tory domain of Raf (L. van Aelst and M. Wigler, personal communication).
26. R. Ruggieri and E. Freed, unpublished observations.
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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43. We have also stripped and re-probed this blot with antibodies to Raf (C20, Santa Cruz Biotech.). Taken together, the data suggest that little 14-3-3 is associated with catalytically inactive Raf. Also, the Raf isolated from cells coinfected with v-*src* may contain more 14-3-3, relative to the Raf present, than does the Raf infected alone.
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45. The Raf gene was first subcloned downstream of the *ADH* promoter into the pADU vector, which is a high–copy number yeast expression vector containing the selectable marker *URA3*. Then, a segment containing the promoter, the Raf gene, and the *ADH* terminator was introduced into the polylinker site of plasmid pRS405 (Stratagene). The linearized plasmid was integrated at the *LEU2* locus in the strain SY1984 (MATα *ste11Δ pep4Δ his3Δ FUS1::HIS3 leu2 ura3 trp1 can1*) (obtained from K. Matsumoto) to generate strain SY1984R-L.
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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Raf-1. Thus, we developed an in vivo system for the assay of Raf activity using the yeast pheromone–induced MAPK pathway. These results not only indicate that Raf is activated by H-Ras in *S. cerevisiae* but also suggest that yeast cells have an activator for mammalian Raf.

To identify yeast components participating in Raf activation, we conducted screens to isolate yeast genes that, when overexpressed, suppressed the transcriptional defect of *ste11ΔSTE7*^{P368} cells expressing c-Raf-1 (*6*). Our expectation was that an excess of a component that functions in Raf activation might enhance Raf activity and, therefore, allow it to substitute for Ste11. We isolated clones from a yeast genomic DNA library on a multicopy plasmid that could activate a mating pathway–responsive reporter gene (*FUS1::HIS3*) in a Raf- and Ste7^{P368}-dependent manner (*7*). We assigned the recovered plasmids to different classes by restriction mapping. The DNA sequence of a representative of one class showed that it contained *RAS1* (*8*). This finding indicates that overproduction of the normal yeast Ras proteins can activate mammalian Raf. A representative of a second class contained *BMH1* (*9*), whose product is more than 60% identical with members of the mammalian 14-3-3 protein family (Fig. 1). Either *RAS1* or *BMH1*, when overexpressed from a multicopy plasmid in the presence of c-Raf-1 and Ste7^{P368}, suppressed the *ste11Δ* mutation as monitored by the histidine phenotype conferred by the *FUS1::HIS3* reporter gene (Fig. 2A) (*10*). These results suggest that overproduction of Ras1 or Bmh1 enhances Raf function.

If Bmh1 has a role in activation of Raf, we reasoned that loss of Bmh1 function would influence the activity of Raf in yeast. We examined the effect of a *BMH1* disruption mutation (*bmh1::LEU2*) (*11*) on Raf activation (Fig. 2B) (*10*). The *bmh1* mutant was viable with nearly wild-type growth rates. However, the *bmh1* mutation blocked the activation of Raf by Ras1. In contrast, the *bmh1* mutation had no effect on the activity of the constitutively activated RafΔN, which lacks the NH_2-terminal regulatory region of Raf (*12*). These results suggest that Bmh1 is required for activation of Raf and that its effect may be mediated through the NH_2-terminal domain of Raf.

To test the interaction of Bmh1 with Raf, we used a LexA-based two-hybrid system (*3*, *13*). One hybrid was a fusion between the LexA DNA-binding domain and Bmh1 (pLexA-BMH1) (*14*). The second hybrid was a fusion between an activation domain of Gal4 and Raf (pACT-RAF) (*14*). These two hybrids were expressed in a *S. cerevisiae* strain that contains an integrated reporter construct in which a binding site for the LexA protein was placed upstream of the yeast *HIS3* coding region. As controls, we used LexA-Ras, which interacts with Gal4-Raf. If the two hybrid proteins interact, then transactivation of the reporter construct occurs and the yeast strain can grow in the absence of histidine. The LexA-Bmh1 fusion alone caused an amount of *HIS3* expression sufficient to allow growth without exogenous histidine. Nevertheless, histidine auxotrophy could be attained by growing cells in the presence of 80 mM 3-aminotriozole (3-AT), a chemical inhibitor of the *HIS3* product, imidazole glycerol phosphate dehydrogenase (IGP) (*15*). Yeast strains transformed with pLexA-BMH1 and pACT-RAF activated the *LexA-HIS3* reporter gene to an extent that the amount of IGP dehydrogenase overcame the inhibition by 3-AT (Fig. 3). Thus, two-hybrid interactions were clearly detected between Bmh1 and Raf.

To define the region of Raf that interacts with Bmh1, we used fusions of either the COOH-terminal (RafΔN) or NH_2-terminal (RafΔC) domains of Raf to Gal4 (*14*). Bmh1 interacted with the NH_2-terminal domain of Raf but not with the COOH-terminal domain (Fig. 3). Because the Ras protein also interacts with the NH_2-terminal domain of Raf (*2*, *3*), we tested whether Bmh1 and Ras interacted with the same region of Raf. We used the RIP51 protein that has the NH_2-terminal two-thirds of Raf (amino acids 48 to 178 in c-Raf-1) fused to a nuclear localization signal and the VP16 acidic activation domain (*3*). The RIP51 fusion interacted with LexA-Ras, but not with LexA-Bmh1, suggesting that Bmh1 and Ras interact with different regions in the NH_2-terminal domain of Raf (Fig. 3). Alternatively, Bmh1 and Ras could interact with the same region but Bmh1 requires other regions of Raf as well.

The above results suggested the possibility that the 14-3-3 protein enhances the kinase activity of Raf by binding to it. To

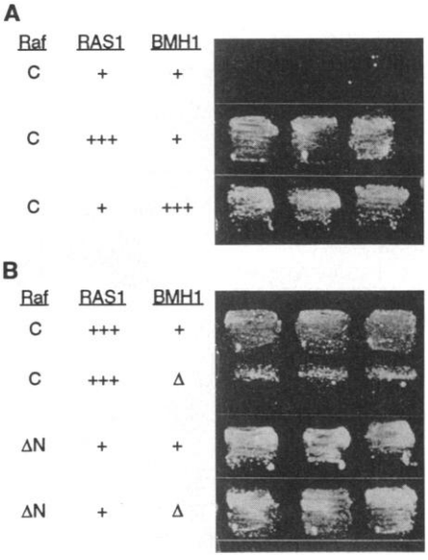

Fig. 2. Effects of Bmh1 on activity of Raf in yeast. The panels on the left specify whether the strains expressed c-Raf-1 (C) or RafΔN (ΔN) and whether *RAS1* and *BMH1* were expressed from the chromosomal copy (+), an overexpressing plasmid (+++), or a disruption allele (Δ). The panels on the right show the growth of cells on synthetic complete (SC)–His plates. (**A**) Suppression of *ste11Δ* by overexpression of *RAS1* and *BMH1* in the presence of c-Raf-1 and Ste7^{P368}. Strains were as follows: row 1, SY1984 + pADU-Raf, pKN7-6; row 2, SY1984 + pADU-Raf, pKNG11; row 3, SY1984 + pADU-Raf, pKNG12. (**B**) Effect of the *bmh1* disruption on Raf. Strains were as follows: row 1, SY1984 + pADU-Raf, pKNG11; row 2, SY1984-43 + pADU-Raf, pKNG11; row 3, SY1984 + pADU-RafΔN, pKN7-6; and row 4, SY1984-43 + pADU-RafΔN, pKN7-6. The plates were incubated for 3 days. Each patch represents an independent transformant.

Fig. 1. Amino acid sequence comparison of mammalian 14-3-3 and yeast Bmh1 proteins (*30*). Amino acid identities between 14-3-3 and Bmh1 sequences are indicated by asterisks. The sequence from the COOH-terminus of annexin is included in the alignment to show a region that has similarity to this segment of annexin. The amino acids that are identical to the annexin sequence are indicated by asterisks. The 14-3-3 sequence is the bovine type η (*21*) and KCIP is the sheep protein kinase C inhibitor protein (*22*).

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14-3-3    MG-DREQLLQRARLAEQAERYDDMASAMKAVTELNEPLSNEDRNLLSVAYKNVVGARRSS   59
          *  **    * ********  *   ** *    ** * ********** **** *
Bmh1      MSTSREDSVYLAKLAEQAERYEEMVENMKTVASSGQELSVEERNLLSVAYKNVIGARRAS   60
          *    *  **********  *  ** *  * *** ************ **** *
KCIP      M--DKSELVQKAKLAEQAERYDDMAAAMKAVTEQGHELSNEERNLLSVAYKNVVGARRSS   58

14-3-3    WRVISSIEQKTMA-DGNEKKLEKVKAYREKIEKELETVCNDVLALLDKFLIKNCNDFQYE  118
          **  ******      *   *    ** *** **      * *  **  **        *
Bmh1      WRIVSSIEQKEESKEKSEHQVELICSYRSKIETELTKISDDILSVLDSHLI--PSATTGE  118
          **  ****** *      *       ** *** **  *  * *  **  **  * **  *
KCIP      WRVISSIEQKTE---RNEKKQQMGKEYREKIEAELQDICNDVLQLLDKYLI--PNATQPE  113

14-3-3    SKVFYLKMKGDYYRYLAEVASGEKKNSVVEASEAAYKEAFEISKEHMQPTHPIRLGLALN  178
          ***** ****** *****  **          **  *** * **       ***********
Bmh1      SKVFYYKMKGDYHRYLAEFSSGDAREKATNASLEAYKTASEIATTELPPTHPIRLGLALN  178
          ***** ****** *** *  ***          *  **  * **   *  ***********
KCIP      SKVFYLKMKGDYFRYLSEVASGDNKQTTVSNSQQAYQEAFEISKKEMQPTHPIRLGLALN  173
                ****   *     **
Annexin           KGDYQKALLYLCGGDD

14-3-3    FSVFYYEIQNAPEQACLLAKQAFDDAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSD--  236
          ********** *  ** ***************** * **********************
Bmh1      FSVFYYEIQNSPDKACHLAKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDMS  238
          ********* *** *** *** *** ****** ************************
KCIP      FSVFYYEILNSPEKACSLAKTAFDEAIAELDTLNEESYKDSTLIMQLLRDNLTLWTSE--  231

14-3-3    QQDEEAGEGN                                                    246
Bmh1      ESGQAEDQQQQQQHQQQQPPAAAEGEAPK                                 267
KCIP      NQGDEGDAGEGEN                                                 244
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test this possibility, Raf was immunoprecipitated from yeast cells expressing c-Raf-1. The immune complexes were mixed with various concentrations of purified recombinant 14-3-3 (type η) protein and assayed for in vitro activation of recombinant *Xenopus* MEK as measured by phosphorylation of *Xenopus* MAPK (Fig. 4). The Raf immune complexes had MEK-kinase activity in the absence of the 14-3-3 protein, but this activity was enhanced by the 14-3-3 protein in a dose-dependent manner. At an optimal concentration of the 14-3-3 protein, three- to fourfold enhancement of the Raf activity was achieved. The immune complexes obtained from the control yeast cells lacking Raf showed essentially no MEK-kinase activity (Fig. 4). These results are consistent with the idea that the 14-3-3 protein binds directly to Raf and enhances its MEK-kinase activity.

The 14-3-3 protein is a soluble acidic protein and belongs to a multigene family (*16*). Members of the 14-3-3 protein family are thought to participate in activation of tyrosine and tryptophan hydroxylases in the presence of Ca^{2+}-calmodulin–dependent kinase II, regulation of protein kinase C (PKC), and activation of Ca^{2+}-dependent exocytosis in permeabilized adrenal chromaffin cells. In these activation processes, two roles for 14-3-3 function have been proposed and each suggests a plausible mechanism for its role in Raf activation.

Binding of the 14-3-3 protein to the regulatory domain of aromatic hydroxylases has been proposed to induce an active conformation. Similarly, 14-3-3 binding to the NH_2-terminal regulatory domain of Raf may induce an active conformation. The NH_2-terminal domain is thought to inhibit the intrinsic kinase activity of Raf (*12*, *17*). Inhibition could result from binding of the NH_2-terminus to the catalytic region in the absence of a positive signal. This negative function may be relieved by a conformational change induced by binding of 14-3-3 and Ras. Our results on in vitro activation of Raf by the 14-3-3 protein are consistent with this hypothesis. However, we cannot eliminate the possibility that other factors are associated with the Raf immunoprecipitates and also participate in Raf activation.

Another possible role for 14-3-3 is to mediate translocation of proteins to the membrane. PKC translocates from the cytosol to the particulate fraction upon activation. This translocation is mediated by RACKs (receptors for activated C kinase) and members of the annexin family that can act as RACKs (*18*). Members of the 14-3-3 family contain a short sequence of 16 amino acids similar to the COOH-terminus of annexins, which corresponds to the PKC binding site (*19*) (see Fig. 1). These observations point to a potential physiological role for 14-3-3 proteins as mediators of PKC translocation.

By extension, 14-3-3 could contribute to Raf translocation to the plasma membrane. Alternatively, it could act to anchor Raf to the membrane once it is localized there by Ras. The requirement for 14-3-3 (Bmh1) in the Ras-induced activation of Raf in yeast suggests that the protein works downstream of Ras. This order favors a model in which 14-3-3 protein contributes to Raf activation after the kinase has been localized to the membrane by Ras. Also consistent with this model, 14-3-3 proteins bind to Raf in the cytosol and follow it to the membrane contingent on localization by Ras or by the addition of lipid modifications to its COOH-terminus (*20*).

Our study identified the 14-3-3 family as proteins capable of activating Raf in vitro and in vivo in the yeast system. Some possible mechanisms for the action of 14-3-3 on Raf include its affects on conformation, membrane localization, stabilization of the Ras-Raf complex, or a combination of these. Important issues for further studies are to determine what mechanisms apply and to resolve the question of whether the 14-3-3–Raf interaction poises Raf for the decisive activation event or directly mediates it.

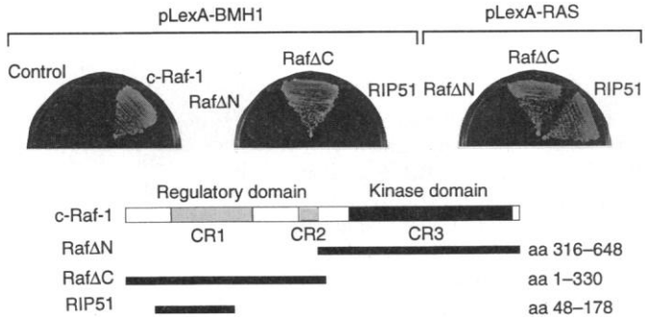

Fig. 3. Interaction of Bmh1 with Raf. The reporter strain L40 (*MAT***a** *LYS2::lexA-HIS3 URA3::lexA-lacZ his3 trp1 leu2*) was transformed with pLexA-BMH1, pLexA-RAS, and the indicated plasmids. An interaction between the fusion proteins expressed by the specified plasmids is demonstrated by the ability of the yeast strain to grow on plates of SC-His containing 80 mM 3-AT. The control plasmid is pACTII (*14*). Full-length human c-Raf-1, RafΔN, and RafΔC were fused to an activation domain of Gal4 (*14*). RafΔN and RafΔC contain amino acids 316 to 648 and amino acids 1 to 330, respectively, of human c-Raf-1. A schematic of these Raf fusions is shown below the plates. CR1 and CR2 indicate the Raf conserved regions in the regulatory domain. CR3 is the kinase domain.

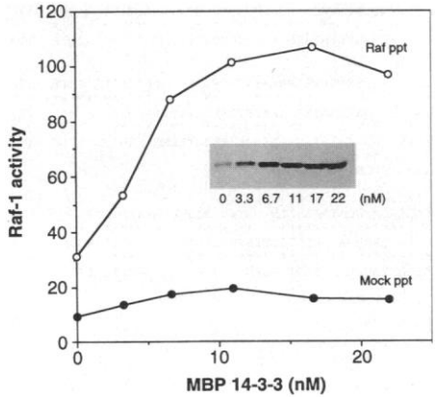

Fig. 4. In vitro activation of Raf by MBP-14-3-3. Immune complexes formed with antibody to human c-Raf-1 (*23*) and extracts of yeast cells expressing c-Raf-1 (strain SY1984-RP) (○) or lacking c-Raf-1 [strain SY1984-VP (*24*)] (●) were incubated with purified recombinant MEK (1.5 μg), together with various amounts of purified MBP-14-3-3 (maltose-binding protein fused to 14-3-3 type η protein) (*25*, *26*). The extent of MEK activation was assayed by its subsequent ability to ^{32}P phosphorylate catalytically inactive GST-MAPK (*26*). After electrophoresis, phosphorylation of the GST-MAPK was detected by autoradiography (inset) or quantified by an image analyzer and shown in arbitrary units; ppt, anti-Raf immunoprecipitates.

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6. The pheromone-stimulated MAPK pathway induces transcription of mating-specific genes such as *FUS1*. The *FUS1::HIS3* reporter gene has the *FUS1* upstream activation sequence joined to the *HIS3* open reading frame. This reporter allows signal activity in a *his3Δ FUS1::HIS3* strain to be monitored by its ability to grow on medium lacking exogenous histidine (His phenotype) (*27*). A *his3Δ ste11Δ FUS1::HIS3 STE7^{P368}* (proline substitution at serine 368) strain has a His$^-$ phenotype. Expression of mammalian RafΔN, but not c-Raf-1, in this strain confers a His$^+$ phenotype. Detailed characterization of Ste7^{P368} will

be presented elsewhere (B. M. Yashar *et al.*, in preparation).
7. Strain SY1984-RP is SY1984 (*ste11Δ his3Δ FUS1::HIS3*) (*27*) transformed with pADU-Raf and pNC318-P368. pADU-Raf contains c-Raf-1 controlled from the *ADH1* promoter. pNC318-P368 carries the *STE7^{P368}* allele controlled from the *CYC1* promoter. SY1984-RP was transformed with a multicopy (YEp13) yeast genomic library. Of the 6800 transformants screened, 43 colonies were His$^+$. Only six of these had a His$^+$ phenotype that was dependent on the presence of pADU-Raf, pNC318-P368, and the library plasmid.
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10. The plasmids pADU-Raf and pADU-RafΔN express c-Raf-1 and a truncated RafΔN lacking the NH_2-terminal domain, respectively, from the *ADH1* promoter. pKN7-6, pKNG11, and pKNG12 express *STE7^{P368}* from the *CYC1* promoter. pKNG11 and pKNG12 also overexpress *RAS1* and *BMH1*, respectively.
11. The *bmh1::LEU2* disruption allele of pG12-14 was constructed by insertion of a 2.7-kb Bgl II fragment of *LEU2* into the Bgl II site of *BMH1*. Strain SY1984-43 is identical to strain SY1984 (*27*) except that the *BMH1* locus is replaced with the *bmh1::LEU2* allele. It was constructed by a gene replacement with the 5.5-kb Xho I–Xba I fragment from pG12-14 (*10*). The resulting disruption was confirmed by Southern (DNA) blot analysis.
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14. pLexA-RAS and its parental vector, pBTM116, were provided by J. A. Cooper (*3*). For creation of an in-frame protein fusion between LexA and Bmh1, the 1.1-kb Hinc II to Bsp HI fragment of *BMH1* was cloned into the Sma I to Bam HI gap of pBTM116. pACTII expresses an activation domain of Gal4 under the control of the *ADH1* promoter. pACT-RAF contains the entire coding sequence of the human c-Raf-1 inserted in-frame to pACTII. pACT-RAFΔC and pACT-RAFΔN contain the NH_2-terminal and COOH-terminal portions, respectively, of human c-Raf-1 inserted in-frame to pACTII. pRIP51 was provided by J. A. Cooper (*3*).
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23. Yeast cells (60-ml culture) were grown to an absorbance at 600 nm (A_{600}) of 0.8, centrifuged, and washed with TBS [20 mM tris-HCl (pH 7.5), 150 mM NaCl]. For preparation of whole-cell protein extracts, the cell pellets were suspended in a lysis buffer [50 mM tris-HCl (pH 7.5), 100 mM NaCl, 1 mM sodium pyrophosphate, 5 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% aprotinin, leupeptin (10 μg/ml), pepstatin (10 μg/ml), chymostatin (10 μg/ml)] and vortexed in the presence of glass beads five times for 1 min each time at 4°C. c-Raf-1–antibody-bound beads (anti-c-Raf-1 beads) were prepared by incubation of 100 μl of antiserum to human c-Raf-1 with 300 μl of a 1:1 slurry of protein A–Sepharose beads. The complexes were washed two times with WB [20 mM tris-HCl (pH 7.5), 20 mM β-glycerophosphate, 15 mM NaF, 10 mM EGTA, 2 mM $MgCl_2$, 50 mM NaCl, 6 mM DTT, 0.1 mM orthovanadate, 1 mM PMSF, 0.5% aprotinin]. One hundred and sixty microliters of a 1:1 slurry of anti-c-Raf-1 beads were added to yeast cell extracts as described above and incubated for 2 hours at 4°C. The resulting complexes were washed with WB three times and used for in vitro assays. Expression of c-Raf-1 and its presence in the immune complexes were verified by immunoblotting with monoclonal antibody to the COOH-terminal half of human c-Raf-1.
24. Strain SY1984-VP is SY1984 with pADU and pNC318-P368 (*7*).
25. The expression plasmid pMAL-14-3-3, encoding the 14-3-3 protein fused to maltose-binding protein (MBP-14-3-3), was provided by Y. Takai and T. Isobe, and MBP-14-3-3 was purified from overexpressing *E. coli* (*28*).
26. The activity of Raf was measured by its ability to activate *Xenopus* MEK. Recombinant histidine-tagged *Xenopus* MEK and catalytically inactive *Xenopus* glutathione-S-transferase (GST)–MAPK (K57D) were produced in *Escherichia coli* and purified as described (*29*). MEK activity was assayed by its ability to phosphorylate catalytically inactive MAPK as described (*29*).
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30. Single-letter abbreviations for the 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; and Y, Tyr.
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Molecular Evidence That the Myxozoan Protists Are Metazoans

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The evolutionary origins of the protistan phylum, Myxozoa, have long been questioned. Although these obligate parasites are like protozoans in many features, several aspects of their ontogeny and morphology have implied a closer relationship to metazoan lineages. Phylogenetic analyses of 18S ribosomal RNA sequences from myxozoans and other eukaryotes, with the use of parsimony, distance, and maximum-likelihood methods, support the hypothesis that myxozoans are closely related to the bilateral animals. These results suggest that the Myxozoa, long considered an assemblage of protozoans, should be considered a metazoan phylum.

The reconstruction of animal phylogeny has engaged scientists for over a century (*1*). With the advent of molecular characters, hypothesized relationships among some taxa have become solidified, whereas others remain contentious (*2*). Most biologists would agree, however, on the set of taxa that constitutes the Metazoa, or multicellular animals. Not since discovery of the Loricifera in the last decade has a phylum been formally added to this taxonomic group (*3*). Here, we present evidence that the Myxozoa, a phylum generally placed in the kingdom Protista (Protoctista) (*4*–*6*), shares its most recent common ancestor with a metazoan, not a protozoan, lineage.

The phylum Myxozoa comprises over 1100 described species of oligocellular, obligate endoparasites and is thus one of the largest protistan assemblages (*4*). Members of the class Myxosporea (the majority of myxozoans) principally infect teleost fishes, and members of the Actinosporea mainly infect aquatic, oligochaete worms (*7*). Myxosporea are either coelozoic within hollow organs or histozoic in solid tissues and cause tissue displacements and destruction and sometimes death of their hosts. For example, infections by the European parasite *Myxobolus cerebralis* in North American salmonid fish can cause serious losses to aquaculture industries. The avirulence of most myxosporeans in their natural hosts, however, implies a long history of association (*8*).

The diagnostic feature of Myxozoa is the infective spore, a dispersal stage composed of distinct gametic (autogamic) cells and polar capsules with coiled, ejectable filaments, enveloped by valve-like somatic cells. Proliferative stages (trophozoites) are often multinuclear and may grow to macroscopic size (*8*). However, the complete life cycle is unknown, because myxozoans have been intractable to

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