

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<sup>338</sup>, 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 co-immunoprecipitated 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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7. Complementary DNAs encoding Flag epitope-tagged d.n.-Raf or c.a.-Raf (containing amino acids 1 to 331 and 331 to 648 of human Raf-1, respectively) (19), hemagglutinin epitope (HA)-tagged c.a.-Akt [U. Franke *et al.*, *Cell* **81**, 727 (1995)] or k.i.-Akt (17), or c.a.-PI3K (15) were subcloned into a bicistronic expression vector consisting of the MCK promoter [J. B. Jaynes, J. E. Johnson, J. N. Buskin, C. L. Gartside, S. D. Haushcka, *Mol. Cell. Biol.* **8**, 62 (1988)] and an IRES-EGFP cassette (Clontech). Subconfluent C2C12 myoblasts were transfected with the use of calcium phosphate (Specialty Media Inc.) as described [D. J. Glass *et al.*, *Cell* **85**, 513 (1996)]. Flow cytometry and cell sorting were performed with a Cytomation MoFlo (Fort Collins, CO) high-speed cell sorter. Laser excitation at 488 nm was performed at a power of 130 mW. Fluorescence emission from GFP was measured with a 530/540-nm band-pass filter. For sorting, cells were collected at a sort rate of 25,000 cells per second.
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20. C2C12 myoblasts were grown at 37°C in high-serum growth medium [Dulbecco's modified Eagle's medium (DMEM) and F12 (3:1, v/v; Specialty Media),

supplemented with 10% fetal bovine serum, insulin-transferrin-selenite (CBR), 4 mM glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml)] under an atmosphere of 10% CO<sub>2</sub>. Myoblasts were switched from high-serum growth medium to low-serum differentiation medium (DMEM, 2% horse serum, and 4 mM glutamine) and 7.5% CO<sub>2</sub> to induce myogenic differentiation. Some myoblasts were also exposed to IGF-1, R3-IGF-1 (10 ng/ml, Sigma), or heregulin-β (5 ng/ml; HRGβ1, R&D Systems).

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 <sup>32</sup>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 <sup>32</sup>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<sup>202</sup> and Tyr<sup>204</sup>, to Akt phosphorylated on Ser<sup>473</sup>, or to MEK1 or MEK2 phosphorylated on Ser<sup>217</sup> and Ser<sup>221</sup> (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<sup>338</sup>, which was provided by R. Marais (16).
24. We thank L. S. Schleifer, P. R. Vagelos, and the remainder of the Regeneron community, particularly S. Bodine and T. Stitt, for support; P. Burfeind for assistance with cell sorting; S. Wang, J. Sikorski, and E. Burova for general technical assistance; E. Burrows and C. Murphy for graphics; A. Bellacosa, P. Tsichlis, D. S. Hauschka, and R. Marais for providing reagents and advice; and E. Hafen for sharing results prior to publication.

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

Sven Zimmermann and Karin Moelling\*

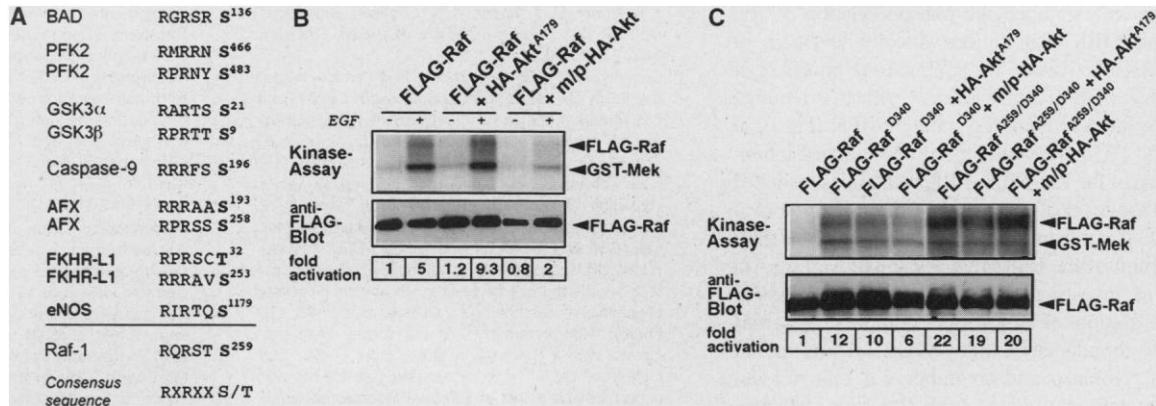
Activation of the protein kinase Raf can lead to opposing cellular responses such as proliferation, growth arrest, apoptosis, or differentiation. Akt (protein kinase B), a member of a different signaling pathway that also regulates these responses, interacted with Raf and phosphorylated this protein 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

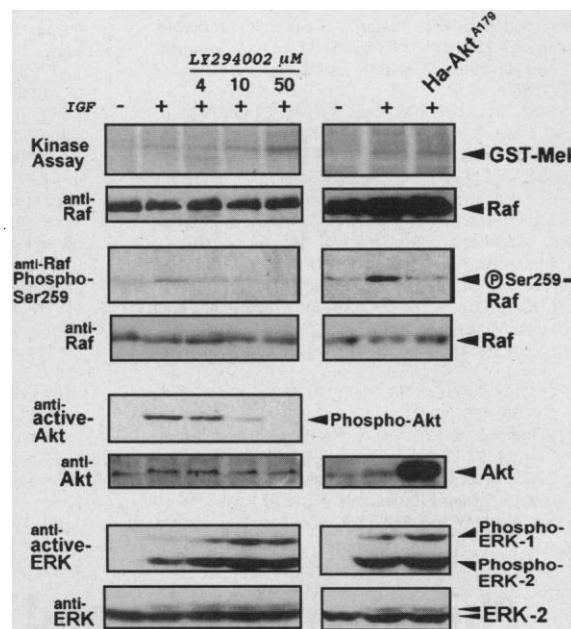
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**Fig. 1.** Inhibition of Raf activation by Akt. (A) Comparison of the putative Akt phosphorylation site in the amino-terminal region of Raf with the sequences of phosphorylation sites of known Akt substrates (18, 19). The phosphorylated residues are shown in bold and a consensus sequence is denoted below. (B and C) Flag-Raf, the activated mutant Flag-Raf<sup>D340</sup>, or the double mutant Flag-Raf<sup>A259/D340</sup> was coexpressed with a constitutively active Akt (m/p-HA-Akt) or a kinase-inactive Akt (HA-Akt<sup>A179</sup>) in HEK293 cells. In (B), the cells were stimulated with EGF (10 ng/ml) for 5 min, and kinase activity of Raf toward a glutathione S-transferase fusion protein of kinase-inactive MEK (GST-MEK) was

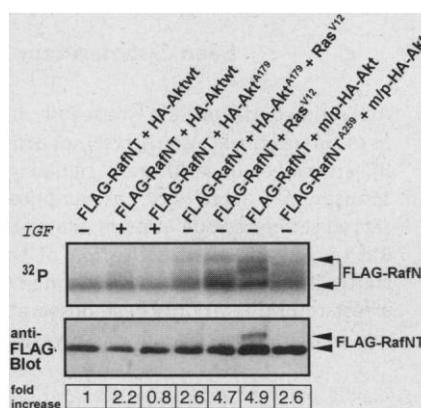


assayed. The radioactivity incorporated into GST-MEK was quantitated by image analysis (Image-Quant) and is shown as fold activation relative to the basal activity of Flag-Raf. Immunocomplexes were also subjected to immunoblot analysis with antibodies to the Flag epitope.

**Fig. 2.** Effects of inhibition of Akt on phosphorylation of Raf and on Raf and ERK activities. HEK293 cells were deprived of serum, incubated for 20 min with the indicated concentrations of LY294002, and then stimulated with IGF (50 ng/ml) for 4 min. Cells expressing kinase-inactive Akt (HA-Akt<sup>A179</sup>) were similarly treated with IGF. Endogenous Raf protein was immunoprecipitated and in vitro kinase assays were performed as in Fig. 1B. Cell lysates were also subjected to immunoblot analysis with antibodies specific for Raf phosphorylated on Ser259, for activated Akt, or for activated ERK, or with antibodies to the corresponding unmodified proteins (20).



**Fig. 3.** Akt-induced phosphorylation of Raf on Ser259 in vivo. HEK293 cells were transfected with the indicated constructs, deprived of serum, and metabolically labeled with [<sup>32</sup>P]-orthophosphate (0.25 mCi/ml) as described (17). They were then stimulated with IGF (100 ng/ml) for 15 min, after which Flag-RafNT proteins were immunoprecipitated, resolved by SDS-polyacrylamide gel electrophoresis, and subjected to image analysis for detection of radioactivity and to immunoblot analysis with antibodies to Flag. The incorporation of <sup>32</sup>P was normalized for protein content and is shown as fold increase compared with the basal phosphorylation of Flag-RafNT. Aktwt, wild-type Akt.



The kinase activity of Raf (5) is regulated by phosphorylation of a highly conserved serine residue (Ser<sup>259</sup>) in the amino-terminal regulatory domain (RafNT) (6). Phosphorylation of Ser<sup>259</sup> mediates binding of the 14-3-3 protein, resulting in Raf inactivation. Mutation of Ser<sup>259</sup> to Ala constitutively activates the kinase activity of Raf (7). The kinases responsible for phosphorylating Ser<sup>259</sup> are unknown but may include members of the protein kinase C family (8).

Because the region surrounding Ser<sup>259</sup> in Raf conforms to a consensus sequence for phosphorylation by the serine-threonine kinase Akt (Fig. 1A) (9, 10), we analyzed the biochemical effect and biological function of Akt-mediated phosphorylation of Raf. Stimulation of human embryonic kidney (HEK293) cells expressing Flag epitope-tagged Raf (7) with epidermal growth factor (EGF) resulted in an increase in the kinase activity of Raf (Fig. 1B). Coexpression of a hemagglutinin epitope (HA)-tagged kinase-inactive mutant of Akt, HA-Akt<sup>A179</sup> (11), enhanced the EGF-induced increase in Raf activity. However, coexpression of an activated Akt mutant that is constitutively targeted to the plasma membrane (m/p-HA-Akt) (11) markedly inhibited the EGF-induced increase in Raf activity (Fig. 1B). A constitutively activated Raf mutant containing the Ser<sup>259</sup>→Ala mutation (7) was not regulated by these Akt proteins (12), suggesting that Akt induces phosphorylation of Raf on Ser<sup>259</sup>. In place of an exogenous stimulus, we expressed another constitutively active mutant of Raf, in which Tyr<sup>340</sup> is mutated to Asp (13), in HEK293 cells. The kinase activity of this mutant Raf was also down-regulated by Akt-dependent phosphorylation of Ser<sup>259</sup> (Fig. 1C).

B) and also regulates these cellular responses, acting either synergistically with (1) or in opposition to (2) the Raf pathway. Coordination

of the two pathways in a single cellular response may depend on cell type or the stage of differentiation (3, 4).

Institute of Medical Virology, University of Zurich, Gloriastrasse 30/32, CH-8028 Zurich, Switzerland.

\*To whom correspondence should be addressed. E-mail: moelling@immv.unizh.ch

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Endogenous Raf in HEK293 cells was also phosphorylated on Ser<sup>259</sup> after stimulation of cells with insulin-like growth factor (IGF). The PI 3-kinase inhibitor LY294002 inhibited IGF-induced phosphorylation of Raf on Ser<sup>259</sup> and increased the extent of activation of Raf and ERK (Fig. 2). The effects of LY294002 were mimicked by expression of the kinase-inactive Akt mutant (HA-Akt<sup>A179</sup>) in HEK293 cells.

In vivo <sup>32</sup>P-labeling experiments revealed that activation of Akt by an oncogenic form of Ras (Ras<sup>V12</sup>) or by IGF resulted in RafNT phosphorylation (4.7- and 2.2-fold increases, respectively) (Fig. 3). However, coexpression of the kinase-inactive Akt mutant inhibited the increase in RafNT phosphorylation in response to either Ras<sup>V12</sup> or IGF. Phosphorylation induced a decrease in the electrophoretic mobility of RafNT. Almost all of the <sup>32</sup>P-labeled phosphate was incorporated into Raf at Ser<sup>259</sup>, as revealed by the observation that the RafNT<sup>A259</sup> mutant was phosphorylated to a much lesser extent than was RafNT (Fig. 3).

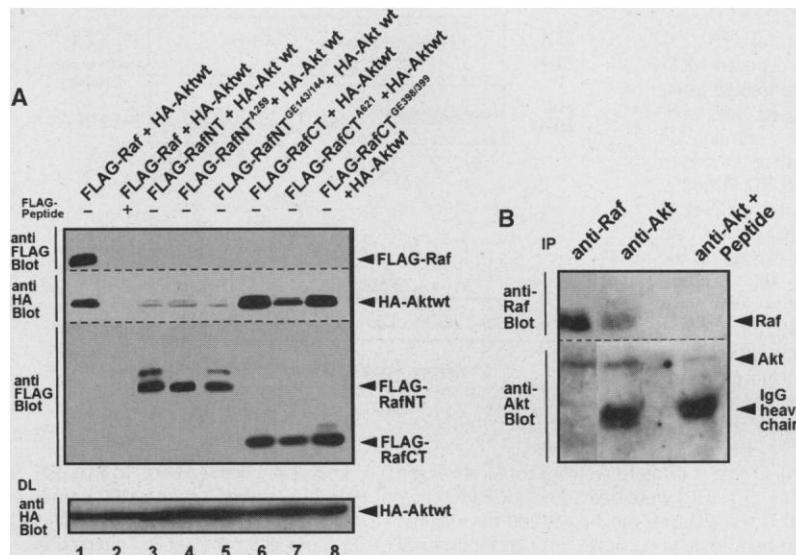
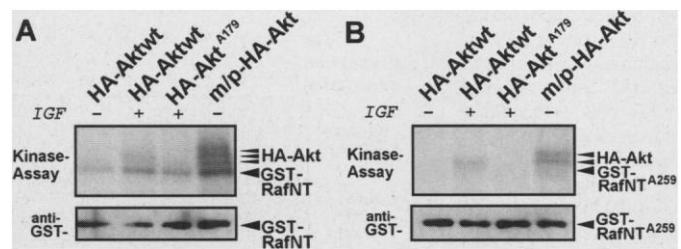
When expressed as a GST fusion protein, RafNT was directly phosphorylated by Akt on Ser<sup>259</sup> in vitro (Fig. 4A); the GST-RafNT<sup>A259</sup> mutant was not phosphorylated by Akt under these conditions (Fig. 4B). The Akt substrate BAD (14) was included in these experiments as a positive control (12). The carboxyl-terminal domain of Raf (RafCT) fused to GST was also not phosphorylated by Akt (12).

Akt and Raf coimmunoprecipitated from HEK293 cells overexpressing these proteins (Fig. 5A). Akt associated with both RafNT and RafCT. Two Raf mutants that do not bind 14-3-3 protein, RafNT<sup>A259</sup> and RafCT<sup>A621</sup> (7), also co-immunoprecipitated with Akt, indicating that 14-3-3 protein does not serve as an adapter between Raf and Akt. In addition, Raf proteins in which residues that constitute putative lipid binding sites are mutated (15), RafNT<sup>G143/E144</sup> and RafCT<sup>G398/E399</sup>, also associated with Akt (Fig. 5A).

Exposure of the MCF-7 breast cancer cell line to IGF induces cell proliferation as a result of activation of the PI 3-kinase–Akt pathway and transient activation of the Raf-MEK-ERK pathway (3). However, prolonged activation of the Raf cascade inhibits growth in these cells (3). Thus, regulation of the Raf-MEK-ERK pathway in MCF-7 cells determines whether the response is proliferation or growth arrest. Endogenous Akt and endogenous Raf co-immunoprecipitated from MCF-7 cells that had been stimulated with IGF for 5 min (Fig. 5B). This interaction was disrupted with a peptide that competed with Akt for the immunoprecipitating anti-Akt antibodies (Fig. 5B). Serum deprivation of the cells or stimulation with IGF for 5 min or 2 hours did not alter the amounts of the co-immunoprecipitating proteins (12).

On exposure of MCF-7 cells to IGF, both

**Fig. 4.** Phosphorylation of Raf on Ser<sup>259</sup> by Akt in vitro. HEK293 cells expressing the indicated HA-Akt proteins were deprived of serum, stimulated for 20 min with IGF (100 ng/ml), lysed, and subjected to immunoprecipitation with antibodies to HA. The resulting precipitates were then subjected to an in vitro kinase assay with GST-RafNT (A) or GST-RafNT<sup>A259</sup> (B) as substrate. Autoradiograms are shown in the upper panels, and immunoblot analysis with antibodies to GST is shown in the lower panels (21). The amounts of Akt in the different reaction mixtures were similar (12).



**Fig. 5.** Association of Akt and Raf. (A) Lysates prepared from HEK293 cells transfected with the indicated constructs were subjected to immunoprecipitation with antibodies to Flag with or without an anti-Flag antibody competing peptide (FLAG peptide), and the resulting precipitates were subjected to immunoblot analysis with antibodies to Flag or to HA. The amount of HA-Akt in direct lysates (DL) was also assessed by immunoblot analysis with antibodies to HA (lower panel) (22). (B) Serum-deprived MCF-7 cells were stimulated with IGF (100 ng/ml) for 5 min, after which endogenous Raf and Akt were immunoprecipitated (IP) with their cognate antibodies in the absence or presence of the immunizing peptide for the antibodies to Akt (22). The resulting immunoprecipitates were then subjected to immunoblot analysis with the same antibodies. IgG, immunoglobulin G.

Raf and ERK activities peaked after 2 to 3 min and then decreased (Fig. 6A). In contrast, Akt was activated rapidly and remained fully active for up to 20 min. Inhibition of Akt activation by LY294002 increased Raf and ERK activities (Fig. 6A).

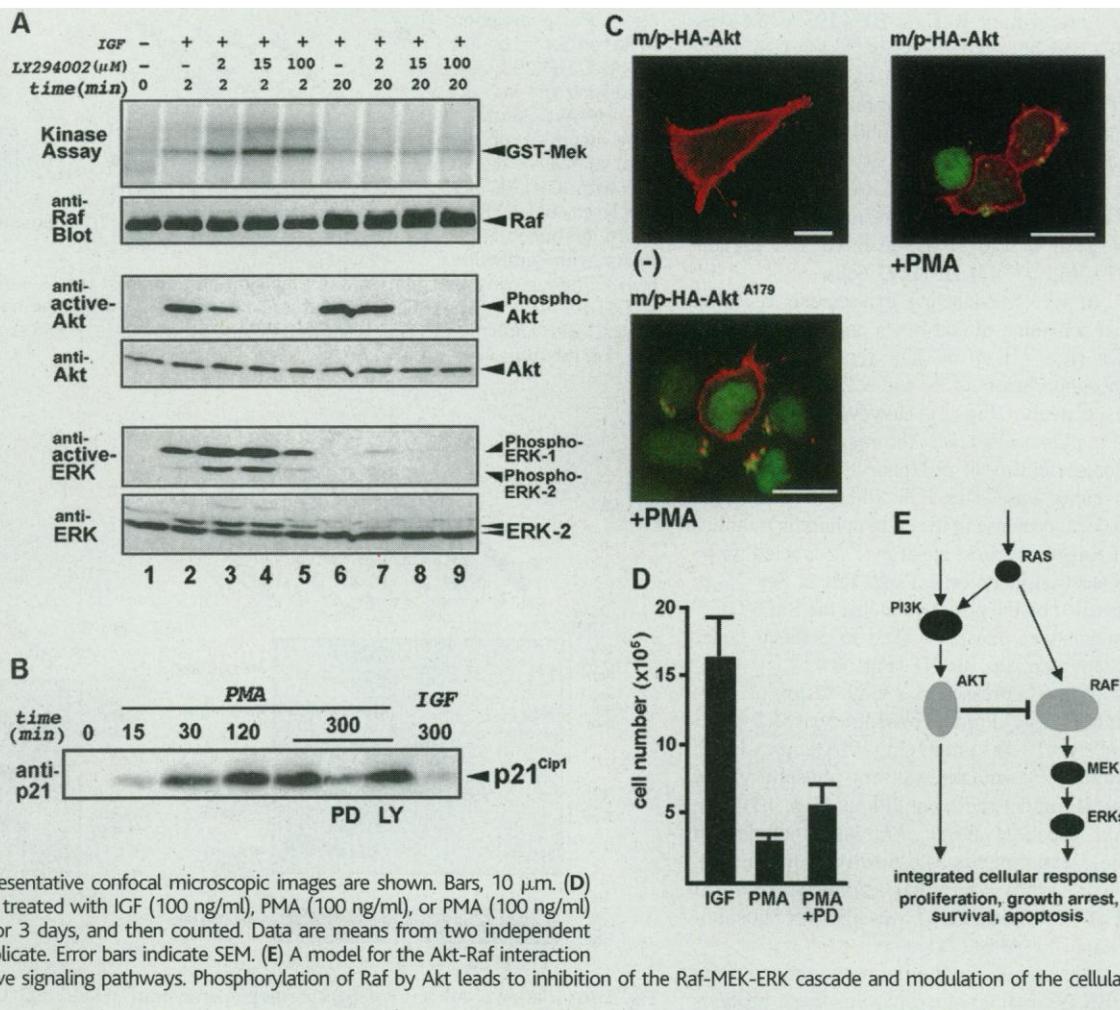
Tumor promoters such as phorbol 12-myristate 13-acetate (PMA) induce sustained activation of ERK as well as the expression of the cyclin-dependent kinase inhibitor p21<sup>Cip1</sup> in MCF-7 cells (3). The MEK inhibitor PD98059 partially inhibited the PMA-induced increase in the amount of p21<sup>Cip1</sup>, whereas LY294002 had no such effect, implicating the Raf-MEK-ERK pathway in regulation of p21<sup>Cip1</sup> expression (Fig. 6B). Expression of membrane-targeted, constitutively active Akt (m/p-HA-Akt) in MCF-7 cells prevented PMA-induced expression of p21<sup>Cip1</sup> in the nucleus; expression of membrane-targeted, kinase-inactive Akt (m/p-HA-Akt<sup>A179</sup>) had no such inhibitory effect (Fig.

6C). The growth-inhibitory effect of PMA on MCF-7 cells was also partially reversed by the MEK inhibitor PD98059 (Fig. 6D) (3).

Our results demonstrate that Akt antagonizes Raf activity by direct phosphorylation of Ser<sup>259</sup>. This modification creates a binding site for 14-3-3 protein, a negative regulator of Raf. Similarly, phosphorylation of BAD or the forkhead transcription factor FKHL1 by Akt also promotes binding of 14-3-3 protein (14, 16). In all three instances, phosphorylation by Akt inactivates the function of its substrate. Cross talk between the Raf-MEK-ERK and the PI 3-kinase–Akt pathways, mediated by direct interaction of Akt with and its phosphorylation of Raf, may switch the biological response from growth arrest to proliferation, as shown for MCF-7 cells, and may also modulate senescence or differentiation as shown for myoblast differentiation (25), depending on the cellular system (Fig. 6E).

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**Fig. 6.** Activation of Raf and ERK promoted by inhibition of PI 3-kinase and Akt. **(A)** Serum-deprived MCF-7 cells were treated with LY294002 and then exposed to IGF (100 ng/ml) or vehicle (dimethyl sulfoxide) for the indicated times. Lysates were subjected to in vitro kinase assays of Raf activity as in Fig. 1 or to immunoblot analysis with antibodies specific for activated ERK or Akt; the blot was stripped and reprobbed with antibodies to the unmodified proteins (20, 23). **(B)** Serum-deprived MCF-7 cells were treated as indicated, lysed, and subjected to indirect double-immunofluorescence analysis to reveal Akt-expressing cells (red) and p21<sup>Cip1</sup> expression (green) (24). Overlays of representative confocal microscopic images are shown. Bars, 10  $\mu$ m. **(D)** MCF-7 cells ( $1.5 \times 10^5$ ) were treated with IGF (100 ng/ml), PMA (100 ng/ml), or PMA (100 ng/ml) plus PD98059 (PD, 20  $\mu$ M) for 3 days, and then counted. Data are means from two independent experiments performed in duplicate. Error bars indicate SEM. **(E)** A model for the Akt-Raf interaction in the context of the respective signaling pathways. Phosphorylation of Raf by Akt leads to inhibition of the Raf-MEK-ERK cascade and modulation of the cellular response.



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18. A. Khwaja, *Nature* **401**, 33 (1999). Abbreviations are PFK2, 6-phosphofructo-2-kinase; GSK3, glycogen synthase kinase 3; FKHL1, Forkhead transcription factor L1; and eNOS, endothelial nitric oxide synthase.
19. Single-letter abbreviations for amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, any amino acid; Y, Tyr.
20. Untransfected HEK293 cells or cells transfected with a vector encoding HA-Akt<sup>A179</sup> were deprived of serum for 20 hours, stimulated with IGF (50 ng/ml) for 4 min, and lysed in radioimmunoprecipitation assay (RIPA) buffer as described (17). Lysate samples were subjected to immunoblot analysis with antibodies specific for Raf phosphorylated on Ser<sup>259</sup> (Biosource International), for activated Akt, or for activated ERK (New England Biolabs). After detection by enhanced chemiluminescence (Amersham Pharmacia Biotech), membranes were stripped and reprobbed with antibodies to Raf (R19120, Transduction Laboratories), or to Akt or ERK (C20 and C14, Santa Cruz Biotechnology). Kinase activity of Raf was assayed as described (17).
21. HEK293 cell transfection and lysis as well as assay of the kinase activity of Akt were performed as described (11, 17).
22. HEK293 cells were lysed 36 hours after transfection and subjected to immunoprecipitation as described (7, 17). For examination of the endogenous Raf-Akt interaction, MCF-7 cells were deprived of serum for 24 hours in phenol red-free medium. After stimulation with IGF (100 ng/ml, 5 min), cells were harvested at 4°C and lysed in NP-40 buffer (7, 17). Cleared lysates were subjected to immunoprecipitation with antibodies to Raf (R19120) or to Akt (C20), and the resulting precipitates were subjected to immunoblot analysis. Immu-

noprecipitation from cell-free lysis buffer did not reveal any nonspecific bands (12).

23. MCF-7 cells were deprived of serum for 24 hours and treated with LY294002 (Calbiochem) for 20 min. After stimulation with IGF (100 ng/ml), the cells were lysed in RIPA buffer, and Raf was immunoprecipitated with 1  $\mu$ g of specific antibodies (R19120) for 3 hours at 4°C, the final 1 hour of which was in the presence of 10  $\mu$ l of protein G-Sepharose (Amersham Pharmacia Biotech). The resulting precipitates were subjected to kinase assays (17).
24. Cells were stimulated for the indicated times, extensively washed, and lysed 5 hours after stimulation. Samples were subjected to immunoblot analysis with antibodies to p21<sup>Cip1</sup> (F5, Santa Cruz Biotechnology). For immunofluorescence studies, MCF-7 cells were cultured on glass cover slips and transfected with HA-Akt expression plasmids. After 24 hours, the cells were deprived of serum for 18 hours and treated with PMA (100 ng/ml) (Calbiochem) or vehicle (dimethyl sulfoxide) for an additional 4 hours. Proteins were visualized with goat antibodies to Akt (C20) and mouse antibodies to p21<sup>Cip1</sup> (F5) and subsequent incubation with rhodamine-conjugated antibodies to goat immunoglobulin or fluorescein-conjugated antibodies to mouse immunoglobulin, respectively (DAKO). Cells were examined under a confocal laser microscope (Bio-Rad/Zeiss-Axioplan) equipped with a Zeiss Neofluar 63 $\times$  objective.
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### <sup>2</sup> **Inhibition of Adipogenesis Through MAP Kinase-Mediated Phosphorylation of PPAR#**

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### <sup>4</sup> **Myogenic Signaling of Phosphatidylinositol 3-Kinase Requires the Serine-Threonine Kinase Akt/Protein Kinase B**

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### <sup>14</sup> **Interleukin-3-Induced Phosphorylation of BAD Through the Protein Kinase Akt**

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### <sup>25</sup> **Differentiation Stage-Specific Inhibition of the Raf-MEK-ERK Pathway by Akt**

Christian Rommel; Brian A. Clarke; Sven Zimmermann; Lorna Nuñez; Roni Rossman; Kristina Reid; Karin Moelling; George D. Yancopoulos; David J. Glass

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