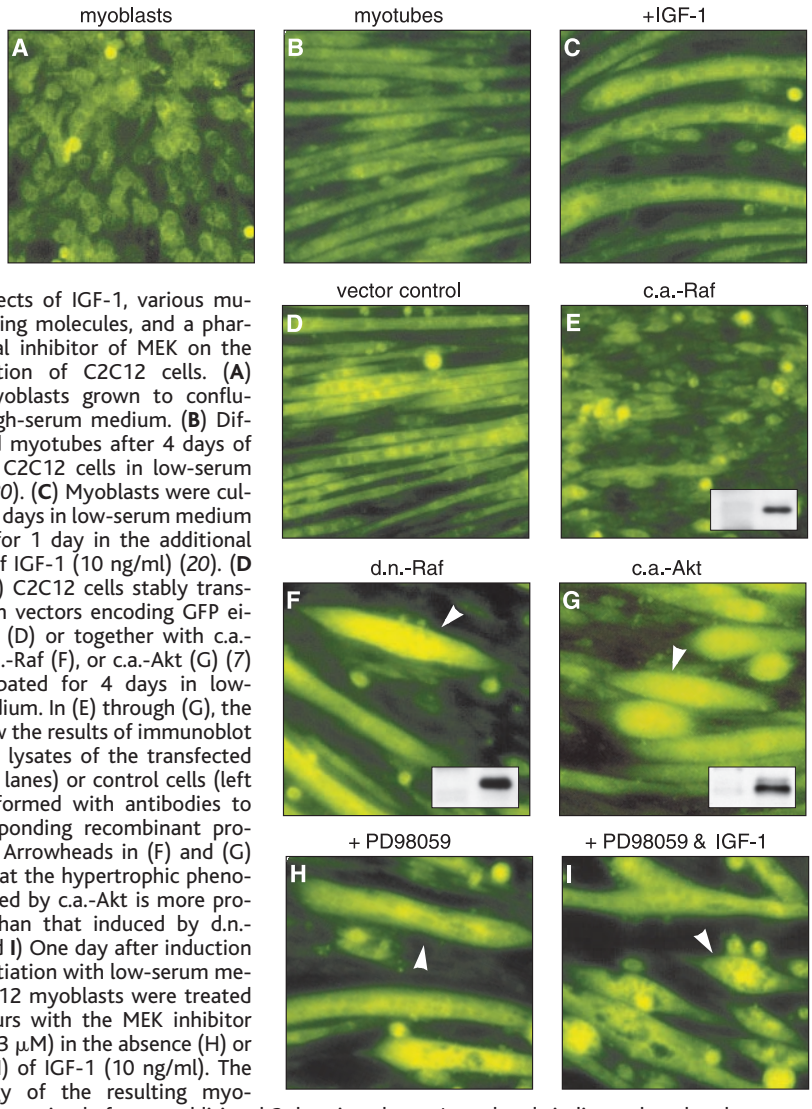

Fig. 1. Effects of IGF-1, various mutant signaling molecules, and a pharmacological inhibitor of MEK on the differentiation of C2C12 cells. (**A**) C2C12 myoblasts grown to confluence in high-serum medium. (**B**) Differentiated myotubes after 4 days of culture of C2C12 cells in low-serum medium (*20*). (**C**) Myoblasts were cultured for 3 days in low-serum medium and then for 1 day in the additional presence of IGF-1 (10 ng/ml) (*20*). (**D** through **G**) C2C12 cells stably transfected with vectors encoding GFP either alone (D) or together with c.a.-Raf (E), d.n.-Raf (F), or c.a.-Akt (G) (*7*) were incubated for 4 days in low-serum medium. In (E) through (G), the insets show the results of immunoblot analysis of lysates of the transfected cells (right lanes) or control cells (left lanes) performed with antibodies to the corresponding recombinant proteins (*21*). Arrowheads in (F) and (G) indicate that the hypertrophic phenotype induced by c.a.-Akt is more pronounced than that induced by d.n.-Raf. (**H** and **I**) One day after induction of differentiation with low-serum medium, C2C12 myoblasts were treated for 24 hours with the MEK inhibitor PD98059 (3 μM) in the absence (H) or presence (I) of IGF-1 (10 ng/ml). The morphology of the resulting myotubes was examined after an additional 2 days in culture. Arrowheads indicate that the phenotype induced by PD98059 (H) is similar to that induced by d.n.-Raf (F), and that the phenotype of cells treated with PD98059 and IGF-1 (I) is similar to that of cells expressing c.a.-Akt (G) [200× magnification].

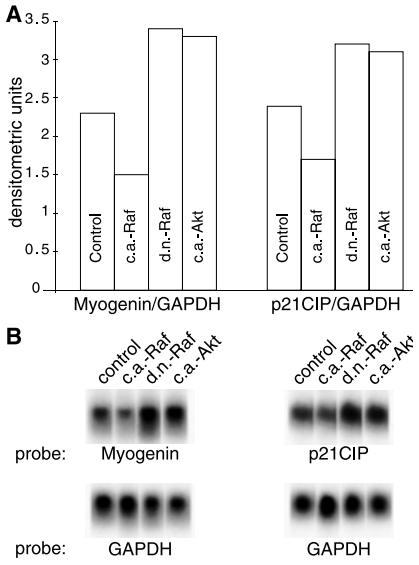

Fig. 2. Effects of transgenes on the abundance of mRNAs encoding myogenin and the cyclin-dependent kinase inhibitor p21. At confluence, total RNA was isolated from C2C12 cells transfected with the control vector or with vectors encoding c.a.-Raf, d.n.-Raf, or c.a.-Akt. The RNA was then subjected to Northern blot analysis with probes specific for myogenin, p21CIP, or glyceraldehyde phosphate dehydrogenase (GAPDH) mRNAs (*22*). Quantitative and original data are shown in (**A**) and (**B**), respectively.

pathways yielded results that were consistent with those of genetic manipulation. Treatment of C2C12 myotubes with PD98059, a pharmacological inhibitor of MEK (*13*), reproduced the hypertrophic phenotype induced by d.n.-Raf (Fig. 1H). Exposure of cells to both this inhibitor and IGF-1 resulted in the generation of many thickened and shortened multinucleated myotubes (Fig. 1I), similar to those produced by cells expressing c.a.-Akt. Therefore, although IGF-1 simultaneously activates both the Raf-MEK-ERK and PI3K-Akt pathways, these two pathways exert opposing effects on myotube hypertrophy.

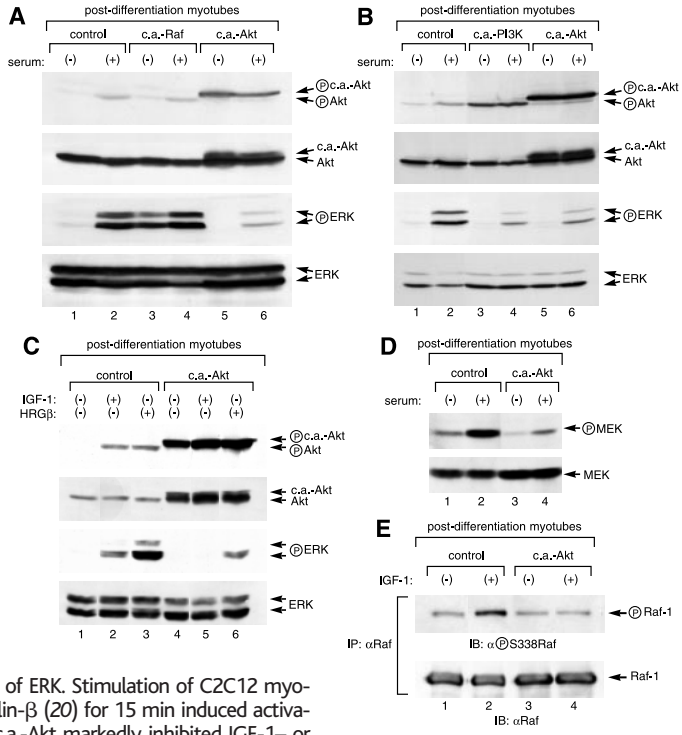

Fig. 3. (**A**) Effects of c.a.-Raf and c.a.-Akt on phosphorylation of ERK. Immunoblot analysis revealed that stimulation of C2C12 myotubes with serum increased the phosphorylation of ERK1 and ERK2 (control). Expression of c.a.-Raf also increased the phosphorylation of ERK in the absence of serum stimulation; ERK phosphorylation was further increased by addition of serum. Expression of c.a.-Akt markedly inhibited serum-induced activation of ERK (*23*). (**B**) Activation of endogenous Akt and inhibition of serum-induced activation of ERK by expression of c.a.-PI3K in C2C12 myotubes. (**C**) Inhibition by c.a.-Akt of IGF-1– or heregulin-β (HRGβ)–induced activation of ERK. Stimulation of C2C12 myotubes with IGF-1 or heregulin-β (*20*) for 15 min induced activation of ERK. Expression of c.a.-Akt markedly inhibited IGF-1– or heregulin-β–induced phosphorylation of ERK. (**D**) Effect of c.a.-Akt on activation of MEK. Stimulation of C2C12 myotubes with serum for 15 min resulted in activation of MEK. Expression of c.a.-Akt inhibited serum-induced phosphorylation of MEK. (**E**) Inhibition by c.a.-Akt of IGF-1–induced phosphorylation of Raf-1 on Ser^{338} (*23*). Raf-1 was immunoprecipitated (IP) from cell lysates and subjected to immunoblot analysis (IB) first with mAb specific for Raf-1 phosphorylated on Ser^{338} (upper panel) and then with Raf-1 mAb (lower panel).

To determine whether inhibition of the ERK pathway by Akt occurred at all stages of muscle differentiation, we compared the effect of activated Akt in differentiated myotubes with that in precursor myoblasts (Fig. 4). Whereas serum-induced activation of ERK was almost completely inhibited by c.a.-Akt in differentiated C2C12 myotubes, no such inhibition of ERK activation was apparent in myoblasts, despite similar levels of expression of c.a.-Akt in the two cell types. Thus, the negative regulatory effect of Akt on the ERK pathway is specific to differentiated muscle cells.

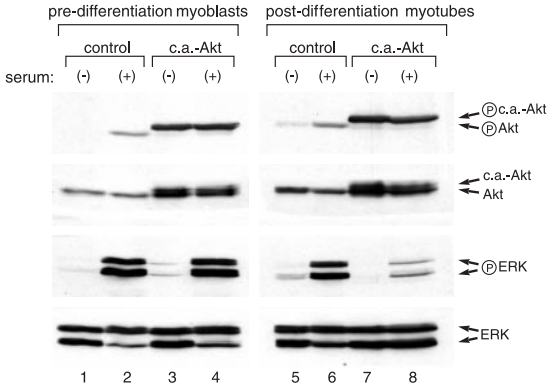

Fig. 4. Differentiation stage specificity of Akt-induced inhibition of ERK activity. Inhibition of serum-induced ERK activation by Akt was examined by immunoblot analysis of subconfluent myoblasts cultured in high-serum growth medium (pre-differentiation myoblasts) or of myotubes 4 days after induction of differentiation (post-differentiation myotubes) (*23*). Expression of c.a.-Akt markedly inhibited serum-induced activation of ERK in differentiated C2C12 cells, but it had no such effect in undifferentiated myoblasts.

To determine at what level of the Raf-MEK-ERK pathway the inhibition by Akt is mediat-

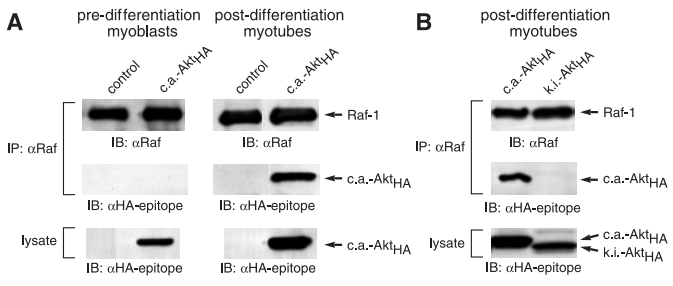

Fig. 5. (**A**) Differentiation stage specificity of the association of Akt with Raf. Cell lysates were prepared from C2C12 myoblasts (left panel) or from myotubes 4 days after induction of differentiation (right panel). Both control myoblasts or myotubes and those expressing c.a.-Akt were analyzed. Raf-1 was immunoprecipitated with a specific mAb, and was subjected to immunoblot analysis with the same antibody as well as with an antibody to HA in order to detect coimmunoprecipitated HA-tagged c.a.-Akt. The extent of expression of c.a.-Akt was also examined by immunoblot analysis of cell lysates with the antibody to HA (*21*, *23*). (**B**) Failure of k.i.-Akt to bind to Raf-1. Raf-1 was immunoprecipitated (*21*) from C2C12 myotubes expressing either c.a.-Akt or k.i.-Akt (*7*, *10*, *11*). The immunoprecipitates were then subjected to immunoblot analysis first with the antibody to HA and then with Raf-1 mAb. Cell lysates were also subjected to immunoblot analysis with the antibody to HA.

ed, we examined the phosphorylation of Raf and MEK, the kinases directly upstream of ERK. Activated Akt inhibited the phosphorylation both of Raf on Ser^{338}, which is required for Raf activation (*16*), and of MEK (Fig. 3, D and E), indicating that the Akt-induced inhibition of the Raf-MEK-ERK pathway is mediated at the level of (or upstream of) Raf.

The mechanism of the stage-specific inhibition of the Raf-MEK-ERK pathway by Akt was investigated by examining the possible formation of a Raf-Akt complex. Whereas the abundance of endogenous Raf was similar in myoblasts and myotubes, c.a.-Akt was coimmunoprecipitated with Raf from differentiated myotubes but not from myoblasts (Fig 5A). A kinase-inactive form of Akt (k.i.-Akt) did not associate with Raf in differentiated myotubes (Fig. 5B), consistent with the notion that either activation or membrane localization of Akt is required for its association with Raf in myotubes.

Zimmermann *et al*., in an accompanying report, show that Akt can phosphorylate Raf in vitro (*17*). However, the cross-regulatory mechanism that we have identified cannot simply be explained by the binding of Akt to Raf followed by Akt phosphorylation of Raf, given that both binding and cross-regulation occur in myotubes but not in myoblasts. Thus, Akt and Raf do not obligately interact. Regulation of the Raf-Akt interaction might be mediated by stage-specific modification of these proteins or by stage-specific accessory proteins.

Signaling molecules are able to induce different phenotypes when expressed in different cell types (*2, 18*). These pleiotropic effects are explained as being dependent on "cellular context," meaning that common signaling mechanisms are at some point interpreted differently by different cell types. It is possible that the cross-regulation between the PI3K-Akt and Raf-MEK-ERK pathways may be important in other cell lineages, in which such crosstalk may similarly depend on differentiation stage. Given that muscle undergoes atrophy in a variety of disease states, the ability to promote muscle hypertrophy would have important clinical implications. Understanding the mechanisms by which the Raf-MEK-ERK and PI3K-Akt pathways regulate muscle hypertrophy may thus contribute to the development of agents that could tip the balance away from atrophy in such disease states.

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21. Immunoprecipitation of the Flag-tagged Raf proteins was performed with antibodies to the Flag epitope (Sigma). HA-tagged Akt proteins were immunoprecipitated with antibodies to the HA tag (Boehringer). Cell lysate preparation, immunoprecipitation, and immunoblot analysis were performed as described (*19*). Endogenous Raf proteins were immunoprecipitated with a monoclonal antibody (mAb) to Raf-1 (Transduction Laboratories).
22. Total RNA was prepared from C2C12 myotubes with the use of a TRI REAGENT kit (Molecular Research Center), and 10 μg were subjected to Northern blot analysis with ^{32}P-labeled DNA probes for myogenin and p21CIP transcripts. Probes were prepared with a random-priming kit (Prime-It II, Stratagene). Equal loading of RNA was confirmed by reprobing the blots with ^{32}P-labeled GAPDH DNA. Blots were subjected to quantitative analysis with a Fujix BAS2000 imaging system (Fuji Medical Systems).
23. Antibodies to ERK1 or ERK2 phosphorylated on Thr^{202} and Tyr^{204}, to Akt phosphorylated on Ser^{473}, or to MEK1 or MEK2 phosphorylated on Ser^{217} and Ser^{221} (NEB) were used to detect exclusively the catalytically activated forms of the kinases. Antibodies to ERK1 or ERK2 (UBI), to Akt (NEB), or to MEK1 or MEK2 (NEB) were used to detect corresponding protein expression levels. Cell lysates were prepared as described (*21*). Endogenous Raf-1 proteins immunoprecipitated with the Raf-1 mAb were subjected to immunoblot analysis with a mAb specific for Raf-1 phosphorylated on Ser^{338}, which was provided by R. Marais (*16*).

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Phosphorylation and Regulation of Raf by Akt (Protein Kinase B)

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Activation of the protein kinase Raf can lead to opposing cellular responses such as proliferation, growth arrest, apoptosis, or differentiation. Akt (protein kinase B), a member of a different signaling pathway that also regulates these responses, interacted with Raf and phosphorylated this protein at a highly conserved serine residue in its regulatory domain in vivo. This phosphorylation of Raf by Akt inhibited activation of the Raf-MEK-ERK signaling pathway and shifted the cellular response in a human breast cancer cell line from cell cycle arrest to proliferation. These observations provide a molecular basis for cross talk between two signaling pathways at the level of Raf and Akt.

The signaling pathway comprising Raf, MEK (mitogen-activated protein kinase, or ERK kinase), and ERK (extracellular signal–regulated kinase) lies downstream of the small guanine nucleotide binding protein Ras and mediates several apparently conflicting cellular responses, such as proliferation, apoptosis, growth arrest, differentiation, and senescence, depending on the duration and strength of the external stimulus and on cell type. Another pathway that lies downstream of Ras includes phosphatidylinositol (PI) 3-kinase and Akt (or protein kinase