MOLECULAR AND CELLULAR BIOLOGY, Nov. 1993, p. 6615–6620
0270-7306/93/116615-06$02.00/0

Vol. 13, No. 11

Reconstitution of the Raf-1–MEK–ERK Signal Transduction Pathway In Vitro

SUSAN G. MACDONALD,[1]* CRAIG M. CREWS,[2] LELIA WU,[1] JONATHAN DRILLER,[1] ROBIN CLARK,[1] R. L. ERIKSON,[2] AND FRANK MCCORMICK[1]

Onyx Pharmaceuticals, Richmond, California 94806,[1] and Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02138[2]

Received 14 June 1993/Returned for modification 30 June 1993/Accepted 6 August 1993

Raf-1 is a serine/threonine kinase which is essential in cell growth and differentiation. Tyrosine kinase oncogenes and receptors and $p21^{ras}$ can activate Raf-1, and recent studies have suggested that Raf-1 functions upstream of MEK (MAP/ERK kinase), which phosphorylates and activates ERK. To determine whether or not Raf-1 directly activates MEK, we developed an in vitro assay with purified recombinant proteins. Epitope-tagged versions of Raf-1 and MEK and kinase-inactive mutants of each protein were expressed in Sf9 cells, and ERK1 was purified as a glutathione *S*-transferase fusion protein from bacteria. Raf-1 purified from Sf9 cells which had been coinfected with v-*src* or v-*ras* was able to phosphorylate kinase-active and kinase-inactive MEK. A kinase-inactive version of Raf-1 purified from cells that had been coinfected with v-*src* or v-*ras* was not able to phosphorylate MEK. Raf-1 phosphorylation of MEK activated it, as judged by its ability to stimulate the phosphorylation of myelin basic protein by glutathione *S*-transferase–ERK1. We conclude that MEK is a direct substrate of Raf-1 and that the activation of MEK by Raf-1 is due to phosphorylation by Raf-1, which is sufficient for MEK activation. We also tested the ability of protein kinase C to activate Raf-1 and found that, although protein kinase C phosphorylation of Raf-1 was able to stimulate its autokinase activity, it did not stimulate its ability to phosphorylate MEK.

The serine/threonine kinase Raf-1 is activated by many growth factors, including epidermal growth factor, platelet-derived growth factor, erythropoietin, and insulin (1, 3, 4, 6, 10, 12, 13, 16, 22, 23) and is believed to play a central role in cell growth. Several studies have shown that Raf-1 functions downstream of and is required for signalling by receptor and nonreceptor tyrosine kinases and Ras in mammalian cells, in *Drosophila melanogaster*, and in *Caenorhabditis elegans*. That Raf-1 acts downstream of Ras is indicated by experiments in which *raf-1* antisense RNA and kinase-defective Raf-1 mutants were able to block cell proliferation and transformation by activated Ras (15). The same study also used the expression of *raf-1* antisense RNA and kinase-defective Raf-1 mutants to show that Raf-1 is required for the normal growth of the cells. Similarly, the involvement of Ras in the mitogen-activated protein (MAP) kinase pathway is well established (20, 24, 26, 27, 31). However, the mechanism by which Src and Ras activate Raf-1 is not known. It has been suggested that the activation of Raf-1 is mediated by yet another kinase whose activity is stimulated by Ras and Src. The hypothesis that Raf-1 is positively regulated by phosphorylation of serine and/or threonine residues is supported by observations that stimulation of growth factor receptors and expression of membrane-bound oncogene products result in the hyperphosphorylation of Raf-1 and an increase in its kinase activity (3, 12, 13, 15, 16, 22, 23). A more recent study showed protein kinase Cα (PKC)-induced phosphorylation of Raf-1 led to increased autokinase activity of Raf-1 (25). It is not yet clear, however, whether the observed phosphorylation on Raf-1 is a consequence, rather than a cause, of its activation.

The study of Raf-1 and the events initiating its activation has been hindered by the lack of sufficient quantities of pure Raf with which to use in vitro assays of its activity and because physiological substrates of Raf-1 have been unavailable. Furthermore, until recently the position of Raf-1, with respect to MAP kinase, in the Ras-MAP kinase pathway was disputed. It has been reported that MAP kinase phosphorylates Raf-1 (2, 19). However, in one study (19), the stoichiometry of phosphorylation was determined to be very low, and because denatured Raf-1 was used in the assays, the effect of this phosphorylation on Raf-1 activity could not be determined. In the other study (2), stoichiometric measurements were not possible and no effect of the MAP kinase phosphorylation on Raf-1 was observed. Several reports have since suggested that MEK (MAP/ERK kinase = MAP kinase kinase = MAP kinase activator), obtained from fractionated cell lysates, is a physiological substrate of Raf-1 (9, 11, 17): in v-*raf*-transformed cells, both MEK and MAP kinase are constitutively active, and in growth factor-stimulated cells, immunoprecipitated Raf-1 is able to stimulate MAP kinase kinase activity in partially purified preparations. These data thus placed Raf-1 upstream of MAP kinase and its activator but could not rule out the presence of an additional kinase, either in the Raf-1 immunoprecipitate or the MEK preparation, that was responsible for the direct activation of MEK via Raf-1. We sought to reconstitute the Raf-1–MEK interaction in vitro by expressing epitope-tagged versions of Raf and MEK in a baculovirus expression system and using immunoaffinity-purified proteins in an in vitro kinase assay. We found that Raf-1, which has been activated by coinfection with either v-*src* or v-*ras* baculovirus, is sufficient to phosphorylate and activate MEK in vitro, thus confirming the identity of MEK as the first physiologically relevant Raf-1 substrate. Furthermore, we

* Corresponding author.

show that PKC is able to phosphorylate, but not activate, Raf-1.

MATERIALS AND METHODS

cDNAs and baculoviruses. Raf-1 was cloned from human placenta by polymerase chain reaction, and the sequence was verified by dideoxynucleotide sequencing. Primers included restriction sites and a peptide epitope tag encoding EYMPME (Glu-Glu tag) on the C-terminal end of the molecule. MEK was cloned as previously described (8). A Glu-Glu epitope tag was placed on MEK by generating a 150-bp fragment of MEK by polymerase chain reaction. The tagged fragment was then subcloned into MEK by fragment switching. Mutations of the catalytic lysines to alanines were generated by single-stranded site-directed mutagenesis and verified by dideoxynucleotide sequencing. Clones were subcloned into pAcC13 and transfected into Sf9 cells to generate recombinant proteins. Coinfections of v-*ras* or v-*src* with *raf-1* were done so that each cell received an approximately 1:1 ratio of each virus. The v-*src* and v-*ras* cDNAs were not epitope tagged.

Immunoaffinity purification of proteins from Sf9 cells. Approximately 48 h after infection of Sf9 cells with recombinant baculovirus, cells were harvested and frozen in liquid nitrogen. Cell pellets were lysed in 5 volumes of hypotonic buffer [20 mM Tris-HCl (pH 8.2), 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N'*-tetraacetic acid containing 10 μg of leupeptin per ml and 1 mM colchicine. The insoluble material was pelleted by centrifugation at 12,000 × *g* at 4°C for 10 min. NaCl and *n*-octylglucoside were added to the supernatant to 80 mM and 0.1%, respectively, and incubated for 10 min at room temperature. The solution was again subjected to centrifugation at 12,000 × *g* at 4°C for 10 min. The resulting supernatant was twice applied to a Glu-Glu monoclonal antibody affinity column. The column was sequentially washed with 10 volumes each of hypotonic lysis buffer (HLB) containing 100 mM NaCl and 0.1% *n*-octylglucoside, HLB containing 0.5% Nonidet P-40, HLB containing 500 mM NaCl and 4 mM β-mercaptoethanol, and HLB. The column was eluted in six one-column volume fractions with 20 mM Tris-HCl (pH 8.2) containing 100 mM NaCl, 2 mM β-mercaptoethanol, and 50 μg of Glu-Glu peptide per ml.

Purification of GST-ERK1. GST-ERK1 was purified from bacterial cells as previously described (7).

In vitro kinase assays. Recombinant Raf-1 was incubated alone or with various combinations of recombinant MEK, GST-ERK, and myelin basic protein (MBP) in kinase buffer (40 mM Tris-HCl [pH 7.5] containing 80 mM NaCl, 8 mM MgCl2, 0.8 mM dithiothreitol, 50 μM ATP, and 5 μCi of [γ-^{32}P]ATP) in a total volume of 50 μl. PKC (0.4 U/μl; Calbiochem) was used in some assays and activated by the inclusion of 2 mM $CaCl_2$ and 10 μM phorbol 12-myristate 13-acetate and 280 μg of phosphatidyl serine per ml. Reaction mixtures were incubated at 30°C for 20 min and were terminated by the addition of Laemmli sample buffer. Proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the gels were stained, dried, and exposed to X-ray film. Some gels were quantitated by using an AMBIS beta scanner.

RESULTS

Expression of recombinant proteins. To purify sufficient quantities of Raf-1 and MEK for use in in vitro kinase assays, peptide epitope (Glu-Glu) tags were engineered into the clones. To allow for distinctions between autokinase activity of Raf-1 and MEK and transphosphorylations, a version of each protein in which the catalytic lysine was mutated to destroy its kinase activity was also made. Figure 1 shows the location of the Glu-Glu epitope in each protein and indicates the mutated residues. Recombinant baculoviruses encoding these clones were used to infect Sf9 cells. Since a previous report had indicated that coexpression of pp60$^{v\text{-}src}$ and p21$^{v\text{-}ras}$ with Raf-1 was necessary to obtain kinase-active Raf-1 (30), Raf-1 was also coexpressed in insect cells with either v-*src* or v-*ras* baculovirus. The recombinant proteins were purified from cell lysates by using Glu-Glu monoclonal antibody columns. It is important to note that the pp60$^{v\text{-}src}$- and p21$^{v\text{-}ras}$-encoding baculoviruses did not contain epitope tags and so were not copurified with

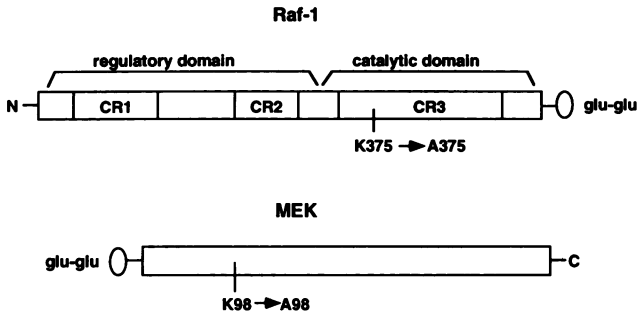

FIG. 1. Structures of the Raf-1 and MEK proteins. Raf-1 contains three consensus regions bearing homologies to regions in other Raf proteins and other unrelated proteins. CR1 contains a zinc finger region homologous to those found in PKC and *N*-chimerin. CR2 is rich in serine and threonine residues which are potential sites for regulatory phosphorylation. CR3 bears a consensus region possessed by all known protein kinases, indicating that this region contains the kinase domain of Raf-1. The amino-terminal half of the protein encodes the regulatory domain of Raf-1. The catalytic lysine and the corresponding mutated residue in each protein are indicated. The locations of the Glu-Glu epitope tags on each protein are also indicated.

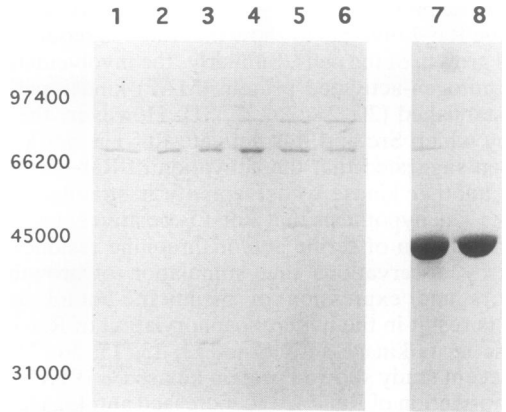

FIG. 2. SDS-PAGE analysis of purified Raf-1 and MEK. Lane 1, Raf-1A; lane 2, Raf-1G; lane 3, Raf-1A that had been coexpressed with v-Src baculovirus; lane 4, Raf-1G that had been coexpressed with v-Src baculovirus; lane 5, Raf-1A that had been coexpressed with v-Ras baculovirus; lane 6, Raf-1G that had been coexpressed with v-Ras baculovirus; lane 7, MEK; lane 8, MEKB. Each preparation was loaded onto an SDS–10% polyacrylamide gel. Molecular mass standards are indicated in daltons.

the Raf-1. Figure 2 shows SDS-PAGE analysis of such immunopurified protein preparations. The identities of the purified Raf-1 and MEK proteins were verified by Western blotting (immunoblotting) with a monoclonal antibody directed against the Glu-Glu epitope tag (not shown). The expression of MEK, as assessed by Coomassie blue staining and Western blotting, was much greater than that of Raf-1. Furthermore, at least 50% of the expressed Raf-1 was found to be insoluble, although sufficient quantities of soluble Raf-1 were easily obtained from cell lysates. Because the Raf-1 was determined, by Western blotting and amino acid sequencing, to be associated with α and β tubulin (20a), colchicine was included in the lysis buffer to disrupt the dimers and their association with Raf-1 and improve the purity of the Raf-1 preparations. This strategy has been successfully used previously by Bollag and colleagues (5) to remove tubulin from baculovirus-expressed neurofibromin. Several other proteins copurified with Raf-1 despite attempts to remove them with stringent washing conditions. No differences were seen, however, either in quantity or composition, in the copurifying proteins when comparing Raf-1 (Raf-1A), kinase-inactive Raf-1 (Raf-1G), Raf-1 that had been coexpressed with v-Src or v-Ras (Raf-1A/v-Src or Raf-1A/v-Ras) and kinase-inactive Raf-1 that had been coinfected with v-Src or v-Ras (Raf-1G/v-Src or Raf-1G/v-Ras).

Activated Raf-1 directly phosphorylates MEK. The purified Raf-1 and MEK were then used in in vitro kinase assays to assess their respective activities. After incubation with magnesium and [γ-^{32}P]ATP, the proteins were resolved by SDS-PAGE and subjected to autoradiography. A typical result is shown in Fig. 3A. When expressed by itself, Raf-1A does not exhibit autokinase activity, nor does it phosphorylate MEK or kinase-inactive MEK (MEKB). MEK itself possesses very little autokinase activity, and MEKB is devoid of kinase activity. In contrast, *raf-1A* that had been coinfected with v-*ras* or v-*src* baculovirus was able to phosphorylate both itself and MEK. The ability of Raf-1 to phosphorylate a kinase-inactive version of MEK suggested that the phosphate incorporation on MEK was due to the activity of the Raf-1 kinase as opposed to the stimulation of MEK autokinase activity by Raf-1. Quantitative analysis of the autoradiogram by using an AMBIS beta scanner showed that MEKB had incorporated more radioactive phosphate than that did MEK, suggesting that it is a better substrate than MEK. But because MEK, and not MEKB, is probably able to autophosphorylate to some extent in the Sf9 cells, this phenomenon may be a reflection of a greater number of sites available for in vitro phosphorylation on MEKB. A similar analysis of the autophosphorylated Raf-1A bands showed that more radioactive phosphate had been incorporated into the Raf-1A purified from cells that had been coinfected with v-Src than the Raf-1A that had been purified from cells coinfected with v-Ras. Correspondingly, similar amounts of Raf-1A/v-Src stimulated more radioactive phosphorylate incorporation into MEK than did Raf-1A/v-Ras. While the reason for this is not known, preliminary kinetic analyses (20a) suggest that it is not a result of an intrinsic difference in the activities of each preparation but rather may reflect differences in the relative amounts of activated Raf-1 in each preparation.

Since other proteins were seen in the Raf-1 preparations, it was possible that a coeluting, comigrating insect cell kinase was responsible for the phosphorylation of both Raf-1 and MEK. To eliminate this possibility, the assay was repeated with kinase-inactive *raf-1* (Raf-1G) that had also been coinfected with either v-*src* or v-*ras* baculovirus (Fig. 3B). While some background phosphorylation is seen on the coinfected Raf-1G, there is no increase in phosphate incorporation in MEK or MEKB by these mutated Raf-1 proteins. It is formally possible that a contaminating kinase, which comigrates only with the kinase-active form of Raf-1, is responsible for the phosphorylation of MEK. However, the data strongly suggest that Raf-1, activated by either v-Src or v-Ras, directly phosphorylates MEK.

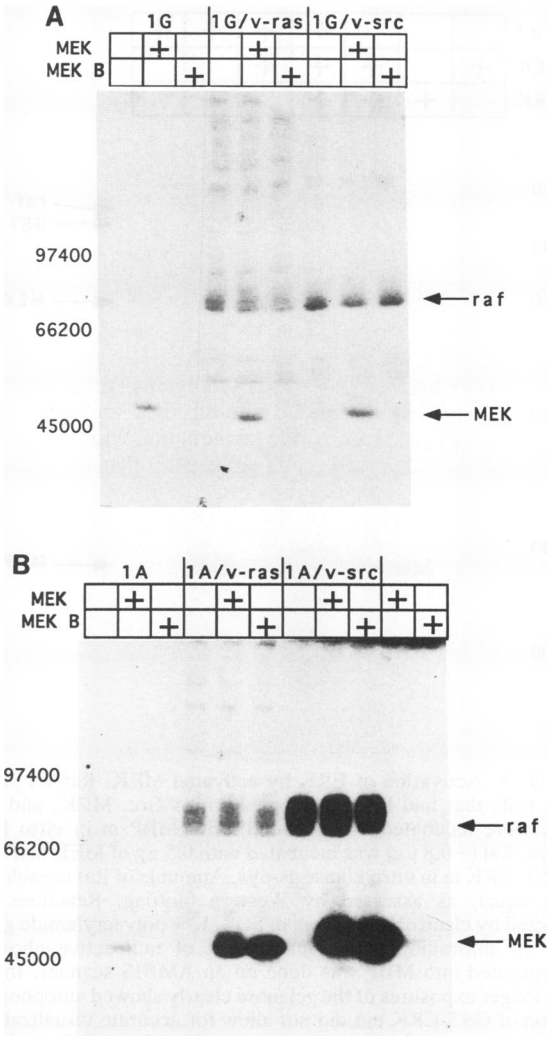

FIG. 3. (A) Phosphorylation of MEK by Raf-1A. 1A, Raf-1A that had been coinfected with v-Src and Raf-1A that had been coinfected with v-Ras were used in in vitro kinase assays with MEK or MEKB. (B) 1G, Raf-1G that had been coinfected with v-Src and Raf-1G that had been coinfected with v-Ras were used in in vitro kinase assays with MEK or MEKB; 0.25 to 0.5 μg of Raf and 0.5 μg of MEK or MEKB was used in each assay. Amounts of Raf in each assay were equal, as assessed by Western blotting. Reactions were analyzed by electrophoresis on SDS–7.5% polyacrylamide gels, Coomassie blue staining, and autoradiography. Molecular mass standards are indicated in daltons.

Raf-1-phosphorylated MEK activates ERK1. Recent studies showing activation of partially purified MEK by immunoprecipitated Raf-1 also linked the phosphorylation of MEK to the activation of ERK (9, 11, 17). In addition, previous work has shown that a bacterially expressed glutathione *S*-transferase (GST)–MEK fusion protein was able to

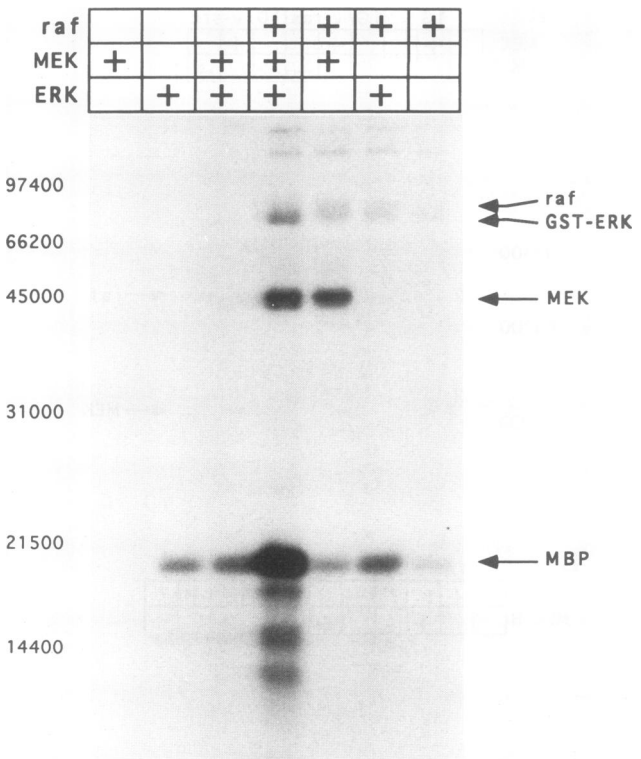

FIG. 4. Activation of ERK by activated MEK. Raf-1A purified from cells that had been coinfected with v-Src, MEK, and GST-ERK were incubated, as indicated, with MBP in in vitro kinase assays. Raf (~0.8 μg) was incubated with 0.5 μg of MEK and 0.4 μg of GST-ERK in in vitro kinase assays. Amounts of Raf in each assay were equal, as assessed by Western blotting. Reactions were analyzed by electrophoresis on an SDS–12% polyacrylamide gel and then by autoradiography. Quantitation of radioactive phosphate incorporated into MBP was done on an AMBIS scanner. In addition, longer exposures of the gel more clearly showed autophosphorylation of GST-ERK but did not allow for accurate visualization of the MBP in the lane containing activated Raf-1, MEK, and GST-ERK, as the linear range of the X-ray film was exceeded. Molecular mass standards are indicated in daltons.

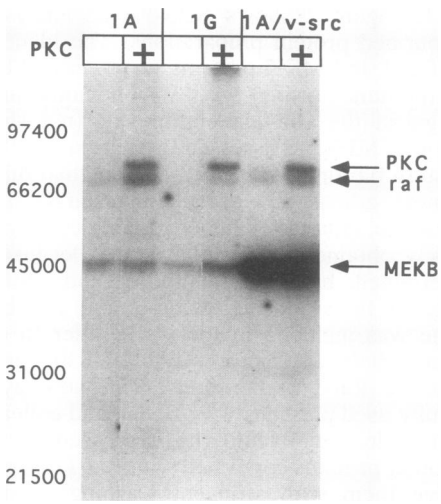

FIG. 5. Phosphorylation of Raf-1 by PKC. Raf-1A, Raf-1G, and Raf-1A/v-Src were purified and incubated with PKC and MEKB, as indicated, in in vitro kinase assays. Amounts of Raf in each assay were equal, as assessed by Western blotting. Reactions were analyzed by electrophoresis on an SDS–10% polyacrylamide gel and then by autoradiography. Similar results were achieved in three separate experiments. The slight apparent increase in the phosphorylation of MEKB in the lane containing PKC, Raf-1G, and MEKB is due to bleed-over from the adjacent lane, in which MEKB is robustly phosphorylated by activated Raf-1. Molecular mass standards are indicated in daltons.

phosphorylate bacterially expressed GST-ERK1 (GST-ERK) to a limited extent (8). Because the bacteria presumably did not express the appropriate MEK-activating molecules, this may not have been representative of the true catalytic potential of the enzyme. In an attempt to extend these observations with purified proteins and show whether the phosphorylation of MEK by activated Raf-1 was of physiological relevance, we reconstituted the putative Raf-1–MEK–ERK pathway in vitro, using MBP as a substrate for GST-ERK (Fig. 4). Although the GST-ERK possesses some catalytic activity by itself (7), only when Raf-1A proteins activated by v-Src, MEK, and GST-ERK were included in the assay was increased phosphorylation on MBP observed. When activated Raf-1, MEK, and GST-ERK were included in the same reaction mixture, 13,927 cpm was associated with MBP, whereas in all other reactions, cpm associated with MBP was 2,092 or less. It is also noteworthy that GST-ERK was not able to increase phosphorylation on Raf-1A coexpressed with v-Src or on Raf-1A that had been expressed alone in insect cells, as it was previously reported that ERK is able to phosphorylate Raf-1 (2, 19). The data then show that Raf-1 directly activates MEK, which subsequently leads to the activation of ERK.

PKC phosphorylates, but does not activate, Raf-1. Since it has been reported that PKC is able to phosphorylate and activate Raf-1 (14, 25), we sought to test this activation by using MEK as a substrate for Raf-1. Figure 5 shows that a mixture of PKCα, -β, and -γ purified from brain tissue was able to stimulate radioactive phosphate incorporation into Raf-1A and to a lesser extent into Raf-1G, confirming that PKC can phosphorylate Raf-1 and stimulate its autokinase activity. Surprisingly, the phosphorylation of these PKC-stimulated modifications of Raf-1A by PKC did not result in a conformation of Raf-1 that was able to phosphorylate MEK. The level of Raf-1A phosphorylation observed was similar to that observed on Raf-1A/v-Src, as assessed by scanning the autoradiogram on an AMBIS beta scanner, showing that the inability of PKC-phosphorylated Raf-1A to phosphorylate MEK was not because of a lesser activation than that of Raf-1A/v-Src. These results suggest that autophosphorylation of Raf-1 may not be a reliable indicator of its physiological activity.

DISCUSSION

Recent reports suggesting that MEK is a physiological substrate of Raf-1 (9, 11, 17) have not been able to eliminate the possibility that another kinase, associated with the MEK fractions or the Raf-1 immunoprecipitates, might be responsible for the direct phosphorylation of MEK. In order to test the ability of Raf-1 to directly phosphorylate MEK, we developed an in vitro system for the assay of Raf-1 activity. We utilized the baculovirus insect expression system to produce recombinant versions of Raf-1 and MEK, which were subsequently used in vitro kinase assays. This expression system, combined with the use of genetically engi-

neered peptide epitope tags, allowed for the simple, rapid purification of large amounts of protein. We found that Raf-1A expressed alone in Sf9 cells possessed no catalytic activity, as judged by its inability to autophosphorylate and to phosphorylate MEK or a kinase-inactive mutant of MEK. When coexpressed with v-Src or v-Ras, however, Raf-1A exhibited autokinase activity and was able to phosphorylate MEK. In addition, when the Raf-1–MEK–ERK kinase cascade was reconstituted in vitro, Raf-1-phosphorylated MEK was demonstrated to activate ERK. Furthermore, attempts to activate Raf-1 in vitro with PKC were not successful, despite previous reports proposing PKC activates Raf-1 (14, 25).

Although the direct mechanism of activation of Raf-1 by Src and Ras is not evident from these studies, the utility of the baculovirus system for the expression of activated Raf-1 through the use of Sf9 cells as a "test tube" is evident. Williams et al. (30) have reported that coexpression of either v-Src or v-Ras with Raf-1 activates Raf-1 autokinase activity only to a limited extent and that triple infection with *raf-1*, v-*ras*, and v-*src* baculoviruses is necessary to fully activate Raf-1 in Sf9 cells. By using the phosphorylation of MEK as a measure of full activation of Raf-1, we found that coexpression of Raf-1 with either v-Src or v-Ras was sufficient to fully activate Raf-1. Although it did appear that the v-Src-coexpressed Raf-1 possessed a higher level of kinase activity than that of the v-Ras-coexpressed Raf-1, it could not be ascertained whether this apparent discrepancy merely reflected differences in the relative amounts of activated Raf-1 in each preparation or whether there was an intrinsic difference in their activities. However, preliminary kinetic data suggest that the intrinsic activity of each form of activated Raf-1 is similar, if not identical. The differences observed in the activity of Raf-1 from each coinfection may simply be a function of the relative expression levels of each oncogene in the preparations or the proportion of cells that are actually coinfected with both viruses. This might also account for the apparent differences, with respect to the determination of full activation of Raf-1, between our data and those of Williams et al. (30). Nonetheless, it is clear that both Ras and Src are capable of activating Raf-1.

The use of immunoprecipitated Raf-1 and partially purified MEK in previous studies (9, 11, 17) left open the possibility that a contaminating kinase was responsible for the phosphorylation of MEK, rather than a direct interaction between MEK and Raf-1. The current studies show that it is unlikely that a contaminating kinase in the Raf-1 preparations is responsible for the phosphorylation of MEK, as kinase-inactive mutants of Raf-1 coinfected with v-Src or v-Ras baculovirus are unable to phosphorylate MEK. In addition, no other radiolabelled bands are seen on autoradiograms of Raf-1–MEK kinase reactions, suggesting that if another kinase is responsible for the phosphorylation of MEK, it must comigrate and associate only with the kinase-competent version of Raf-1. In order to designate the phosphorylation of MEK by Raf-1 as a physiological regulatory event, it was important to show that the phosphorylation of MEK by Raf-1 led to the activation of MEK. Therefore, purified active Raf-1 was incubated in vitro with MEK, GST-ERK, and MBP in an attempt to stimulate the ability of ERK to phosphorylate MBP. It was found that MEK, phosphorylated by activated Raf-1, was able to stimulate the incorporation of phosphate into MBP by ERK. Together, these data show that MEK is a substrate for activated Raf-1 and that MEK thus phosphorylated can activate ERK. In comparison, Williams et al. (29) have used the baculovirus system to reconstitute the Ras-to-ERK pathway in Sf9 cells. Using double or triple infections with combinations of v-*src*, v-*ras*, v-*raf*, *raf-1*, and ERK baculoviruses, they conclude that the activation of ERK by Ras can occur via Raf-1-dependent and Raf-1-independent pathways. However, MEK was not included in these infections, but rather the insect cell homolog of MEK was relied upon to feed into ERK, thus not allowing the assessment of its activity under different conditions. In addition, because the sequential activations were achieved in the insect cells themselves and then the activities of Raf-1 and ERK were assessed, there exists the possibility that some of the activities have been altered by feedback in the cells, as was noted by the authors, thus obscuring the primary responses of Raf-1 and ERK to stimulation by Ras and Src. The cloning of a MEK kinase that is distinct from Raf-1 (18), however, suggests that there may indeed exist multiple pathways that lead to the activation of MEK and, perhaps, ERK. Reconstitution of these pathways in vitro will answer some of these questions.

Because we were able to activate Raf-1 in insect cells by coinfection with baculoviruses encoding $pp60^{v\text{-}src}$ and $p21^{v\text{-}ras}$, the data presented further support the model that Raf-1 lies downstream of both $pp60^{src}$ and $p21^{ras}$. The activation of Raf-1 by $pp60^{v\text{-}src}$, however, does not appear to be via a direct mechanism, as both phosphoamino acid analyses and antiphosphotyrosine blotting of Raf-1 purified from insect cells coinfected with that baculovirus have shown no phosphotyrosine (20a). Furthermore, $p21^{v\text{-}ras}$ alone also does not appear to directly activate Raf-1, as experiments in which we have included purified, processed Ras in in vitro assays with Raf-1 have not resulted in the activation of Raf-1, nor is there any immunodetectable Ras in our active Raf-1 preparations (20a).

We also show that Raf-1 is not activated by phosphorylation by PKC. The recent conclusions that PKC directly phosphorylates and activates Raf-1 (14, 25) were based on the observation that Raf-1, phosphorylated by PKC, was able to autophosphorylate. Whether PKC alone can activate Raf-1, however, is another question, since different studies have shown that the activation of Raf-1 appears to be dependent on PKC in some systems and independent of PKC in other systems. While our data do not conflict with the observation that PKC can phosphorylate Raf-1, the use of the physiological substrate, MEK, to assess the activation state of Raf-1 allowed us to discriminate between autophosphorylation and activation of Raf-1. As many studies have relied upon the autophosphorylation of Raf-1 as a measure of its activity, this is an important result, suggesting that earlier studies using Raf-1 autophosphorylation as a readout of Raf-1 activation may require reassessment. From our data, we conclude that autophosphorylation of Raf-1 may not be a reliable measure of the activation of Raf-1. Alternatively, phosphorylation by PKC may activate Raf-1 for a specific, as yet unidentified, substrate, thereby rendering autophosphorylation a relevant phenomenon. In the recent report by Kolch et al. (14), it is suggested that PKC activation of Raf-1 is mechanistically different from the activation of Raf-1 by receptor tyrosine kinases. If this is true, then perhaps the phosphorylation of Raf-1 by PKC does allow it to phosphorylate a substrate other than MEK. The identification of other Raf-1 substrates will answer this question. The agent(s) responsible for the activation of Raf-1, then, is not discernible from these studies. Our own data, in conjunction with others', indicates, however, that the activation of Raf-1 may involve more than one event. For instance, several reports have recently demonstrated a direct interaction

between RasGTP and Raf-1 (21, 28, 32). Thus, Ras may interact permissively with Raf-1, allowing another regulatory molecule (kinase?) to activate the Raf-1 kinase. Current studies in our laboratory are addressing these issues.

ACKNOWLEDGMENTS

We thank Walter Crosier for expert technical assistance.

S.G.M. is a fellow of the National Cancer Institute (CA60443). This work was supported, in part, by NIH grant CA42580 (R.L.E.) and NIH Genetics training grant GM07620-14 (C.M.C.).

REFERENCES

1. **Ambrosio, L., A. P. Mahowald, and N. Perrimon.** 1989. Requirement of the *Drosophila raf* homologue for *torso* function. Nature (London) **342**:288–290.
2. **Anderson, N. G., P. Li, L. A. Marsden, N. Williams, T. M. Roberts, and T. W. Sturgill.** 1991. Raf-1 is a potential substrate for mitogen-activated protein kinase *in vivo*. Biochem. J. **277**: 573–576.
3. **App, H., R. Hazan, A. Zilberstein, A. Ullrich, J. Schlessinger, and U. Rapp.** 1991. Epidermal growth factor (EGF) stimulates association and kinase activity of raf-1 with the EGF receptor. Mol. Cell. Biol. **11**:913–919.
4. **Baccarini, M., G. N. Gill, and E. R. Stanley.** 1991. Epidermal growth factor stimulates phosphorylation of RAF-1 independently of receptor autophosphorylation and internalization. J. Biol. Chem. **266**:10941–10945.
5. **Bollag, G., F. McCormick, and R. Clark.** 1993. Characterization of full-length neurofibromin: tubulin inhibits Ras GAP activity. EMBO J. **12**:1923–1927.
6. **Carroll, M. P., J. L. Spivak, M. McMahon, N. Weich, U. R. Rapp, and W. S. May.** 1991. Erythropoietin induces raf-1 activation and raf-1 is required for erythropoietin-mediated proliferation. J. Biol. Chem. **266**:14964–14969.
7. **Crews, C. M., A. Alessandrini, and R. L. Erikson.** 1991. Mouse *Erk-1* gene product is a serine/threonine protein kinase that has the potential to phosphorylate tyrosine. Proc. Natl. Acad. Sci. USA **88**:8845–8849.
8. **Crews, C. M., A. Alessandrini, and R. L. Erikson.** 1992. The primary structure of MEK, a protein kinase that phosphorylates the *ERK* gene product. Science **258**:478–480.
9. **Dent, P., W. Haser, T. A. J. Haystead, L. A. Vincent, T. M. Roberts, and T. W. Sturgill.** 1992. Activation of mitogen-activated protein kinase kinase by v-raf in NIH 3T3 cells and in vitro. Science **257**:1404–1407.
10. **Dickson, B., F. Sprenger, D. Morrison, and E. Hafen.** 1992. Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. Nature (London) **360**:600–602.
11. **Howe, L. R., S. J. Leevers, N. Gomez, S. Nakielny, P. Cohen, and C. J. Marshall.** 1992. Activation of the MAP kinase pathway by the protein kinase raf. Cell **71**:335–342.
12. **Izumi, T., H. Tamemoto, M. Nagao, T. Kadowaki, F. Takaku, and M. Kasuga.** 1991. Insulin and platelet-derived growth factor stimulate phosphorylation of the c-*raf* product at serine and threonine residues in intact cells. J. Biol. Chem. **266**:7933–7939.
13. **Kizaka-Kondoh, S., K. Sato, K. Tamura, H. Nojima, and H. Okayama.** 1992. Raf-1 protein kinase is an integral component of the oncogenic signal cascade shared by epidermal growth factor and platelet-derived growth factor. Mol. Cell. Biol. **12**:5078–5086.
14. **Kolch, W., G. Heidecker, G. Kochs, R. Hummel, H. Vahidi, H. Mischak, G. Finkenzeller, D. Marme, and U. R. Rapp.** 1993. Protein kinase Cα activates RAF-1 by direct phosphorylation. Nature (London) **364**:249–252.
15. **Kolch, W., G. Heidecker, P. Lloyd, and U. R. Rapp.** 1991. Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. Nature (London) **349**:426–428.
16. **Kovacina, K. S., K. Yonezawa, D. L. Brautigan, N. K. Tonks, U. R. Rapp, and R. A. Roth.** 1990. Insulin activates the kinase activity of the raf-1 proto-oncogene by increasing its serine phosphorylation. J. Biol. Chem. **265**:12115–12118.
17. **Kyriakis, J. M., H. App, X.-F. Zhang, P. Banerjee, D. L. Brautigan, U. R. Rapp, and J. Avruch.** 1992. Raf-1 activates MAP kinase-kinase. Nature (London) **358**:417–421.
18. **Lange-Carter, C. A., C. M. Pleiman, A. M. Gardner, K. J. Blumer, and G. L. Johnson.** 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science **260**:315–319.
19. **Lee, R.-M., M. H. Cobb, and P. J. Blackshear.** 1992. Evidence that extracellular signal-regulated kinases are the insulin-activated Raf-1 kinase kinases. J. Biol. Chem. **267**:1088–1092.
20. **Leevers, S. J., and C. J. Marshall.** 1992. Activation of extracellular signal-regulated kinase, ERK2, by p21*ras* oncoprotein. EMBO J. **11**:569–574.

20a. **Macdonald, S. G., and F. M. McCormick.** Unpublished data.

21. **Moodie, S. A., B. M. Willumsen, M. J. Weber, and A. Wolfman.** 1993. Complexes of RasGTP with Raf-1 and mitogen-activated protein kinase kinase. Science **260**:1658–1662.
22. **Morrison, D. K., D. R. Kaplan, J. A. Escobedo, U. R. Rapp, T. M. Roberts, and L. T. Williams.** 1989. Direct activation of the serine/threonine kinase activity of Raf-1 through tyrosine phosphorylation by the PDGF β-receptor. Cell **58**:649–657.
23. **Morrison, D. K., D. R. Kaplan, U. Rapp, and T. M. Roberts.** 1988. Signal transduction from membrane to cytoplasm: growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. Proc. Natl. Acad. Sci. USA **85**:8855–8859.
24. **Reed, J. C., S. Yum, M. P. Cuddy, B. C. Turner, and U. R. Rapp.** 1991. Differential regulation of the p72-74 RAF-1 kinase in 3T3 fibroblasts expressing *ras* or *src* oncogenes. Cell Growth Diff. **2**:235–243.
25. **Sozeri, O., K. Vollmer, M. Liyanage, D. Frith, G. Kour, G. E. I. Mark, and S. Stabel.** 1992. Activation of the c-Raf protein kinase by protein kinase C phosphorylation. Oncogene **7**:2259–2262.
26. **Thomas, S. M., M. DeMarco, G. D'Arcangelo, S. Halegoua, and J. S. Brugge.** 1992. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. Cell **68**:1031–1040.
27. **Tsuda, L., Y. Inoue, M.-A. Yoo, M. Mizuno, M. Hata, Y.-M. Lim, T. Adachi-Yamada, H. Ryo, Y. Masamune, and Y. Nishida.** 1993. A protein kinase similar to MAP kinase activator acts downstream of the Raf kinase in Drosophila. Cell **72**:407–414.
28. **Warne, P. H., P. R. Viciana, and J. Downward.** 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 *in vitro*. Nature (London) **364**:352–355.
29. **Williams, N. G., H. Paradis, S. Agarwal, D. L. Charest, S. L. Pelech, and T. M. Roberts.** 1993. Raf-1 and p21v-ras cooperate in the activation of mitogen-activated protein kinase. Proc. Natl. Acad. Sci. USA **90**:5772–5776.
30. **Williams, N. G., T. M. Roberts, and P. Li.** 1992. Both $p21^{ras}$ and $pp60^{v\text{-}src}$ are required, but neither alone is sufficient to activate the Raf-1 kinase. Proc. Natl. Acad. Sci. USA **89**:2922–2926.
31. **Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis.** 1992. *ras* mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1 and RSK. Cell **68**:1041–1050.
32. **Zhang, X.-F., J. Settleman, J. M. Kyriakis, E. Suzuki-Takeuchi, S. J. Elledge, M. S. Marshall, J. T. Bruder, U. R. Rapp, and J. Avruch.** 1993. Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature (London) **364**:308–313.