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Regulation of Raf-1 and Raf-1 Mutants by Ras-Dependent and Ras-Independent Mechanisms In Vitro

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The serine/threonine kinase Raf-1 functions downstream from Ras to activate mitogen-activated protein kinase kinase, but the mechanisms of Raf-1 activation are incompletely understood. To dissect these mechanisms, wild-type and mutant Raf-1 proteins were studied in an in vitro system with purified plasma membranes from v-Ras- and v-Src-transformed cells (transformed membranes). Wild-type $(His)_6$- and FLAG-Raf-1 were activated in a Ras- and ATP-dependent manner by transformed membranes; however, Raf-1 proteins that are kinase defective (K375M), that lack an in vivo site(s) of regulatory tyrosine (YY340/341FF) or constitutive serine (S621A) phosphorylation, that do not bind Ras (R89L), or that lack an intact zinc finger (CC165/168SS) were not. Raf-1 proteins lacking putative regulatory sites for an unidentified kinase (S259A) or protein kinase C (S499A) were activated but with apparently reduced efficiency. The kinase(s) responsible for activation by Ras or Src may reside in the plasma membrane, since GTP loading of plasma membranes from quiescent NIH 3T3 cells (parental membranes) induced de novo capacity to activate Raf-1. Wild-type Raf-1, possessing only basal activity, was not activated by parental membranes in the absence of GTP loading. In contrast, Raf-1 Y340D, possessing significant activity, was, surprisingly, stimulated by parental membranes in a Ras-independent manner. The results suggest that activation of Raf-1 by phosphorylation may be permissive for further modulation by another membrane factor, such as a lipid. A factor(s) extracted with methanol-chloroform from transformed membranes or membranes from Sf9 cells coexpressing Ras and Src^{Y527F} significantly enhanced the activity of Raf-1 Y340D or active Raf-1 but not that of inactive Raf-1. Our findings suggest a model for activation of Raf-1, wherein (i) Raf-1 associates with Ras-GTP, (ii) Raf-1 is activated by tyrosine and/or serine phosphorylation, and (iii) Raf-1 activity is further increased by a membrane cofactor.

The proto-oncogene product Raf-1 is a member of a family of serine/threonine protein kinases (Raf-1, B-Raf, and A-Raf) that function in protein kinase cascades important for mitogenic signaling (31). Raf-1 phosphorylates and activates mitogen-activated protein (MAP) kinase kinase (MKK, also known as MEK), the specific activator of MAP kinase (23). MAP kinase in turn phosphorylates several regulatory proteins (4, 21) in the cytoplasm (e.g., PHAS1, $p90^{rsk}$, and $cPLA_2$) and nucleus (e.g., $p62^{TCF}$ and c-Myc) to alter the program of transcription and translation required for mitogenesis. Raf-1 exhibits restricted substrate specificity in vitro (12). Although other in vivo substrates for Raf-1 than MKK may exist, none have been definitively established.

The mechanism of Raf-1 activation is incompletely understood. Activation of Raf-1 in vivo occurs at the plasma membrane (20, 28) and is dependent upon association with Ras-GTP (24). Association of purified Raf-1 and Ras proteins in the presence of ATP in vitro is not sufficient to cause activation. These results suggest the presence of another factor(s) for Raf-1 activation in the plasma membrane, such as protein kinases and/or lipid cofactors.

Raf-1 contains three domains conserved among members of the Raf kinase family, termed CR1, CR2, and CR3 (30). CR1 (residues 62 to 134), near the NH_2 terminus, contains the major region (residues 51 to 131) required for Ras-Raf interaction (14, 34). Mutation of a single arginine (Arg-89) in CR1 to leucine abolishes Ras-Raf binding in vitro and in vivo (9). In addition, CR1 contains two pairs of cysteine residues (Cys-X_2-Cys) which bind zinc and are identically spaced to paired cysteines in members of the protein kinase C (PKC) family (15). The paired cysteines are required for phorbol ester binding and activation of PKC, an observation that has raised the possibility of allosteric interactions of Raf-1 with a lipid cofactor(s). Mutation of one of the paired cysteines, Cys-168, in CR1 to Ser reduces Ras-Raf binding in vitro and in the two-hybrid binding assay (34), suggesting that the paired cysteines may also contribute to Ras-Raf interaction. No functions have been assigned to the short serine- and threonine-rich CR2 domain (32). CR3 is the catalytic domain, comprising the COOH-terminal half of the protein (31).

Raf-1 is phosphorylated in quiescent cells, and increased phosphorylation occurs at several sites in response to stimuli that cause enzymatic activation (9, 19, 23). Ser-259 and Thr-268 in the CR2 domain and Ser-499, Ser-621, and Tyr-340/341 in the CR3 domain have been identified as in vivo Raf-1 phosphorylation sites. Stimulation of fibroblasts with platelet-derived growth factor or phorbol esters has been reported to increase phosphorylation of serines 259 (25) and 499 (19), respectively. Tyrosine 340 and/or 341 becomes phosphorylated when Raf-1 is coexpressed with c-Ha-Ras and Src^{Y527F} in Sf9 cells (9). Serine 621 appears to be phosphorylated constitutively in fibroblasts and Sf9 cells (25).

The ability of phosphorylation site mutants of Raf-1 to be activated in vivo has been examined by using the Sf9 cell system. Raf-1 S621A (10, 25) and YY340/341FF (9) mutants are incapable of being activated in vivo, consistent with but not proving a requirement for phosphorylation at these sites for

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enzymatic activation. Mutation of either serine 259 (25) or serine 499 (19) to alanine does not inhibit the activation of Raf-1 by coexpressed Ras and Src^{Y527F}; mutation of serine 499, however, has been reported to inhibit activation by PKC in this cell system (19).

The activation potential of Raf-1 mutants containing alterations within the CR1 domain has also been analyzed in Sf9 cells. Raf-1 R89L, which does not bind Ras-GTP in vitro, can be activated by coexpression with Src^{Y527F} but not by coexpression with Ras (11). Mutation of Cys-168 to serine blocks activation by Ras, assayed by measuring the Raf-dependent increase of transcription of an AP1/Ets-driven chloramphenicol acetyltransferase reporter, possibly by reducing its affinity for Ras-GTP (34). However, a C165S/C168S double mutant of Raf-1 retains sufficient affinity for Ras-GTP to permit activation of Raf-1 enzymatic activity by Ras in Sf9 cells (26).

Recently, we demonstrated that Raf-1 can be activated in vitro by purified plasma membranes, isolated from cells transformed with v-Ras or v-Src, by an ATP- and Ras-dependent mechanism (7). Studies in such a defined system permit biochemical dissection of the Raf-1 activation mechanisms. Herein, we have dissected mechanisms of Raf-1 activation by systematic examination of a panel of functionally relevant Raf-1 mutants. These studies strongly support a requirement for tyrosine and/or serine phosphorylation for Raf-1 activation by membrane-associated kinase(s) and also suggest that Raf-1 phosphorylation may be permissive for further modulation of its enzymatic activity by Ras-independent interaction with a putative lipid cofactor.

MATERIALS AND METHODS

Materials. Recombinant baculoviruses for expression of tagged wild-type and mutant Raf-1 proteins were generated as described previously (25, 26); FLAG and $(His)_6$ tags, added to facilitate purification, were placed at the NH_2 and the COOH termini, respectively. FLAG is a trademark of IBI-Kodak, New Haven, Conn., for an octapeptide with the sequence DYKDDDDK. Anti-FLAG M2 immunoglobulin G agarose and FLAG peptide were obtained from the same manufacturer. $(His)_6$-MKK1 was expressed in *Escherichia coli* as described previously (13), using a plasmid kindly provided by G. Johnson, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo. Sources of other reagents and cell lines have been given previously (7).

Purification of MKK and Raf-1. The MKK substrate used herein was $(His)_6$-MKK1 from bacterial expression. $(His)_6$-Raf-1 wild-type and mutant proteins, expressed in Sf9 cells by using recombinant baculoviruses (5), and $(His)_6$-MKK1 were purified by sequential Ni^{2+}-chelate and Mono Q chromatography and stored as described previously (5). FLAG-tagged Raf-1 proteins were expressed in Sf9 cells with recombinant baculoviruses and were affinity purified in a single step with anti-FLAG M2 immunoglobulin G linked to agarose. Briefly, Sf9 cells were infected with FLAG-Raf-1 or (His_6)-Raf-1 (5) recombinant baculoviruses (estimated multiplicity of infection, 10), with or without recombinant baculoviruses for c-Ha-Ras and Src^{Y527F} (multiplicity of infection, 2 each), to produce nonactivated or activated Raf-1 proteins, respectively. Sf9 cells expressing FLAG-Raf-1 proteins were homogenized 48 h postinfection in 40 ml of chilled buffer A [500 mM Tris/Cl (pH 7.9, 4°C), 1% (vol/vol) Nonidet P-40, 0.1% (vol/vol) sodium deoxycholate, 0.05% (vol/vol) sodium dodecyl sulfate (SDS), 0.5 mM EDTA, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10% (vol/vol) glycerol, 0.1% (vol/vol) 2-mercaptoethanol] containing a cocktail of protease inhibitors (5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg of egg white trypsin inhibitor per ml, and 40 μg each of *N*-*p*-tosyl-lysine chloromethyl sulfonyl ketone, leupeptin, pepstatin A, L-1-tosylamido-2-phenylethyl chloromethyl ketone, aprotinin, E64 [Sigma-Aldrich], and α_2-macroglobulin per ml) in a motorized Teflon-glass homogenizer. All subsequent steps were either on ice or at 4°C. The supernatant obtained from centrifugation of the homogenate (5,000 × *g* for 10 min) was batch adsorbed to 0.5 ml of anti-FLAG immunoglobulin G-agarose for 2 h. The anti-FLAG agarose was washed twice with 50 ml of buffer A, twice with 50 ml of buffer B (25 mM Tris/Cl [pH 7.9, 4°C], 0.1 mM EDTA, 0.1 mM EGTA, 0.01% [vol/vol] Nonidet P-40, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 10% [vol/vol] glycerol, 0.1% [vol/vol] 2-mercaptoethanol). Adsorbed FLAG-Raf-1 protein was eluted by gentle overnight mixing of the anti-FLAG agarose with 5 ml of buffer B containing 0.1 mg of FLAG peptide per ml. The agarose beads were washed with a further 5 ml of buffer B. The elutate and washings were pooled, dialyzed against buffer B containing 50% (vol/vol) glycerol (omitting Nonidet P-40), and stored at −20°C. FLAG-tagged Raf-1, in contrast to $(His)_6$-tagged Raf-1, was not stable to freezing and thawing. FLAG-Raf-1 was at least 50 to 75% pure as judged by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue or silver staining (see Fig. 3).

Plasma membrane isolation. Parental NIH 3T3, v-Ras-transformed NIH 3T3, and v-Src-transformed Swiss mouse 3T3 fibroblasts were grown to confluence and serum starved as described previously (7). Cells (from 10 100-mm-diameter plates) were washed and scraped into 50 ml of phosphate-buffered saline. The cells were pelleted (1,000 × *g* for 5 min), and the cell pellet was resuspended and homogenized with a motorized Teflon-glass homogenizer in 5 ml of buffer C (25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.6 at 4°C], 8.6% [wt/vol] sucrose, 10 mM EDTA, 0.2% [vol/vol] 2-mercaptoethanol, 10 mM EGTA) containing the same cocktail of protease inhibitors as in buffer A. The homogenate was layered onto 7 ml of buffer C containing 39% (wt/vol) sucrose and centrifuged (31,000 × *g* for 30 min at 4°C) in an SW41 rotor (Beckman, Palo Alto, Calif.). The plasma membrane fraction was collected from the top of the 39% sucrose interface. Collected membranes were washed by being mixed with an equal volume of 1 M Tris/HCl (pH 7.9 at 4°C), diluted (1:35 with chilled water), and reisolated by centrifugation (40,000 × *g* for 30 min at 4°C) in a 50.2 Ti rotor (Beckman). Plasma membranes were resuspended to a final protein concentration of 1 mg/ml in buffer C containing the same cocktail of protease inhibitors as in buffer A. Plasma membranes were kept on ice and used immediately; unused membranes were discarded. Equal portions of plasma membranes, based on total membrane protein, from v-Ras- and v-Src-transformed cells were sonicated (three times for 20 s on ice) as described previously (7).

In vitro Raf-1 activation. Procedures for activation of Raf-1 in vitro, including necessary controls and analysis, were as previously described (7) except for the following modification: MKK substrate (3 μg) was recombinant $(His)_6$-MKK1 expressed in bacteria. Reactions were terminated with sample buffer for SDS-PAGE (11% polyacrylamide gel) to permit quantification of ^{32}P incorporation into excised, Coomassie blue-stained MKK1 protein bands. Control assays were performed to measure MAP kinase kinase kinase (MAPKKK) activity of each individual component (MKK1, Raf-1 protein, and membranes) to calculate expected values of MAPKKK activity, predicted from additivity. ^{32}P incorporation into MKK1 from autophosphorylation was in the range of 500 to 1,000 cpm. Data were expressed as fold increases in MKK1 phosphorylation to permit comparison between experiments (n = 4 to 10) with several membrane and $(His)_6$-Raf-1/FLAG-Raf-1 preparations. In each experiment with Raf mutants, a positive control with wild-type Raf-1 was always included. A two-tailed Student t test was used for statistical comparisons; error bars indicate the standard deviation. In some experiments, activation of MKK by Raf-1 was measured by phosphorylation of kinase-defective MAP kinase (2 μg), with an additional 2-min incubation.

GTP loading of plasma membrane preparations. Portions (0.1 ml) of plasma membranes (0.1 mg of total protein) in buffer C were incubated (15 min at 30°C) with or without 1 mM GTP and also with or without protein phosphatase inhibitors (final concentrations, 10 μM Microcystin-LR [Calbiochem, San Diego, Calif.], 0.2 mM sodium orthovanadate, and 25 mM sodium β-glycerophosphate). Membranes from each condition were repelleted in an Airfuge (Beckman) at approximately 100,000 × *g* for 15 min at 4°C. Special care was taken to remove all the supernatant. Plasma membranes were then resuspended in ice-cold buffer C to the original volume and kept chilled until use. In parallel, portions of the membranes prior and after GTP loading were processed for SDS-PAGE and protein immunoblotting with anti-Raf-1 SP63 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.); endogenous membrane-bound Raf-1 remained membrane bound after this procedure. Plasma membranes from each condition were then assayed as above for the ability to activate wild-type FLAG-Raf-1.

Isolation and assays of FLAG-Raf-1 proteins after incubation with plasma membranes. Membrane treatment and assays of FLAG-Raf-1 wild-type protein for MAPKKK activity and kinase-defective FLAG-Raf-1 K375M for ^{32}P incorporation were performed in two stages. FLAG-Raf-1 and membranes [without $(His)_6$-MKK1] were first incubated as described for the standard in vitro activation, except that either 0.2 mM unlabelled ATP or 1 mM AMPPNP (5′-adenylylimidodiphosphate) was included or ATP was omitted. The reaction was terminated by the addition of EDTA (final concentration, 20 mM), and the mixture was diluted to 1 ml with buffer A. FLAG-Raf-1 was purified from the detergent-solubilized reaction mixtures by adsorption to 0.05 ml of anti-FLAG agarose as described for isolation of FLAG-Raf-1 from Sf9 cells, with a 50-fold scaled-down protocol for washing. FLAG-Raf-1 was not eluted from the agarose beads and was assayed for MAPKKK activity directly with $(His)_6$-MKK1. FLAG-Raf-1 K375M was incubated with membranes as above, except that 0.2 mM [γ-^{32}P]ATP (20 cpm/fmol) was used, isolated as above, released from the beads into SDS-PAGE sample buffer, and subjected to SDS-PAGE (11% polyacrylamide 12- by 15-cm gel) to determine ^{32}P incorporation into FLAG-Raf-1 K375M.

Phosphoamino acid analysis of Raf-1 proteins phosphorylated by plasma membranes. FLAG-Raf-1 K375M was incubated with parental and transformed plasma membranes as described for the standard in vitro activation, except that 5 μg of FLAG-Raf-1 K375M was present in the incubations, $(His)_6$-MKK1 was omitted, and 0.2 mM [γ-^{32}P]ATP (20,000 cpm/pmol) was used. Assays were terminated by addition of SDS-PAGE sample buffer followed by heating (100°C for 2 min). Stopped samples were then subjected to SDS-PAGE (11% polyacryl-

amide 12- by 15-cm gel), which was continued until the prestained bovine serum albumin molecular weight standard was 1 cm from the bottom of the gel. Resolved proteins were transferred onto Immobilon P (Millipore, Bedford, Mass.). The FLAG-Raf-1 K375M protein band was visualized by protein immunoblotting on Immobilon P and excised; regions corresponding to the position of K375M in lanes for control reactions without K375M were also excised. Partial hydrolysis (with 6 N HCl for 1 h at 110°C) was performed on the excised pieces as described previously (17). Phosphoamino acid analysis was performed at pH 2.5 as described previously (16) to better separate phosphoserine and phosphothreonine. (His_6)-Raf-1 YY340/341FF preparation (20 μg of total protein) and purified FLAG-Raf-1 Y340D (5 μg) were phosphorylated and analyzed identically.

Assays of Raf-1 in the presence of organic-soluble factors extracted from purified plasma membranes. Plasma membranes from parental and transformed fibroblasts were prepared as above. Plasma membranes from Sf9 cells infected with c-Ha-Ras and Src^{Y527F} baculoviruses or with wild-type baculovirus (estimated multiplicities of infection, 10 each) were prepared by a similar procedure 48 h after infection. Equal protein amounts (0.1 mg in 1 ml of buffer C) of purified plasma membranes from fibroblasts or Sf9 cells were transferred to glass test tubes. All subsequent steps were performed on ice. An equal volume of reagent-grade methanol-chloroform (2:1) was added, and the mixture was vigorously vortexed four times. After 5 min on ice, the mixture was centrifuged ($1,000 \times g$ for 5 min at 4°C), and the organic and aqueous layers were carefully separated from the precipitate and transferred to glass tubes. Both fractions were snap-frozen in liquid N_2 and immediately lyophilized in a Speed-Vac (Savant Instruments, Farmingdale, N.Y.). When the organic and aqueous fractions were dry, they were briefly subjected to further drying by a gentle stream of helium. Each dried fraction was resuspended in 2 ml of cold water by immersion of the tube in a bath (filled with ice-cold water) sonicator (model G112SP1G; Laboratory Supply, Hicksville, N.Y.) three times for 20 s each. The sonicated lipid fraction appeared slightly opalescent. Assays for lipid cofactor stimulation of Raf-1 activity were performed immediately by first incubating (for 10 min at 30°C) 20 μl (~ 1 μg) of Raf-1 protein (one of FLAG-Raf-1 expressed alone or together with Ras, with Src^{Y527F}, or with Ras and Src^{Y527F}; FLAG-Raf-1 Y340D; or FLAG-Raf-1 K375M) with 20 μl (~ 1% of the total fraction) of either organic or aqueous extract and then assaying Raf-1 enzymatic activity (total final volume, 100 μl; 15 min at 30°C) by additions of (final concentrations) MKK1 (30 ng/μl), 0.2 mM [γ-^{32}P]ATP (5,000 cpm/pmol), 15 mM $MgCl_2$, 0.5 mM $MnCl_2$, 25 mM sodium β-glycerophosphate (pH 7.4 at 30°C), 0.1 mM sodium orthovanadate, leupeptin (10 μg/ml), and 0.1 mM phenylmethylsulfonyl fluoride. Reaction was initiated with [γ-^{32}P]ATP and quenched with sample buffer for SDS-PAGE (11% polyacrylamide gel) to determine ^{32}P incorporation into MKK.

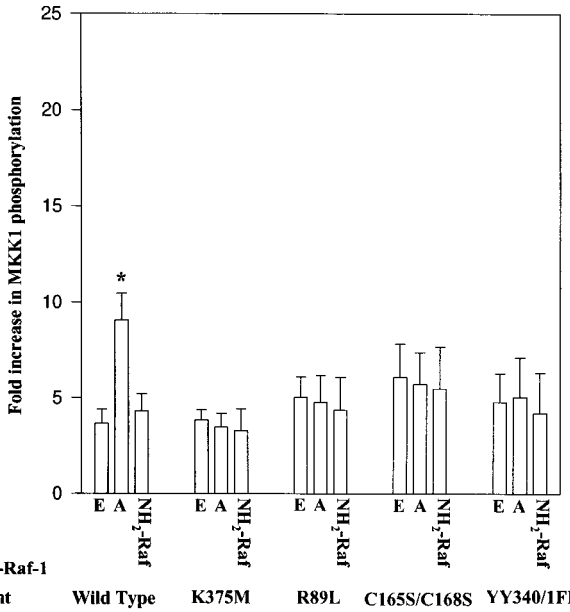

FIG. 1. Activation of $(His)_6$-Raf-1 mutants by plasma membranes from v-Ras- and v-Src-transformed fibroblasts. Transformed membranes were incubated with the indicated $(His)_6$-Raf-1 wild-type and mutant proteins to assess activation of the exogenous Raf protein (see Materials and Methods). Data were expressed as fold increases in MKK1 phosphorylation to permit comparison between experiments with several membrane and $(His)_6$-Raf-1 preparations. MKK1 autophosphorylation was 950 ± 100 cpm. Error bars given are standard deviations of the mean. E, expected value for MAPKKK activity predicted from additivity of all individually assayed components; A, actual observed MAPKKK activity value. Plasma membranes that had been pre-incubated with 1.0 μg of an NH_2-terminal fragment (amino acids 1 to 257) of Raf-1 (7) for 15 min on ice to block association between $(His)_6$-Raf-1 and Ras in the plasma membranes are labelled NH_2-Raf. *, $P < 0.05$ comparing actual and expected MKK1 phosphorylation.

RESULTS

Activation of $(His)_6$-Raf-1 wild-type and mutant proteins. A sonicated mixture of plasma membranes purified from v-Ras- and v-Src-transformed fibroblasts, referred to here as "transformed membranes" for brevity, were previously found to be more efficacious in activating Raf-1 in vitro than was either membrane preparation alone (7). Activation of Raf-1 wild-type and mutant proteins by plasma membranes was examined by measurement of ^{32}P incorporation into its specific substrate $(His)_6$-MKK1 (see Materials and Methods). Control assays were performed to quantitate ^{32}P incorporation into $(His)_6$-MKK1 due to $(His)_6$-MKK1 autophosphorylation, due to endogenous MAPKKK activity of the membranes, and due to $(His)_6$-Raf-1 protein. Activation data were expressed, as previously (7) as the actual or observed MKK1 phosphorylation versus the expected value of ^{32}P incorporation predicted from additivity (Fig. 1) of the components assayed individually. Transformed membranes stimulated the activity of wild-type $(His)_6$-Raf-1, and the stimulation was abrogated by blocking association of Raf and Ras with an NH_2-terminal fragment of Raf-1 (NH_2-Raf), as previously reported (7). Phosphorylation of $(His)_6$-MKK1 correlated with an increase in MKK1 enzymatic activity, assayed by phosphorylation of K52R MAP kinase protein (reference 7 and data not shown). No difference in expected and actual MKK1 phosphorylation was observed when a kinase-defective mutant of Raf-1, K375M, was used, further validating the analytical method (Fig. 1).

YY340/341FF $(His)_6$-Raf-1, which lacks the tyrosine phosphorylation sites, was not activated by transformed membranes in vitro. Thus, data from both in vitro and Sf9 cell studies suggest that tyrosine phosphorylation of YY340/341 may play a critical role in the activation of the Raf-1 protein kinase (see Discussion) (9).

R89L and C165S/C168S Raf-1 mutants, which have reduced affinities for Ras-GTP, were not activated by transformed membranes, in contrast to previous reports that both were activated in Sf9 cells coexpressing either Src^{Y527F} or c-Ha-Ras and Src^{Y527F} (9, 11, 26). Because these results were discrepant and because previous reports had not precisely quantitated the extent of activation of these mutants (11, 26), the abilities of R89L and C165S/C168S to be activated in Sf9 cells were reexamined (Table 1). Raf-1 R89L and C165S/C168S were activated in Sf9 cells coexpressing c-Ha-Ras and Src^{Y527F}, but the activation was less efficient than that of Raf-1. Coexpression with c-Ha-Ras alone did not stimulate R89L but did stimulate both C165S/C168S and wild-type activities. R89L, C165S/C168S, and wild-type Raf-1 were all activated to a similar extent by coexpression with Src^{Y527F} alone. Thus, the cell-free system may be more sensitive to perturbations that reduce the ability of Raf-1 to be activated by Ras, at least for these specific mutations, than the Sf9 cell system is.

Activation of FLAG-Raf-1 wild-type and mutant proteins. Additional experiments were performed to assess the ability of membranes to activate Raf-1 proteins with mutation of known in vivo sites of phosphorylation (Fig. 2). These experiments used a series of Raf-1 proteins generated with the FLAG epitope at the NH_2 terminus, which permitted their purification to near homogeneity by immunoaffinity adsorption (Fig. 3). In contrast, preparations of (His_6)-Raf-1 are impure and

TABLE 1. Activation of (His_6)-tagged Raf-1, Raf-1 R89L, and Raf-1 C165S/C168S in Sf9 cells expressed alone or together with Ras, with Src^{Y527F}, or with Ras and Src^{Y527F} proteins[a]

Raf-1 protein expressed	% of Raf-1 kinase activity[b]			
	Raf-1 expressed alone	Raf-1 co-expressed with c-Ha-Ras	Raf-1 co-expressed with Src^{Y527F}	Raf-1 co-expressed with c-Ha-Ras and Src^{Y527F}
Wild type	0.2 ± 0.1	25 ± 4	36 ± 7	100[c]
R89L	0.3 ± 0.1	0.8 ± 0.3	37 ± 5	40 ± 6
C165S/C169S	0.1 ± 0.1	14 ± 5	47 ± 5	66 ± 9

[a] (His_6)-Raf-1 and mutant proteins were expressed in Sf9 cells in the presence or absence of upstream activating oncogenes and purified as described in Materials and Methods (7). Equal amounts of each Raf-1 preparation (2 μg), containing approximately equal amounts of Raf-1 proteins as assessed by immunoblotting, were assayed for MAPKKK activity as described in Materials and Methods.

[b] Data (mean ± standard deviation, $n = 3$) are expressed as the percentage of the Raf-1 kinase activity versus MKK1 in comparison with (His_6)-Raf-1 activated by coexpression with both c-Ha-Ras and Src^{Y527F}.

[c] By definition.

contain Raf-1-associated proteins hsp90 and p50 (27a) and 14-3-3 (12a), as well as many unidentified proteins.

Activation of FLAG-Raf-1 was reliably greater than that of (His_6)-Raf-1. FLAG-Raf-1 enzymatic activity was increased ~50-fold, comparing MAPKKK activities attributable to FLAG-Raf-1 incubated with and without membranes and ATP/Mg. (Expected versus actual values given are values for total ^{32}P incorporation, and the fold stimulation of FLAG-Raf-1 is actually greater than the fold increase of actual versus expected values.) The improvement may result from either the amount or the purity of the FLAG-Raf-1, compared with (His)$_6$-Raf-1, used in the assays.

FLAG-Raf-1 mutants S259A (25), S499A (19), and S301A (not previously reported) were all activated in vitro, in general agreement with their ability to be activated in the Sf9 cell system. The extents of activation of wild-type, S259A, S499A, and S301A Raf-1 proteins by coexpression with c-Ha-Ras and Src^{Y527F} in the Sf9 cell system did not differ statistically (data not shown). However, we observed differences in the extents of activation of these mutants by transformed membranes. S259A and S499A were activated to a statistically lower level than were S301A and the wild type (Fig. 2). Although these data are from different experiments, care was taken to ensure that approximately equal amounts of Raf-1 proteins and transformed membranes were used by Western immunoblotting and protein assays, respectively (data not shown). Serine 259 and serine 499 are putative sites of regulatory phosphorylation by an unidentified protein kinase and by PKC, respectively (1, 19). We can conclude that neither phosphorylation is absolutely required for activation in vitro. The data obtained are also compatible with a potential regulatory role for phosphorylation of S259 and S499, because the extent of activation of these mutants was less than that of wild-type Raf-1. However, the latter inference must be viewed cautiously.

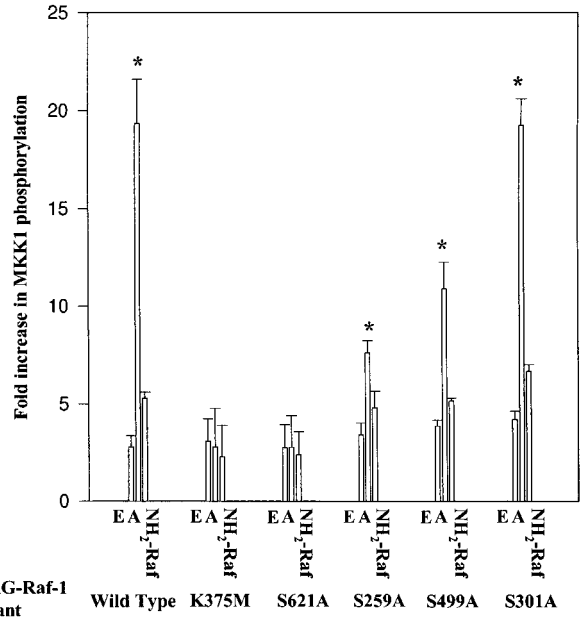

FIG. 2. Activation of FLAG-Raf-1 proteins by plasma membranes from v-Ras- and v-Src-transformed fibroblasts. Transformed membranes were incubated with the indicated FLAG-Raf-1 wild-type and mutant proteins to assess activation of the exogenous Raf protein (see Materials and Methods). Labelling is as for Fig. 1. MKK1 autophosphorylation was 720 ± 150 cpm ($n = 8$). *, $P < 0.05$ comparing actual and expected MKK1 phosphorylation.

FLAG-Raf-1 S621A was not activated by transformed membranes. Serine 621 is highly phosphorylated in quiescent fibroblasts and undergoes no apparent change in ^{32}P content in NIH 3T3 cells labeled with $^{32}P_i$ and stimulated with platelet-derived growth factor (25). The inability of S621A to be activated in vitro or in Sf9 cells strongly suggests that phosphorylation of Ser-621 is obligatory for Raf-1 activity, even though phosphorylation of Ser-621 may play no role in the stimulation of Raf-1 by mitogens.

Activation of FLAG-Raf-1 in vitro requires ATP. The effect of replacing ATP with AMPPNP was studied to determine whether a transferable γ phosphate was necessary for activation of Raf-1 (Table 2). Omission of ATP from the activation reaction mixture resulted in no Raf-1 activation. Inclusion of the nonhydrolyzable ATP analog, AMPPNP, in the reaction mixture instead of ATP (Table 2) also resulted in no observable stimulation of Raf-1 activity, consistent with a requirement for phosphate transfer.

Phosphorylation of kinase-inactive Raf-1 K375M in vitro by transformed membranes. To confirm the role of phosphorylation in the activation of Raf-1 by membranes, incorporation of ^{32}P into FLAG-Raf-1 K375M under activating conditions in the in vitro system was analyzed after reisolation via immunoadsorption to anti-FLAG M2 agarose and SDS-PAGE. FLAG-Raf-1 K375M is kinase defective and autophosphorylates weakly (25). Incubation of FLAG-Raf-1 K375M with transformed membranes and [γ-^{32}P]ATP/Mg resulted in a fivefold increase in K375M phosphorylation. K375M phosphorylation appeared to be dependent upon association with Ras in the plasma membrane, because phosphorylation was greatly reduced when membranes were first incubated with NH_2-Raf (Fig. 4A and B). Wild-type FLAG-Raf-1 was activated by the same transformed membrane preparation(s) (data not shown). To maximize quantities of [^{32}P]FLAG-Raf-1 K375M recovered for phosphoamino acid analysis, reactions were quenched with SDS-PAGE sample buffer and phosphorylated K375M was resolved by electrophoresis until it was nearly at the bottom of the gel; it was then electrophoretically transferred to Immobilon P. Controls omitting K375M but including transformed membranes were processed to ensure that phosphoamino acids obtained were ascribable to K375M protein. ^{32}P incorporation into FLAG-Raf-1 K375M was increased into both serine and tyrosine but not threonine residues (Fig. 4C). The increases in [^{32}P]phosphoserine and [^{32}P]phosphotyrosine levels were approximately 25- and 15-fold, respectively, as

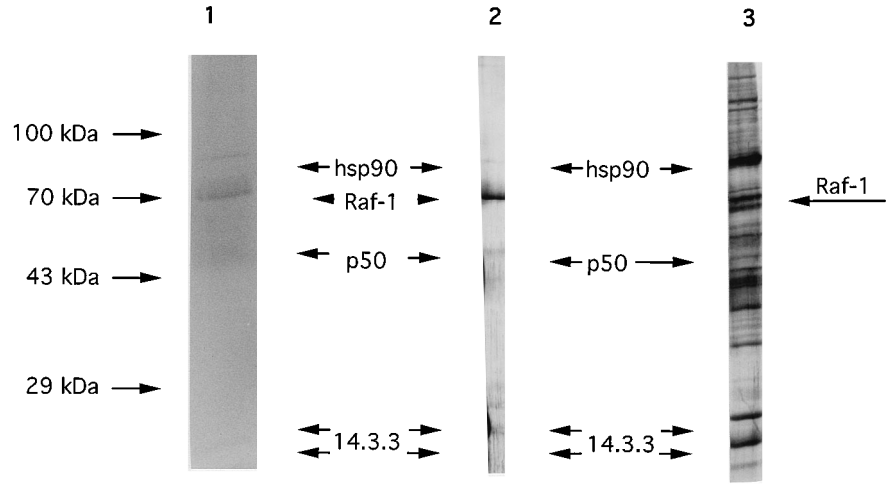

FIG. 3. Coomassie blue- and silver-stained gels from SDS-PAGE of $(His)_6$-tagged Raf-1 and FLAG-tagged Raf-1 proteins. A 1-μg portion of each protein [10 μg for $(His)_6$-Raf-1 protein] was subjected to SDS-PAGE on 11% polyacrylamide gels and either Coomassie blue stained (lane 1) or silver stained (lanes 2 and 3). Each preparation is from a single affinity purification step on either anti-FLAG agarose (lanes 1 and 2) or Ni^{2+} agarose (lane 3). Lanes: 1, Coomassie blue-stained FLAG-Raf-1; 2, silver-stained FLAG-Raf-1; 3, silver-stained $(His)_6$-Raf-1. Raf-1-associated proteins Hsp90, p50, and 14-3-3 are indicated in the preparations by arrows.

quantitated by both Cerenkov radiation and PhosphoImager (Molecular Dynamics) analysis (data not shown). FLAG-Raf-1 Y340D also underwent tyrosine phosphorylation; however, $(His)_6$-Raf-1 YY340/341FF was not phosphorylated on tyrosine residues when the same procedures were used as for phosphoamino acid analysis of K375M (Fig. 4D). Thus, activation of Raf-1 in vitro is accompanied by tyrosine and serine phosphorylation. Since the double F mutant is not tyrosine phosphorylated by the transformed plasma membranes which contain activated Src^{Y527F}, the data obtained are consistent with assignment of Y340 and Y341 as the sites of Raf tyrosine phosphorylation (9). Additional site studies by peptide mapping and/or mass spectrometry are to be part of a future study.

GTP-loaded parental plasma membranes activate exogenous FLAG-Raf-1 in vitro. Membranes from v-Ras- and v-Src-transformed cells but not from parental cells activate Raf-1 (7). Plasma membranes from v-Ras-transformed fibroblasts contain elevated levels of both diacyglycerol (33) and translocated PKC (8), and PKC has been reported to phosphorylate directly and to activate Raf-1 (19). We asked whether GTP loading of plasma membranes from quiescent parental cells, in the absence of cytosolic components, induced a capacity for activation. GTP loading of parental membranes permitted them to activate Raf-1 (Table 3). GTP loading of transformed plasma membranes further stimulated their ability to activate exogenous Raf-1 (Table 3). These data indicate that quiescent, parental membranes constitutively contain factors required to activate Raf-1 in vitro and suggest that additional factors do not have to be recruited from the cytosol to permit Raf-1 activation. However, a mechanism(s) of Raf-1 activation may exist wherein recruitment of other cytosolic factors, such as PKC isoforms, is required.

TABLE 2. Activation of $(His)_6$-Raf-1 requires phosphate transfer from ATP[a]

Membrane type and ATP or AMPPNP status	Fold increase in MKK1 phosphorylation[b]		
	Estimated	Actual	Actual (preincubation of membranes with N-terminal fragment of Raf-1)
Transformed + ATP	2.51 ± 0.25	6.18 ± 0.13[c]	2.38 ± 0.44
Transformed − ATP	1.40 ± 0.30	1.38 ± 0.28	1.26 ± 0.30
Transformed + AMPPNP	1.54 ± 0.32	1.37 ± 0.10	1.38 ± 0.25

[a] Membrane treatment and assay of MAPKKK activity was performed in two stages as described in Materials and Methods.
[b] Data are given as the fold increase in ^{32}P incorporation into MKK1 above that due to MKK1 autophosphorylation (490 ± 130 cpm).
[c] $P < 0.05$ comparing actual and expected MKK1 phosphorylation.

Unexpectedly, we also observed that GTP loading of either parental or transformed plasma membranes resulted in a significant reduction in the endogenous MAPKKK activity associated with these membranes (Table 3). The reduction was not due to loss of Raf-1 protein upon loading (6). GTP-stimulated inactivation was blocked by protein phosphatase inhibitors, both serine/threonine and tyrosine phosphatase inhibitors being required (6). Purified active FLAG-Raf-1, but not less pure active $(His)_6$-Raf-1, can be inactivated by treatment with either protein tyrosine or serine/threonine protein phosphatases (6). These studies strongly indicate that Raf-1 activity can be regulated by protein phosphorylation/dephosphorylation.

Ras-independent enhancement of FLAG-Raf-1 Y340D activity by membranes. Mutation of Raf-1 Tyr-340, an identified site of phosphorylation by Src^{Y527F} in vivo and in vitro, to Asp causes a very significant but partial activation of Raf-1 enzymatic activity (>20-fold) (9). Analyses of FLAG-Raf-1 Y340D in the in vitro system surprisingly revealed that its enzymatic activity was enhanced by parental membranes in a Ras-independent manner (Fig. 5A). This result was unexpected, since parental membranes do not cause any appreciable change in the activity of wild-type Raf-1 unless these membranes are first loaded with GTP (Table 3). Transformed plasma membranes, in contrast to parental membranes, enhanced the activity of Y340D by both Ras-dependent and Ras-independent mechanisms (Fig. 5A). Note that prior incubation of transformed membranes with NH_2-Raf reduces the observed fold increase in MKK phosphorylation to approximately that obtained with parental membranes. Also note that prior incubation of parental membranes with NH_2-Raf did not block activation of FLAG-Raf-1 Y340D.

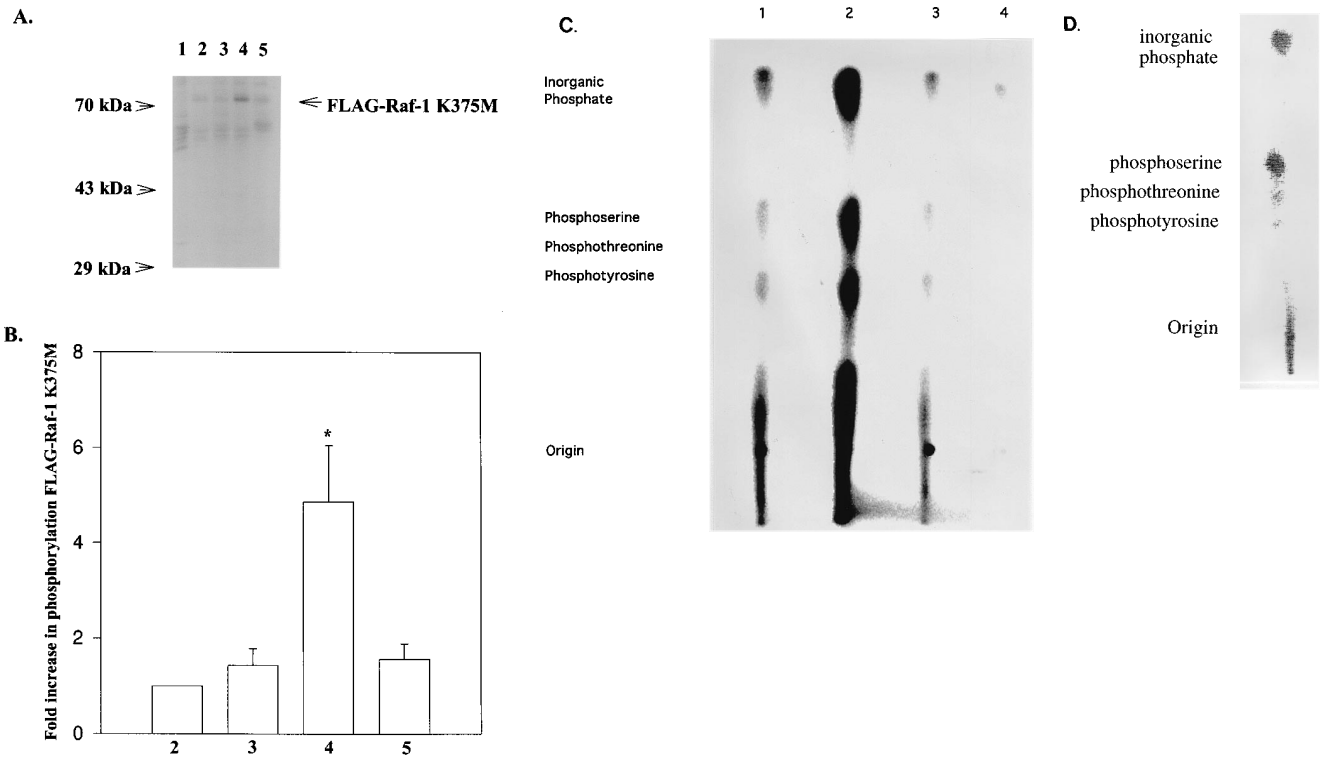

FIG. 4. Ras-dependent phosphorylation of FLAG-Raf-1 K375M and (His_6)-Raf-1 YY340/341FF by plasma membranes from v-Ras- and v-Src-transformed fibroblasts. (A) FLAG-Raf-1 K375M (5 μg) was incubated (15 min at 30°C) with transformed plasma membranes (5 μg) in the presence of 0.2 mM [γ-^{32}P]ATP/Mg (20,000 cpm/pmol), and the reaction was terminated by addition of EDTA. FLAG-Raf-1 K375M was reisolated with anti-FLAG agarose (see Materials and Methods) and subjected to SDS-PAGE and autoradiography (16 h with an intensifying screen at −70°C). Results from a representative experiment are shown. Lanes: 1, transformed membranes omitting FLAG-Raf-1 K375M; 2, autophosphorylation of FLAG-Raf-1 K375M alone; 3, parental membranes plus FLAG-Raf-1 K375M; 4, transformed membranes plus FLAG-Raf-1 K375M; 5, transformed membranes preincubated with an NH_2-terminal fragment of Raf-1 (amino acids 1 to 257) as described previously (7) plus FLAG-Raf-1 K375M. (B) FLAG-Raf-1 K375M was phosphorylated in vitro by transformed membranes, isolated as described for panel A, and proteins were resolved by SDS-PAGE for Western blotting and determination of ^{32}P incorporation ($n = 5$). For ease of comparison, numbering for lanes in panel B is the same as for panel A. ^{32}P incorporation: bar 2, autophosphorylation FLAG-Raf-1 K375M (460 ± 130 cpm, $n = 5$); bar 3, parental membranes plus FLAG-Raf-1 K375M; bar 4, transformed membranes plus FLAG-Raf-1 K375M; bar 5, transformed membranes preincubated with an NH_2-terminal fragment of Raf-1 (amino acids 1 to 257) as described previously (7) plus FLAG-Raf-1 K375M. *, $P < 0.05$ comparing parental and transformed membranes. (C) [^{32}P]phosphoamino acid analyses of FLAG-Raf-1 K375M phosphorylated in vitro by transformed membranes and purified from other membrane components by SDS-PAGE to near the bottom of the gel (see Materials and Methods), followed by immunoblotting of the FLAG-Raf-1 K375M protein. The immunostained Raf-1 protein bands were excised for phosphoamino acid analysis. The panel shows FLAG-Raf-1 K375M protein with (lane 1) or without (lane 2) preincubation with an NH_2-terminal fragment of Raf-1 (amino acids 1 to 257) (7) and of controls: lane 3, endogenous Raf-1 associated with transformed plasma membranes (omitting exogenous Raf-1), and migrating on SDS-PAGE at the same position as K375M; lane 4, autophosphorylated FLAG-Raf-1 K375M (see Materials and Methods). The figure is a photograph of a PhosphorImager-generated image on heat-sensitive paper from a representative experiment ($n = 5$). (D) [^{32}P]phosphoamino acid analysis of (His_6)-Raf-1 YY340/341FF (20 μg, equal immunoblotting to FLAG-Raf-1 K375M) phosphorylated in vitro by transformed membranes and purified from other membrane components by SDS-PAGE as for panel C, followed by immunoblotting of the (His_6)-Raf-1 YY340/341FF protein. Autophosphorylation of (His_6)-Raf-1 YY340/341FF was negligible. The figure is a photograph of a PhosphorImager-generated image from a representative experiment, printed by a laser printer ($n = 2$).

These properties of Y340D are likely to reflect a conformational change, possibly mimicking one caused by regulatory tyrosine phosphorylation at this site. Since active FLAG-Raf-1 prepared by coexpression with c-Ha-Ras and Src^{Y527F} is phosphorylated at Tyr340/341 (9), we examined the ability of transformed membranes to enhance its activity (Fig. 5B). The activity of active FLAG-Raf-1, like that of Y340D, could be further enhanced in vitro by both parental and transformed plasma membranes via a Ras-independent mechanism. However, the activity of active FLAG-Raf-1, unlike that of Y340D, was not further enhanced by transformed membranes via a Ras-dependent mechanism. These data suggest that plasma membranes per se may contain a factor(s) that enhances the activity of partially active or active, but not inactive, Raf-1 proteins. The enhancement, although modest when expressed as a fold increase of actual versus expected values, represents a large increment in total units of MAPKKK activity. To illustrate, the FLAG-Raf-1 used in these experiments, activated in situ by coexpression with c-Ha-Ras and Src^{Y527F}, transferred

TABLE 3. GTP-loaded, but not unloaded, parental plasma membranes activate exogenous FLAG-Raf-1[a]

Membrane type and loading status	Fold increase in MKK1 phosphorylation[b]		
	Estimated	Actual	Actual (preincubation of membranes with N-terminal fragment of Raf-1)
Parental + GTP	1.51 ± 0.57	9.29 ± 1.28[c]	1.67 ± 0.49
Parental − GTP	2.34 ± 0.66	2.39 ± 1.08	2.05 ± 1.45
Transformed + GTP	2.02 ± 0.80	13.20 ± 0.52[c]	1.97 ± 0.70
Transformed − GTP	4.94 ± 0.59	12.48 ± 0.73[c]	4.99 ± 0.60

[a] Parental NIH 3T3 and transformed v-Ras/v-Src membranes were incubated with and without GTP in the presence or absence of protein phosphatase inhibitors as described in Materials and Methods and assayed for the ability to stimulate exogenous FLAG-Raf-1.

[b] Data are expressed as expected and actual values of MKK1 phosphorylation (mean ± standard deviation, $n = 3$). Values for plasma membranes that had been preincubated with 1.0 μg of an NH_2-terminal fragment (amino acids 1 to 257) of Raf-1 (7) for 15 min on ice to block association between FLAG-Raf-1 and Ras in the plasma membranes are in the third column.

[c] $P < 0.05$ comparing actual and expected MKK1 phosphorylation.

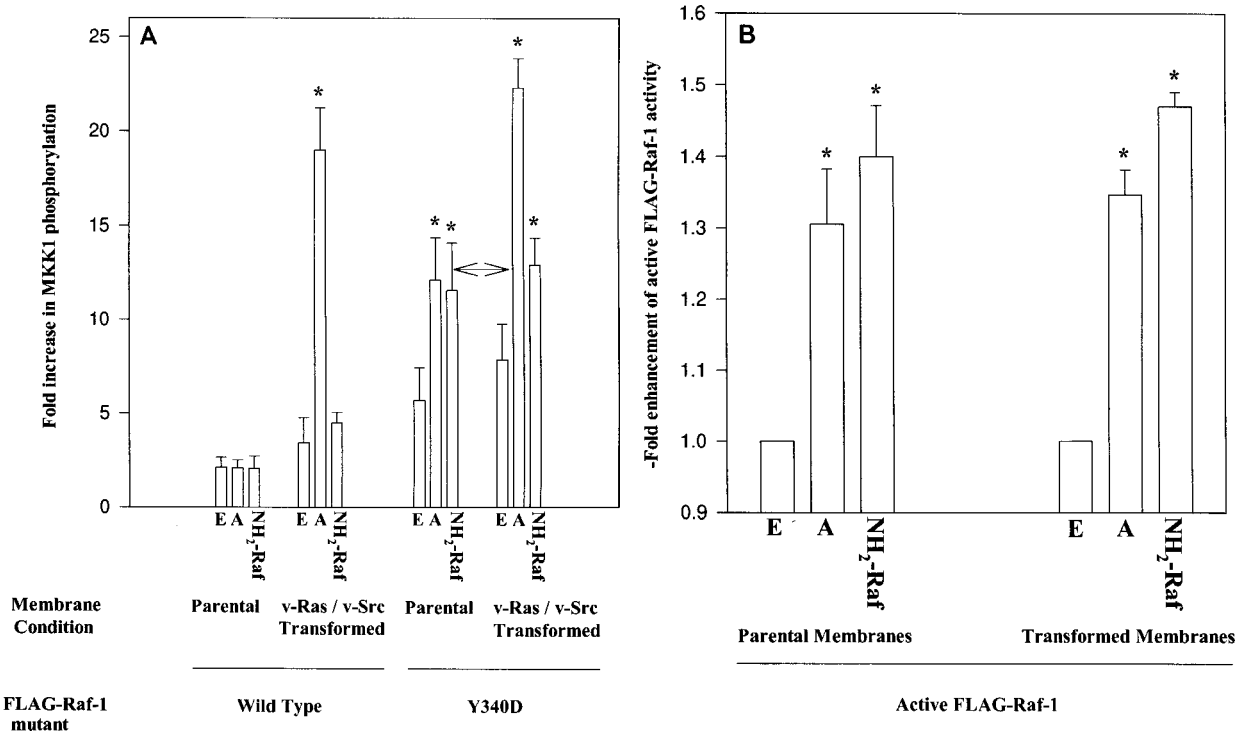

FIG. 5. Activation of FLAG-Raf-1 Y340D and active FLAG-Raf-1 by parental and transformed plasma membranes. (A) Parental NIH 3T3 membranes and transformed membranes were incubated with either FLAG-Raf-1 wild type or FLAG-Raf-1 Y340D to assess activation of the exogenous Raf protein (see Materials and Methods). E, expected value; A, actual observed value (see Fig. 1 legend for a description of the labels). MKK1 autophosphorylation was 610 ± 190 cpm (n = 5). *, $P < 0.05$ comparing actual and expected MKK1 phosphorylation. (B) Activation of exogenous active FLAG-Raf-1, prepared from Sf9 cells coinfected with Ras and Src^{Y527F}, by parental and transformed membranes. Labeling is as in panel A. *, $P < 0.05$ comparing actual and expected MKK1 phosphorylation.

approximately 50 pmol of phosphate into MKK1 per μg in the standard assay, in contrast to 0.1 pmol/μg for "inactive" Raf-1 from singly-infected Sf9 cells. Thus, the ~1.4-fold increase for active FLAG-Raf-1 represents a 20-pmol/μg increase in activity whereas a 50-fold increase for inactive FLAG-Raf-1 catalyzed by transformed membranes (e.g., Fig. 2) represents only a 5-pmol/μg increase in activity.

As an initial step to identify the membrane factor(s) that stimulates Raf-1 activity, plasma membranes from Sf9 cells infected with c-Ha-Ras and Src^{Y527F} baculoviruses or with wild-type baculovirus were purified and extracted with methanol-chloroform to separate the cellular contents into organic- and water-soluble phases. Both fractions were then frozen, lyophilized, resuspended in water, and sonicated, and portions were assayed for their ability to enhance the activities of FLAG-tagged Raf-1, Raf-1 activated in Sf9 cells by Ras and Src, Raf-1 Y340D, and Raf-1 K375M proteins (Fig. 6). Water-soluble factors did not affect the activity of any of the Raf-1 proteins tested (data not shown). Organic-soluble factors caused a modest (1.5-fold) enhancement of FLAG-Raf-1 activity and had no stimulatory effect upon kinase-defective K375M (Fig. 6). In contrast to the result with inactive FLAG-Raf-1, organic-soluble factors caused a sixfold enhancement of Y340D activity (Fig. 6) as well as a three- to fourfold enhancement of the activity of FLAG-Raf-1 activated by coexpression with Ras and Src^{Y527F} (Fig. 6). Organic-soluble factors isolated from plasma membranes of Sf9 cells infected with wild-type baculovirus also stimulated the activities of activated FLAG-Raf-1 and Y340D proteins, although possibly to a lesser extent than did factors from Ras- and Src-infected cells (Fig. 6). Addition of either fraction did not alter MKK1 autophosphor-

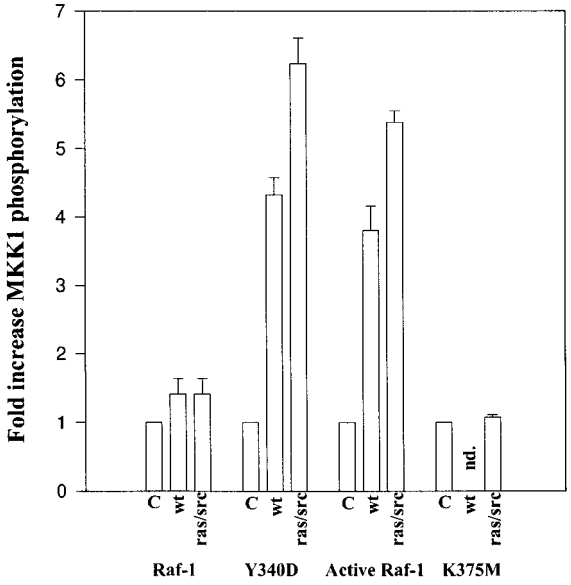

FIG. 6. Activation of FLAG-Raf-1 Y340D and active FLAG-Raf-1 by factors extracted from purified Sf9 cell plasma membranes purified from cells infected with either wild-type baculovirus or recombinant baculoviruses expressing c-Ha-Ras and Src^{Y527F}. Shown is the MKK1 phosphorylation by 1 μg of either FLAG-Raf-1, FLAG-Raf-1 Y340D, active FLAG-Raf-1, or FLAG-Raf-1 K375M in the presence or absence (control, C) of organic-soluble factors extracted with methanol-chloroform (2:1) from membranes from Sf9 cells infected with either wild-type baculovirus or c-Ha-Ras and Src^{Y527F} baculoviruses (see Materials and Methods). Active FLAG-Raf-1 was prepared by coexpression with Ras and Src^{Y527f}; the other FLAG-tagged Raf-1 proteins were purified from singly infected Sf9 cells. Data (n = 3 to 6) are expressed as the fold increase of MAPKKK activity induced by the tested fraction. nd., not determined.

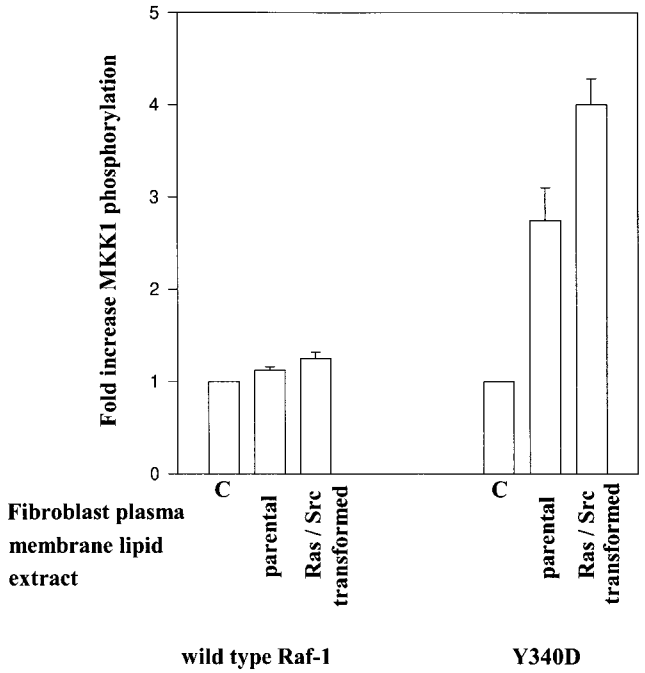

FIG. 7. Activation of FLAG-Raf-1 Y340D by organic-soluble factors extracted from purified parental or transformed fibroblast plasma membranes. Shown is the MKK1 phosphorylation by either FLAG-Raf-1 wild type or FLAG-Raf-1 Y340D in the presence or absence (control, C) of organic-soluble factors extracted with methanol-chloroform (2:1) from purified parental or transformed fibroblast plasma membranes (see Materials and Methods). Data (n = 3) are expressed as the fold increase of MAPKKK activity induced by the tested fraction in comparison with control values, defined as 1. Addition of either fraction did not alter MKK1 autophosphorylation.

ylation (7a), indicating an effect on Raf-1 and not its substrate. The most likely organic-soluble factors responsible for this effect are cellular lipids, but our initial findings do not exclude other possibilities, such as fortuitous refolding of an extracted, denatured protein component. These data suggest that inactive Raf-1 is very poorly modulated by lipid cofactors, in contrast to partially active Y340D or active wild-type Raf-1, whose activities were markedly enhanced.

To determine whether the Ras-independent activation of FLAG-Raf-1 Y340D by parental and transformed plasma membranes was also due to a organic-soluble factor, plasma membranes from these sources were extracted with methanol-chloroform (Fig. 7). Both parental and transformed plasma membranes contained organic-soluble factors which stimulated the activity of FLAG-Raf-1 Y340D; parental membranes appeared to contain less of the activating factor (Fig. 7). These results strongly implicate the extracted factor(s) as the cause of the Ras-independent activation observed (Fig. 5).

Full activation of the Raf-1 protein kinase in Sf9 cells requires its coexpression with the upstream activating oncogene products c-Ha-Ras and Src^{Y527F}. Coexpression of Raf-1 with either Ras or Src^{Y527F} leads to a partial Raf-1 enzymatic activation in this system. Consequently, we determined whether the manner and extent to which Raf-1 was activated in Sf9 cells affected its susceptibility to stimulation by organic-soluble factors from transformed plasma membranes (Fig. 8). Expression of FLAG-Raf-1 with Ras alone or Src^{Y527F} alone increased FLAG-Raf-1 enzymatic activity, but expression with both Ras and Src^{Y527F} caused a larger activation, in agreement with previous reports (9). Organic-soluble factors stimulated the activity of each activated Raf-1 independently of the manner of activation, but they did not stimulate the activity of nonactivated Raf-1. Organic-soluble factors extracted from the bacterium *E. coli* did not stimulate activated Raf-1 (Fig. 8), indicating that the factors and phenomenon described are specific for eukaryotic membranes.

Additional studies, including purification and identification of the responsible factor(s), are necessary to determine the role of lipid cofactors in Raf-1 activation. These data are the first to demonstrate any significant degree of activation of Raf-1 in vitro by a mechanism(s) other than phosphorylation.

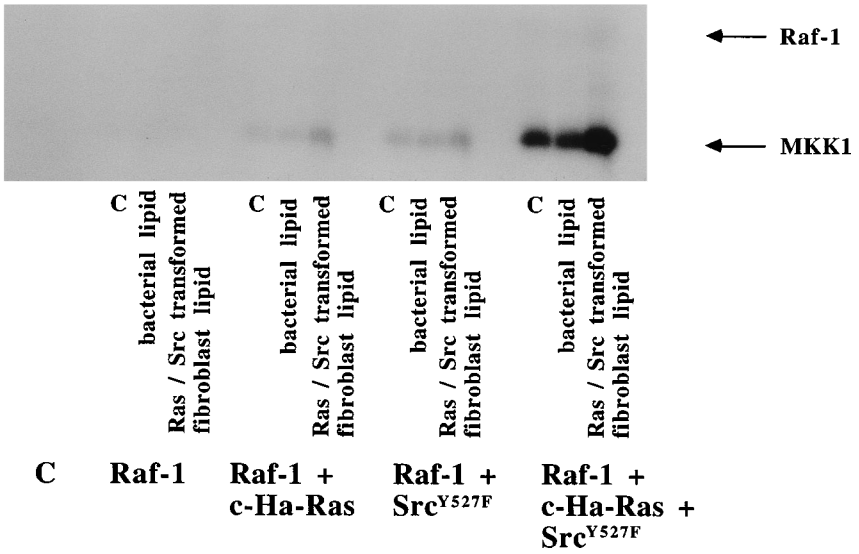

FIG. 8. Activation of active FLAG-Raf-1 (from coexpression with c-Ha-Ras, with Src^{Y527F} or Ras and Src^{Y527F}) by organic-soluble factors extracted from purified transformed plasma membranes. The autoradiograph shows MKK1 phosphorylation by FLAG-Raf-1 and the activated FLAG-Raf-1 proteins in the presence and absence (control, C) of organic-soluble factors extracted with methanol-chloroform (2:1) from purified transformed fibroblast plasma membranes (see Materials and Methods). Organic-soluble factors were extracted from an amount of *E. coli* containing an equal amount of total protein to that of the membranes being extracted. A representative experiment is shown (n = 3). Addition of either fraction did not alter MKK1 autophosphorylation.

DISCUSSION

One-third of human cancers are associated with mutations in the gene encoding Ras, resulting in Ras proteins that are predominantly GTP loaded and activated (22). This causes constitutive translocation of a portion of the cellular Raf-1 protein to the plasma membrane and activation of its enzymatic activity (23). Activation of Raf-1 by oncogenic Ras is critical for transmission of signals that deregulate cell replication (29). Thus, understanding the regulation of the Raf-1 protein kinase is vital to determining how the *ras* oncogene transforms cells. To this end, we studied the activation of Raf-1 and a series of functionally relevant Raf-1 mutants by a mixture of plasma membranes from transformed and parental cells.

Mimicking the in vivo situation, activation of Raf-1 in vitro by transformed membranes requires association with Ras-GTP. Two specific reagents that interfere with Ras-Raf binding, a neutralizing Ras antibody and the NH_2-terminal fragment of Raf-1, both block in vitro activation (7). Furthermore, a Raf-1 mutant protein, R89L, that shows no appreciable binding to Ras was incapable of activation in vitro. These data argue strongly that the activation mechanisms occurring in vitro are pertinent to the in vivo mechanisms.

Raf-1 contains a cysteine-rich region (His-139–X_{12}-Cys–X_2–Cys–X_6–Gly–X_2–Cys–X_4–His–X_2–Cys–X_7–Cys-176) which binds Zn^{2+} by coordination to the paired Cys-X_2-Cys sequences therein (15). Other signaling proteins contain similar motifs, termed zinc fingers for their chelation of Zn^{2+} or butterfly motifs for their predicted shape, including Vav, diacylglyerol kinase, and members of the PKC family (18). For classical PKCs, the zinc fingers are essential for phorbol ester binding, providing the basis for speculation that Raf-1 might interact with a regulatory lipid (15). Other evidence, chiefly that Raf-1 C-168 has reduced affinity for Ras, has pointed to a role for this motif in Ras binding (34).

We evaluated the activation of a Raf-1 protein containing a double mutation in the zinc finger and observed significant differences between activation of this mutant in Sf9 cells by coexpressed oncogenes and activation in vitro by transformed membranes. Raf-1 C165S/C168S was not activated in vitro but was activated by coexpression with Ras. The extent of activation of C165S/C168S by Ras in Sf9 cells, however, was appreciably smaller than that of wild-type Raf-1 by Ras. Overexpression in Sf9 cells may have obscured functional differences, e.g., in Ras-binding affinity, between C165S/C168S and its parent that were revealed by the in vitro system. This caveat concerning overexpression applies to other conclusions drawn from coexpression experiments in Sf9 cells. Our in vitro data are consistent with an obligatory requirement for association of Raf-1 with Ras for Raf-1 activation. Raf-1 R89L, which shows no appreciable Ras binding in vitro, is robustly activated in Sf9 cells coexpressing Src^{Y527F}, implying that activation by Src is Ras independent in this context (11). However, any activation of Raf-1 by tyrosine kinases of the Src family in physiologic contexts, such as in immune cells (30), may require Raf-1 association with Ras-GTP. This specific question has not been studied, but in fibroblasts anti-Ras reagents block v-Src-induced activation of MAP kinase (29) as well as v-Src-induced stimulation of mitogenesis (27).

Phosphorylation of Raf-1 has been proposed to play an essential role in the activation of the enzyme (25). Our in vitro data strongly support this hypothesis. Activation of Raf-1 by transformed membranes requires incubation with ATP/Mg, and activation does not occur in the presence of AMPPNP/Mg, consistent with a requirement for phosphoryl transfer. Kinase-defective Raf-1, K375M, becomes phosphorylated on both serine and tyrosine residues when incubated with transformed membranes and [γ-^{32}P]ATP/Mg, also consistent with regulation by phosphorylation. Furthermore, phosphorylation of K375M was blocked by prior incubation of the membranes with the NH_2-terminal domain of Raf-1.

The data obtained with Raf-1 proteins containing mutations of putative regulatory phosphorylation sites are consistent with regulation by both tyrosine and serine phosphorylations. Raf-1 S621A was not activated in vitro, consistent with the working hypothesis that phosphorylation of Ser-621 is required constitutively (24). More provocative was the finding that Raf-1 YY340/341FF was not activated by the mixture of membranes from v-Ras and v-Src cells or phosphorylated on tyrosine residues in the presence of transformed membranes containing v-Src. Since either v-Ras or v-Src membranes activate Raf-1 (7), the mixture should exhibit the regulatory potential of both types of oncogenic signals. Thus, our data raise the possibility that phosphorylation of Raf-1 at tyrosine residues 340/341 is an obligatory event for activation of Raf-1 by Ras or Src and that Ras and Src work in concert.

The current model for Raf-1 activation, derived principally from Sf9 coexpression studies, is that Ras and Src produce independent signals that activate Raf-1, since some activation is obtained by coexpression of Raf-1 with either Ras (usually c-Ras) or an activated form of Src. However, optimal activation of Raf-1 in Sf9 cells requires coexpression with both Ras and Src (32). YY340/341FF Raf-1, in contrast, is not activated in Sf9 cells by Ras or Src or both (9). While the defect in activation of the YY340/341FF mutant could be due to a deleterious effect of the mutations on the protein structure, the fact that Y340 and Y341 are sites of in vivo phosphorylation and the fact that Y340D is an activating mutation suggest otherwise (9). In addition, mutation of Asp-337 to Ala abolishes tyrosine phosphorylation and activation of Raf-1 by Src^{Y527F} in Sf9 cells (9). Previous studies of Raf-1 tyrosine phosphorylation have all used Raf-1 immunoprecipitated from total-cell lysates, disproportionally containing inactive Raf-1. Studies of the phosphoamino acid content of membrane-associated Raf-1 are warranted.

Raf-1 proteins containing an Ala mutation at Ser-259 or Ser-499 were activated in vitro but possibly with a decreased efficiency in comparison with the wild type. Ser-259 is phosphorylated by an unidentified protein kinase. Recently, Beimling et al. reported adsorption of unidentified Ser-259 kinase activities from A431 cell lysates to the NH_2-terminal Raf-1, fused to glutathione-*S*-transferase (1). The Ser-259 kinase activity adsorbed, however, was not detectably different between lysates from epidermal growth factor-treated or untreated cells. We do not know if our preparations of transformed membranes contain Ser-259 kinase activity. Ser-259 was an apparent site of increased phosphorylation in fibroblasts treated with platelet-derived growth factor. Thus, Ser-259 phosphorylation could be involved in Raf-1 activation, but there is as yet no conclusive evidence.

S499A was activated by transformed membranes, albeit less well than was the wild type, indicating that Ser-499 phosphorylation is not required for activation by Ras or Src. This conclusion agrees with the results of studies of the mutant in Sf9 cells (19). Ras-transformed membranes contain increased amounts of both diacylglycerol and some isozymes of PKC (8, 33). PKC α has been reported to directly activate Raf-1 by phosphorylation of Ser-499 (19). Inclusion of pseudosubstrate inhibitors of either PKC α or PKC ζ (each at 100 μM), however, did not detectably alter activation of Raf-1 by transformed membranes (data not shown), suggesting that translo-

cated forms of these PKC isozymes are not responsible for activation of Raf-1 by transformed membranes.

Translocation of factors from the cytoplasm to the membrane does not appear to be required for activation by Ras, on the basis of the following argument. Membranes isolated from quiescent, parental cells neither contain nor activate Raf-1 in vitro but acquire the ability to enhance Raf-1 enzymatic activity after GTP loading and incubation at 30°C. We cannot as yet conclude that this effect results from GTP loading of Ras; GTP loading may also have activated other G proteins that caused or contributed to the response.

Recently, we have demonstrated that phosphorylation of Raf-1 is part of the activation mechanism by Ras and Src (6). Highly active Raf-1, purified from Sf9 cells coexpressing Ras and Src^{Y527F} so as to remove Raf-associated proteins such as 14-3-3 and Hsp90, is nearly completely inactivated by treatment with either protein tyrosine or serine/threonine phosphatases (6). In addition, GTP loading appears to activate, or otherwise allow, membrane-associated protein phosphatases to deactivate endogenous MAPKKK activity in membranes as well as that of exogenous Raf-1 (6). This GTP-stimulated inactivation with membranes was first noted in control experiments required for studies reported herein (Table 3).

Regulation of Raf-1 by phosphorylation does not preclude participation of lipid cofactors in the in vivo mechanism. In support of this hypothesis, the cysteine-rich domain of Raf-1 discussed above binds to liposomes. Furthermore, addition of phosphatidylcholine phospholipase C from *Bacillus cereus* to NIH 3T3 cells activates Raf-1 (2, 7) and a putative inhibitor (D609) of phosphatidylcholine phospholipase C abrogates activation of Raf-1 in these cells by growth factors (2). Force et al., however, found no significant enhancement in vitro of Raf-1 by a panel of available lipids (12). Our experimental rationale to search for lipid cofactors is to use purified plasma membranes from Sf9 cells coinfected with Ras and Src^{Y527F} in the hope that these membranes may contain increased amounts of any lipid cofactors involved in Raf-1 activation, should these exist. Factors, presumably lipids, extractable with methanol-chloroform did not alter the basal activity of highly purified preparations of inactive Raf-1, in general agreement with data from Force et al. (12). In sharp contrast, the extracted factors markedly enhanced the enzymatic activity of partially active Y340D or active wild-type proteins. The activating factors were destroyed by treatment with 0.1 M KOH in methanol, compatible with the enhancing factors being glycerophospholipids (7a). Identification as such may provide a link between observations that have suggested a role for activation of a phosphatidylcholine phospholipase C (2) or phosphatidylcholine phospholipase D (2a) in Raf-1 activation.

Our findings suggest a model, offered solely to motivate further investigation, wherein (i) Raf-1 is first recruited to the plasma membrane by binding to Ras-GTP; (ii) Raf-1 is phosphorylated on tyrosine and/or serine residues, inducing conformational changes that increase enzymatic activity; and (iii) activated Raf-1 binds lipid cofactors that further enhance its activity. Steps (i) and (ii) seem unequivocal. In a recent review, Daun et al. proposed a model in which Raf interacts with a putative lipid cofactor in a Raf-1–Ras-GTP complex prior to phosphorylation of Raf-1 on regulatory tyrosine or serine sites (3). These workers also cited unpublished data for an unidentified lipid activator from brain membranes. The demonstration here of a sixfold enhancement of Y340D activity by a mixture of crude lipids isolated from membranes of cells expressing Ras and Src^{Y527F} is an important first step in evaluating the role, if any, of lipid cofactors in Raf-1 activation.

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