Binding of 14-3-3 Proteins to the Protein Kinase Raf and Effects on Its Activation

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To identify proteins that may participate in the activation of the protein kinase Raf, proteins that interact with Raf were selected in a two-hybrid screen. Two members of the 14-3-3 protein family were isolated that interacted with both the amino terminal regulatory regions of Raf and the kinase domain of Raf, but did not compete with the guanine nucleotide–binding protein Ras for binding to Raf. 14-3-3 proteins associated with Raf in mammalian cells and accompanied Raf to the membrane in the presence of activated Ras. In yeast cells expressing Raf and MEK, mammalian 14-3-3 β or 14-3-3 ζ activated Raf to a similar extent as did expression of Ras. Therefore, 14-3-3 proteins may participate in or be required for the regulation of Raf function. These findings suggest a role for 14-3-3 proteins in Raf-mediated signal transduction.

Activation of the mitogen-activated protein (MAP) kinase pathway is a central event in the response of cells to mitogenic factors. MAP kinase activation is essential for proliferation of fibroblasts in response to growth factors (*1*) and is in many cases dependent on activated Ras (*2*). The c-Raf-1 (Raf) protein kinase is a downstream effector of Ras, which in turn phosphorylates and activates the enzyme known as MAP kinase kinase or MEK [MAP kinase or extracellular signal–regulated kinase (ERK) kinase] (*3*), the activator of the MAP kinases ERK1 and ERK2 (*4*). Raf appears to function downstream of Ras because v-Raf can transform cells in which Ras function is inhibited (*5*). Also, antisense Raf RNA and dominant negative mutants of Raf interfere with transformation by activated Ras (*6*). Genetic evidence both in *Drosophila* and in *Caenorhabditis elegans* also indicates that Raf is a downstream element in Ras-mediated signaling (*7*). Ras and Raf interact directly both in vitro and in vivo (*8*, *9*). This association occurs with higher affinity if Ras is in the guanosine triphosphate–bound form and is mediated by the Ras effector domain and a region of Raf containing part of its CR1 (conserved region 1) in the NH_2-terminal regulatory domain of the protein. Although Raf can be activated in insect cells by expression with either v-Src or v-Ras (*10*, *11*), simple association between Ras and Raf in vitro does not activate Raf (*12*).

Activated Ras directs Raf kinase to the plasma membrane (*13*, *14*). A modified form of Raf that contains a CAAX motif [C, cysteine; A, aliphatic amino acid; X, any amino acid (*15*)] and is thereby directed to the plasma membrane (*14*), becomes activated without a requirement for activated Ras. Ras may therefore be dispensable after plasma membrane localization has been achieved. However, the mechanism by which Raf becomes activated has not yet been elucidated.

To identify proteins that might participate in Raf activation, we used a two-hybrid screen (*16*, *17*) of a HeLa complementary DNA (cDNA) library fused with the Gal4 activation domain, with full-length Raf protein kinase fused with the Gal4 DNA-binding domain as target. Of 10 clones identified as specific positives, 2 were distinct members of the 14-3-3 protein family, designated 14-3-3 β (human 1054) (*18*) and 14-3-3 ζ (*19*, *20*). To address the specificity of these interactions, we tested other Gal4 DNA-binding domain fusions. We observed no interaction, as judged by growth on medium lacking histidine (−his) and expression of β-galactosidase (β-Gal) activity, of either of the 14-3-3 proteins with the adenomatous polyposis coli (APC) protein amino acids 1034 to 2130 (Fig. 1B), with p53, with Ras (Ser^{186}) (*21*), or with the Gal4 DNA-binding domain alone (*22*). We have, however, detected an interaction between 14-3-3 β and the Bcr protein (*23*), which itself has endogenous protein kinase activity (*24*).

For determining the region of Raf that participates in the interaction with 14-3-3 proteins, constructs encoding portions of Raf (Fig. 1A) were transformed into yeast with either of the two 14-3-3 family members or with Ras (Ser^{186}). The Ras (Ser^{186}) fusion interacted with full-length Raf and with the NH_2-terminal region including CR1, but not with the middle region containing CR2 or with the COOH-terminal kinase domain (Fig. 1B). Both of the 14-3-3 proteins also interacted with the NH_2-terminal domain of Raf, and 14-3-3 β displayed a similar interaction with the middle portion of Raf and with the COOH-termi-

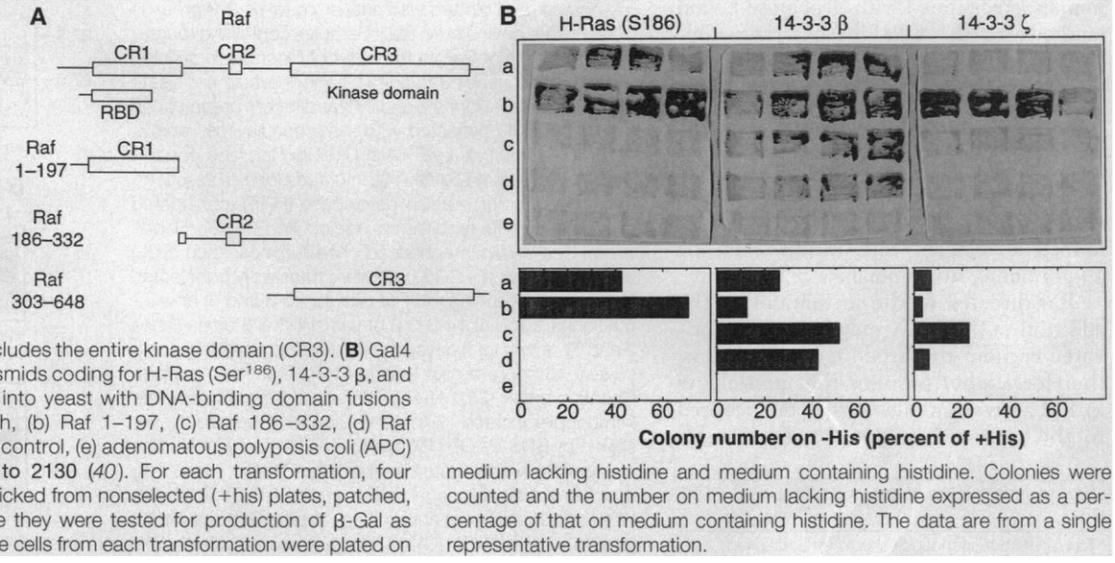

Fig. 1. Interaction between portions of Raf and 14-3-3 proteins in the two-hybrid system (*39*). (**A**) Schematic diagram of the portions of the Raf kinase used for determining the regions of interaction. Raf 1–197 includes the Ras-binding domain (amino acids 51 to 131) and the zinc finger–like region, which together constitute CR1. Raf 186–332 includes the CR2 region. Raf 303–648 includes the entire kinase domain (CR3). (**B**) Gal4 activation domain fusion plasmids coding for H-Ras (Ser^{186}), 14-3-3 β, and 14-3-3 ζ were transformed into yeast with DNA-binding domain fusions containing (a) Raf full-length, (b) Raf 1–197, (c) Raf 186–332, (d) Raf 303–648, and as a negative control, (e) adenomatous polyposis coli (APC) protein amino acids 1034 to 2130 (*40*). For each transformation, four independent colonies were picked from nonselected (+his) plates, patched, and grown for 3 days before they were tested for production of β-Gal as described (*41*). Portions of the cells from each transformation were plated on medium lacking histidine and medium containing histidine. Colonies were counted and the number on medium lacking histidine expressed as a percentage of that on medium containing histidine. The data are from a single representative transformation.

nal kinase domain. 14-3-3 ζ interactions with both the middle portion of Raf and the COOH-terminal kinase domain, although barely detectable with the β-Gal assay, were evident with the more sensitive measure of growth on medium lacking histidine (Fig. 1B) (*25*). Thus, taking into account the overlap between the Raf fusions, we conclude that the interaction between Raf and 14-3-3 proteins is mediated by at least two distinct regions of the Raf protein.

Given the interaction of the 14-3-3 proteins with the CR1 region, it was possible that these proteins interacted with the Ras-binding domain and might compete for Ras binding. In an in vitro binding assay, recombinant 14-3-3 β did not bind directly to the Ras-binding domain (RBD) of Raf, nor did it compete with Ras for binding to the RBD (*26*). We found no evidence for association between 14-3-3 β and K-Ras in the in vitro assay. These data suggest that the binding site on Raf for 14-3-3 proteins is distinct from the Ras-binding domain and that Raf molecules bound to 14-3-3 protein may simultaneously be bound to Ras.

For investigating in vivo association between Raf and 14-3-3 proteins, Raf was immunoprecipitated from insect Sf9 cells expressing catalytically inactive Raf, wild-type Raf, and wild-type Raf with v-Src, and the resultant samples were analyzed by protein immunoblotting with antiserum to 14-3-3 proteins. We observed 14-3-3 proteins in all of these Raf preparations (Fig. 2A). Preparations of unrelated proteins, such as H-Sos and human papilloma virus (HPV) E2, similarly isolated from Sf9 cells, did not contain 14-3-3 proteins.

For determining whether 14-3-3 proteins associated with Raf in mammalian cells, Raf tagged with the epitope MEYMPME [(Met-Glu-Tyr-Met-Pro-Met-Glu), Glu-Glu Raf] was expressed in COS cells or coexpressed with 14-3-3 β, and immunoprecipitated with Sepharose-bound antibody to the Glu-Glu tag (anti–Glu-Glu). Cells that expressed Glu-Glu Raf yielded coimmunoprecipitated 14-3-3 proteins whether or not 14-3-3 β was also overexpressed (Fig. 2B). Cells that expressed 14-3-3 β alone did not yield Raf or 14-3-3 proteins in the anti–Glu-Glu immunoprecipitates (Fig. 2B). Thus, Raf also formed a complex with members of the 14-3-3 family in mammalian cells.

Raf directed to the membrane by the addition of a CAAX motif becomes activated without the need for activated Ras; therefore, other proteins that participate in Raf activation must either be localized in the plasma membrane or be translocated there under conditions that translocate Raf. The association between 14-3-3 proteins and inactive Raf suggested that 14-3-3 proteins might accompany Raf to the plasma membrane upon Ras activation. We addressed the localization of 14-3-3 in COS cells by immunoprecipitation of Raf overexpressed alone or in combination with activated Ras. When Raf was expressed alone, it was found mainly in the soluble or supernatant fraction (S100) and was accompanied by 14-3-3 protein (Fig. 3). When Raf was expressed with activated Ras, a larger proportion was recovered in the membrane or pellet fraction (P100) and was also accompanied by 14-3-3 protein (Fig. 3) (*27*). Consistent with these fractionation data, immunofluorescence microscopy indicated that when 14-3-3 β was expressed alone in MDCK cells it was localized in the cytosol, whereas when it was expressed together with activated Ras, partial redistribution to the plasma membrane was observed (*28*). These data indicate that 14-3-3 protein accompanies Raf to the plasma membrane and is therefore associated with Raf in the membrane where activation occurs.

To address the functional consequences of the interaction between Raf and 14-3-3 proteins, we used a yeast system in which Raf activation is coupled to growth. In *Saccharomyces cerevisiae*, mating signals are transduced through a series of kinases that are homologous to the members of the MAP kinase pathway. They include Ste11, homologous to MEK kinase (*29*), Ste7, the MEK counterpart, and two redundant MAP kinases, Fus3 and Kss1 (*30*). One target of the Fus3 kinase is the Ste12 transcription factor, which promotes transcription from promoters upstream of mating-inducible genes (*31*). Fusion of one such promoter, that of the *FUS1* gene, to the *HIS3* gene renders the cells dependent on activation of the mating pheromone pathway for viability in the absence of exogenous histidine (−his). We used a strain that is also mutated in one of the signaling components, the *STE11* gene, and is therefore impaired for signaling and growth on medium lacking histidine. This defect can be complemented by introduction of an active form of Raf, such as its constitutively active kinase domain, together with MEK (*32*). Full-length Raf does not signal in this system, but can be activated by overexpression of Ras (Fig. 4A). Introduction of the 14-3-3 β or 14-3-3 ζ gene into the *ste11* mutant strain carrying full-length Raf integrated into the genome and the MEK gene on a plasmid, resulted in stimulation of growth on −his medium (Fig. 4A). This phenotype was not observed in cells that contained either Raf or MEK alone, indicating a specific effect on Raf. The degree of growth observed with the 14-3-3 clones was comparable to that obtained with Ras. Raf purified from cells expressing 14-3-3 proteins had a kinase activity that was two to three times that of Raf from cells not expressing 14-3-3 (Fig.

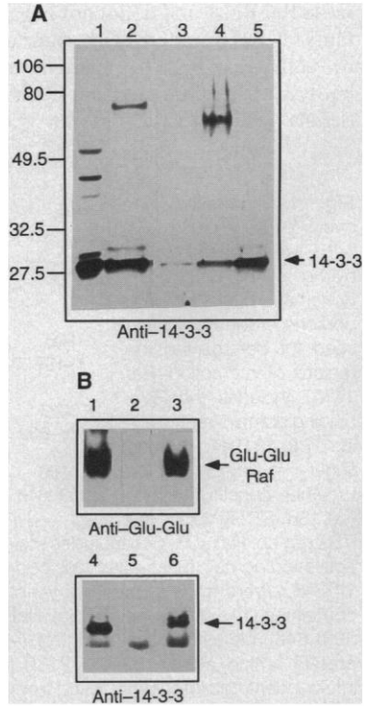

Fig. 2. Binding of Raf to 14-3-3 proteins in insect cells and COS cells. (**A**) Raf tagged at the COOH-terminus with the sequence MEYMPME [(Met-Glu-Tyr-Met-Pro-Met-Glu), Glu-Glu] was purified from insect Sf9 cells with antibody to Glu-Glu coupled to Sepharose (anti–Glu-Glu Sepharose) as described (*11*) and eluted with Glu-Glu peptide. Proteins were resolved by polyacrylamide gel electrophoresis (PAGE) on 10% gels (NOVEX), transferred to Immobilon membrane, and blotted with antiserum to 14-3-3 proteins purified from bovine brain (*42*). Samples containing roughly equal amounts of Raf on the basis of Coomassie staining were loaded in lanes 3 to 5 (*43*). Lane 1, whole-cell lysate from MDCK cells; lanes 2 and 5, two different preparations of Glu-Glu Raf coinfected with v-*src* baculovirus; lane 3, catalytically inactive (Lys^{375}Ala) Glu-Glu Raf; lane 4, catalytically competent Glu-Glu Raf infected alone. The second antibody was horseradish peroxidase (HRP)–conjugated goat antibody to rabbit immunoglobulin G (IgG). Bands were visualized with enhanced chemiluminescence (ECL) reagents (NEN). (**B**) COS cells were transiently transfected (*44*) with Glu-Glu–tagged Raf with (lanes 3 and 6) or without (lanes 1 and 4) 14-3-3 β or with 14-3-3 β alone (lanes 2 and 5), grown for 2 days, and serum-deprived for approximately 16 hours before lysis in MAP kinase immunoprecipitation buffer (*38*). After centrifugation the lysates were immunoprecipitated with anti–Glu-Glu Sepharose, resolved by SDS-PAGE (10% gels), and blotted with alkaline phosphatase–conjugated antibody to Glu-Glu (lanes 1 to 3) or with rabbit polyclonal antiserum to 14-3-3 followed by HRP-conjugated goat antibody to rabbit IgG (lanes 4 to 6) as in (A). The anti–Glu-Glu blot was visualized with the Western light protein detection kit (Tropix, Inc.).

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4B). This amount of activation was sufficient for a biological stimulation of the pathway and was similar to the approximately twofold stimulation of Raf activity observed with Ras.

The 14-3-3 proteins comprise a well-conserved family of proteins present in mammalian cells as well as in flies, yeast, and plants (*33*). Several members of the family have been described in mammalian cells. These proteins have been implicated in biochemical processes such as inhibition of protein kinase C (PKC), activation of tyrosine and tryptophan hydroxylases, and stimulation of exocytosis from adrenal chromaffin cells. Distinct members of the 14-3-3 family may serve diverse regulatory roles in the cell.

Our data suggest that one of the regions of Raf that interacts with 14-3-3 proteins may be the zinc finger–like region just COOH-terminal to the RBD. PKC, which contains similar zinc finger–like regions, binds to receptors for activated PKC (RACKs) (*34*), to annexin I, and to kinase C inhibitor protein (KCIP), a member of the 14-3-3 protein family (*33*, *34*). The region of these proteins thought to mediate this interaction is similar to a region present in all the members of the 14-3-3 protein family and to a sequence in the COOH-terminus of proteins of the annexin family (*35*). Therefore, this annexin-like domain may take part in an association with the zinc finger–like region of Raf.

Association with 14-3-3 proteins alone is not sufficient to activate the kinase activity of Raf. 14-3-3 proteins are associated with both inactive Raf in the cytosol and active Raf in the membrane fraction, and thus may be a necessary structural component of the Raf kinase. When BMH1 (*36*) (a yeast 14-3-3 homolog) is deleted, Ras can no longer stimulate the activity of Raf in the yeast system (*37*). These observations suggest that Ras and 14-3-3 may both be required in a common process of Raf activation. In the yeast system, overexpression of 14-3-3 β or 14-3-3 ζ is sufficient to activate Raf kinase. When Raf is expressed in yeast, in which no known homolog exists, Raf plasma membrane localization and activation may be limited by the endogenous levels of Ras and 14-3-3 proteins, and therefore increased when exogenous Ras and 14-3-3 proteins are expressed. In mammalian cells, 14-3-3 proteins may participate in the Raf activation process after membrane localization has been achieved.

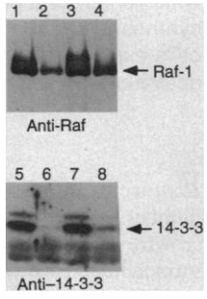

Fig. 3. Association of 14-3-3 protein with Raf at the plasma membrane. COS cells transiently transfected with either Glu-Glu–tagged Raf alone (lanes 1, 2, 5, and 6) or Glu-Glu–tagged Raf in combination with H-Ras (Val^{12}) (lanes 3, 4, 7, and 8) were subjected to hypotonic lysis and fractionation (*27*). The soluble or supernatant (S100) (lanes 1, 3, 5, and 7) fraction and the detergent-soluble pellet (P100) (lanes 2, 4, 6, and 8) fraction were immunoprecipitated with anti–Glu-Glu Sepharose, and proteins were resolved by SDS-PAGE on 10% gels (NOVEX), transferred to Immobilon membrane, and blotted with either polyclonal antibody to c-Raf (C20, Santa Cruz Biotech.) (lanes 1 through 4) or with polyclonal antibodies to 14-3-3 proteins (lanes 5 through 8). Both blots were incubated with HRP-conjugated goat antibody to rabbit IgG and bands were visualized with ECL reagents (NEN).

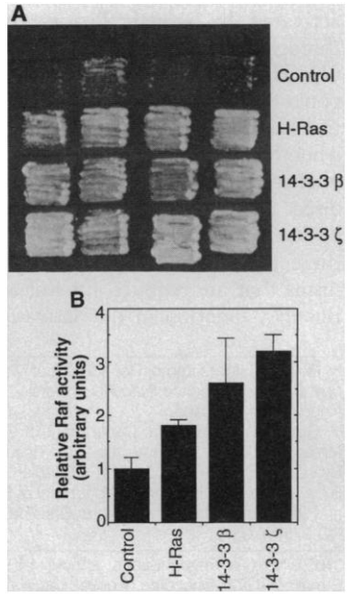

Fig. 4. Activation of Raf by 14-3-3 proteins in yeast. Strain SY1984R-L (MATα *ste11Δ pep4Δ his3Δ FUS1::HIS3 leu2 ura3 trp1 can1 RAF::LEU2*) (*45*) was transformed with plasmid YEpMEK (*46*). The resulting strain was transformed with one of the following plasmids: the AAH5 vector (control), AAH5-Ha-Ras (H-Ras) (*47*), AAH5-14-3-3 β, or AAH5-14-3-3 ζ. (**A**) Four independent isolates of each derivative were plated on medium lacking histidine, and growth was monitored after 3 days. (**B**) The Raf protein was affinity purified on anti–Glu-Glu Sepharose from solubilized P100 fractions (*48*) prepared from each of the above derivatives. A portion of each preparation was incubated with saturating amounts of recombinant MEK (8 μg) and [γ^{32}P]adenosine triphosphate (5 μCi) for 20 min at 30°C. The reactions were stopped by addition of Laemli buffer and boiled for 5 min. The phosphorylated products were separated by SDS-PAGE and then quantitated with an AMBIS radioisotope detector. The counts incorporated into MEK were corrected for the amount of Raf proteins as measured by protein immunoblotting as described (Fig. 2) (*49*). The relative Raf activity was normalized to the basal activity of the control. Vertical bars represent standard errors calculated on means of three experiments.

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25. In general, we observed a stronger interaction of Raf with 14-3-3 β than with 14-3-3 ζ. The two assays used (growth on medium lacking histidine and β-Gal activity) resulted in different impressions regarding the relative strength of binding of 14-3-3 proteins to different regions of Raf. We observed fewer colonies on medium lacking histidine when using constructs containing the Ras-binding domain of Raf. This was likely due to toxicity of these constructs, because they would be expected to inhibit endogenous Ras. Our data do not justify conclusions about the relative strength of 14-3-3 interactions with different regions of Raf. Also, other investigators have isolated 14-3-3 β using the Raf COOH-terminus as target, and have shown a stronger interaction with the NH_2-terminal regula-

tory domain of Raf (L. van Aelst and M. Wigler, personal communication).
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27. Cells were lysed in hypotonic lysis buffer [10 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, 25 mM NaF, 1 mM EGTA, 1 mM dithiothreitol (DTT), 100 mM Na_3VO_4, aprotinin (10 μg/ml), soybean trypsin inhibitor (10 μg/ml), 20 μM leupeptin, 1 mM Pefabloc] and nuclei were removed by centrifugation at 1500*g*. The supernatant was subjected to dounce homogenization and centrifuged again at 100,000*g*. The resulting supernatant is the S100 fraction. The sedimented material (P100 fraction) was solubilized in MAP kinase immunoprecipitation buffer (*38*) and centrifuged to remove Triton-insoluble material. Both S100 and detergent-soluble P100 fractions were subjected to immunoprecipitation.
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38. Twenty millimolar tris-HCl (pH 8.0), 137 mM NaCl, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 100 μM Na_3VO_4, 50 mM NaF, aprotinin (10 μg/ml), 20 μM leupeptin, 1 mM Pefabloc (AEBSF, Boehringer Mannheim).
39. The Raf coding sequence was isolated with the polymerase chain reaction (PCR) as described (*11*). For use in the two-hybrid screen (*16*), the Raf gene was fused to the sequence encoding the Gal4 DNA-binding domain by subcloning into the pGBT8 plasmid (*34*) at Nco I and Sac I sites. Yeast (*S. cerevisiae*) strain YGH-1 (*34*) was used. The YGH-1 strain contains two reporter genes, *HIS3* and *lacZ*, which allow selection of cDNAs encoding proteins that interact with the product of the gene of interest. Raf 1–197 and Raf 186–333 were constructed as PCR products with pGEM Raf as template and primers including restriction linkers for cloning into pGBT8 at Nco I and Sac I. The Raf 303–648 plasmid was constructed by insertion of a PCR product coding for Raf amino acids 303 through 381 into pGBT8-Raf digested with Eco RI and Sal I. The 14-3-3 fusions isolated in the two-hybrid screen, included the sequence encoding the full-length 14-3-3 protein in frame with the Gal4 sequence. 14-3-3 β began at position −250 and had a deletion from −97 to −3. 14-3-3 ζ contained 80 bp of upstream sequence and had a deletion that ended at position −13.
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46. Plasmid YEpMEK contains the MEK gene, expressed off the constitutive promoter of the glyceraldehyde dehydrogenase gene, and the *TRP1* gene for selection.
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48. Yeast cells were broken with glass beads in a buffer containing 10 mM tris-HCl (pH 7.5), 10 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 10 μg each per milliliter of buffer of leupeptin and aprotinin, E64 (2 μg/ml), 1 mM Pefablock, 0.25 mM Na_3VO_4, and 5 mM NaF. Cell lysates were centrifuged at 100,000*g* and the sedimented fractions (P100) were solubilized in a buffer containing 50 mM tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and protease and phosphatase inhibitors as in the lysis buffer. Glu-Glu–tagged Raf proteins were immunoprecipitated from the solubilized P100 fractions with anti–Glu-Glu Sepharose. Immune complexes were washed as described (*11*) and portions were assayed for kinase activity and protein immunoblot analysis.
49. The autoradiogram was scanned with a UMAX UC630 scanner and the Adobe Photoshop program. The density of the Raf bands was quantitated with the National Institutes of Health Image 1.44 computer program. The linearity range was established with a calibration curve.
50. We thank K. Matsumoto for communicating results before publication; T. Isobe for providing antiserum to the 14-3-3 proteins; S. Cook and J. Hancock for critical reading of the manuscript; B. Rubinfeld for providing pGBT8 APC; M. Callow and T. Vuong for technical assistance; and J. Huang, D. Ramirez, and J. Fitzsimmons for secretarial assistance.

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Stimulatory Effects of Yeast and Mammalian 14-3-3 Proteins on the Raf Protein Kinase

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Intracellular signaling from receptor tyrosine kinases in mammalian cells results in activation of a signal cascade that includes the guanine nucleotide–binding protein Ras and the protein kinases Raf, MEK [mitogen-activated protein kinase (MAPK) or extracellular signal–regulated kinase (ERK) kinase], and MAPK. MAPK activation that is dependent on the coupling of Ras and Raf was reconstituted in yeast. Yeast genes were isolated that, when overexpressed, enhanced the function of Raf. One of them is identical to *BMH1*, which encodes a protein similar to members of the mammalian 14-3-3 family. Bacterially synthesized mammalian 14-3-3 protein stimulated the activity of Raf prepared from yeast cells expressing c-Raf-1. Thus, the 14-3-3 protein may participate in or be required for activation of Raf.

Extracellular mitogenic agents, including a number of growth factors, impart their growth signal to the cell by stimulating an intracellular pathway consisting of kinases that are members of the Raf, MEK, and MAPK families. Raf activation appears to occur downstream of Ras, which is in turn activated by growth factors that signal through receptor protein tyrosine kinases (*1*). Thus, Ras and Raf are essential components of the MAPK pathway that controls cell proliferation. Raf binds to Ras when Ras is in its active, guanosine triphosphate–bound state, suggesting that Raf is a direct effector of Ras (*2*, *3*). However, Ras by itself does not activate Raf in vitro, so there must be other molecules or mechanisms that are required for Raf activation. Ras may function in the activation of Raf by recruiting Raf to the membrane (*4*).

Elements of the MAPK signaling pathway appear to have been conserved during evolution, because Ras and homologs of each of the kinases, except Raf, have been identified in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (*5*). One of the MAPK pathways in *S. cerevisiae* controls the response to mating pheromone (*5*). This signaling cascade consists of the Ste11, Ste7, and Fus3 or Kss1 kinases whose equivalents in mammals are MEK kinase (or Raf), MEK, and MAPK, respectively. Ste11, Ste7, and Fus3 or Kss1 act sequentially to transmit a signal to the transcription factor Ste12, which is required for the expression of mating-specific genes.

We identified a gain-of-function mutation of *STE7*, *STE7*^{P368} (*6*). Without pheromone stimulation, Ste7^{P368} has increased kinase activity and induces some mating responses. However, this activity is still dependent on the presence of the upstream kinase, Ste11. An activated form of mammalian Raf (RafΔN), but not normal c-Raf-1, can substitute for Ste11 activity in vivo (*6*). However, when c-Raf-1 and Ste7^{P368} are expressed together with mammalian H-Ras, the Ste11 deficiency is rescued by c-

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