Ras-Induced Activation of Raf-1 Is Dependent on Tyrosine Phosphorylation

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Although Rafs play a central role in signal transduction, the mechanism(s) by which they become activated is poorly understood. Raf-1 activation is dependent on the protein’s ability to bind Ras, but Ras binding is insufficient to activate Raf-1 in vitro. Ras expression in vivo is able to activate Raf-1, and we have investigated the importance of Raf-1 tyrosine phosphorylation to this Ras-induced activation, in the absence of an overexpressed tyrosine kinase. We demonstrate that Raf-1 purified from Sf9 cells coinfected with baculovirus Ras but not Src could be inactivated by protein tyrosine phosphatase PTP-1B. 14-3-3 and Hsp90 proteins blocked both the tyrosine dephosphorylation and inactivation of Raf-1, suggesting that Raf-1 activity is phosphotyrosine dependent. In Ras-transformed NIH 3T3 cells, a minority of Raf-1 protein was membrane associated, but essentially all Raf-1 activity and Raf-1 phosphotyrosine fractionated with plasma membranes. Thus, the tyrosine-phosphorylated and active pool of Raf-1 constitute a membrane-localized subtraction, which could also be inactivated with PTP-1B. By contrast, B-Raf has aspartic acid residues at positions homologous to those of the phosphorylated tyrosines (at 340 and 341) of Raf-1 and displays a high basal level of activity. B-Raf was not detectably tyrosine phosphorylated, membrane localized, or further activated upon Ras transformation, even though B-Raf has been shown to bind to Ras in vitro. We conclude that tyrosine phosphorylation is an essential component of the mechanism by which Ras activates Raf-1 kinase activity and that steady-state activated Ras is insufficient to activate B-Raf in vivo.

The Raf family of serine/threonine kinases consists of Raf-1, A-Raf, and B-Raf, all of which have an amino-terminal domain which can specifically associate with Ras:GTP (20, 38, 39, 43, 51–53, 57) and a carboxy-terminal kinase domain (7). Rafs can phosphorylate and activate MAP kinase kinase (MEK) (5, 8, 21, 23, 30, 43) and are key regulators of the MAP kinase cascade. Raf family members are thought to be major effectors of Ras not only because of their physical association but also because genetic data suggest that a Raf protein functions downstream of Ras (21, 23, 30, 43) and are key regulators of the MAP kinase cascade. Raf family members are thought to be major effectors of Ras not only because of their physical association but also because genetic data suggest that a Raf protein functions downstream of Ras (21, 23, 30, 43).

Association of Raf-1 with Ras:GTP is insufficient by itself to cause Raf-1 activation, but binding to activated p21ras causes Raf-1 to translocate to the membrane (34, 49, 54). Mechanisms for Raf-1 activation are present in cell membranes (10), and targeting of Raf-1 to cell membranes causes cell transformation (32, 47). While these experiments established that Raf-1 activation occurs at the membrane, the biochemical mechanism(s) by which Raf-1 is activated remains unclear.

The system for which Raf-1 regulation has been described in most detail is Sf9 cells triply infected with recombinant baculoviruses expressing Raf-1, Ras, and Src (referred to here as Raf-1[Ras/Src]) (55). Under these conditions, Raf-1 becomes highly activated and phosphorylated on several sites, including tyrosines 340 and 341 (14). Mutation of tyrosines 340 and 341 to phenylalanine creates a Raf-1 protein which does not become activated in Sf9 cells, while mutation of tyrosine 340 to aspartic acid converts Raf-1 to a transforming protein (14). We have recently demonstrated that Raf-1[Ras/Src] purified to near homogeneity could be substantially inactivated by the tyrosine-specific phosphatase PTP-1B (9). Inactivation of Raf-1 by PTP-1B strongly implicates tyrosine phosphorylation in the activation of Raf-1, since the catalytic cleft of PTP-1B is too deep to dephosphorylate serine or threonine residues (25). Thus, it seems very probable that in this triple-overexpression system, tyrosine phosphorylation of Raf-1 is important for its maximal activation. However, it is not clear whether independent activation of Raf-1 by either Ras or Src alone occurs by the same mechanism or whether Ras and Src activate Raf-1 by distinct mechanisms, with a synergy when Ras and Src are simultaneously overexpressed, as has been suggested (55).

It is similarly unclear—and a subject of controversy—whether tyrosine phosphorylation of Raf-1 plays a role in its activation in the absence of an overexpressed tyrosine kinase. This uncertainty has arisen at least in part because the stoichiometry of Raf-1 tyrosine phosphorylation in fibroblasts stimulated with growth factors or serum is too low to correlate with an appreciable degree of activity (1, 3, 4, 29). Activation of Raf-1 under these circumstances has also been disputed (26, 43, 58).

Other phosphorylation events on Raf-1 are thought to correlate with its activation (2, 27, 31, 40), but no site of serine/threonine phosphorylation has been identified which is not appreciably phosphorylated constitutively, whose phosphorylation correlates temporally with Raf-1 activation, and whose mutation renders the kinase inactive. However, serine 621 (S-621), which is reportedly a constitutive site of phosphorylation, appears to be required for Raf-1 activity (40). Therefore, while dephosphorylation of S-621 could render Raf-1 inactive, phosphorylation of S-621 does not appear to be an activation mechanism.

Another Raf family member, B-Raf, has recently been determined by biochemical analysis to be the major MEK activator in neuronal tissue (5, 23) and also in fibroblasts (43). Like Raf-1, B-Raf is able to bind Ras:GTP (38, 43), and the inter-
action is inhibited by agents which elevate cyclic-AMP levels (43). The mechanisms of B-Raf regulation have not been described, but a significant predictive difference between B-Raf and Raf-1 is that amino acids homologous to tyrosines 340 and 341 of Raf-1 are replaced by negatively charged aspartic acid residues in B-Raf.

Raf-1 exists in a complex with several other proteins, and there has been considerable interest in determining whether they play a role in regulating Raf-1 activity. Hsp50 and Hsp90 associate with Raf-1 (46), as do members of the multifunctional 14-3-3 family of proteins (15–17, 22). There is not general agreement on the role of 14-3-3 proteins in Raf-1 activation, but any activation mediated by 14-3-3 is at most slight. It was recently shown that association with 14-3-3 proteins is not essential for Raf-1 function (37), suggesting that 14-3-3 proteins have a function distinct from the direct activation of Raf-1. This agrees with our recent report that 14-3-3α is able to block the inactivation of Raf-1[Ras/Src] by phosphatases (9), suggesting that Raf-1-associated proteins may maintain the active state of Raf-1. However, as with studies of the activation of Raf-1, it has been uncertain whether the effects of these stabilizing proteins occurred by regulation of the state of tyrosine phosphorylation (i.e., protecting Raf-1 from phosphorylation) or by some other mechanism (e.g., allosterically, causing Raf-1 to maintain an active conformation following dephosphorylation).

Thus, it seems clear that Raf-1[Ras/Src] kinase activity is dependent on both tyrosine and serine/threonine phosphorylations and that inactivation of Raf-1[Ras/Src] by phosphatases can be inhibited by 14-3-3 or Hsp90. To determine the importance of tyrosine phosphorylation of Raf-1 to its activation by Ras, in the absence of overexpressed or constitutively active Src, we assessed the activity and tyrosine phosphorylation of Raf-1 activated only by Ras expression. Raf-1 isolated from SF9 cells infected with recombinant baculoviruses expressing only Raf-1 and c-Ha-Ras (referred to here as Raf-1[const]) was also susceptible to inactivation by PTP-1B. Inactivation correlated with decreased immunostaining with anti-phosphotyrosine antibodies, and both inactivation and dephosphorylation were blocked by 14-3-3 or Hsp90. Furthermore, in Ras-transformed NIH 3T3 cells, the Raf-1 molecules which localized to cell membranes, although a minority of the total Raf-1, constituted essentially all Raf-1 kinase activity in these cells, were phosphorylated on tyrosine, and were sensitive to inactivation by PTP-1B. Cytosolic Raf-1 was not detectably tyrosine phosphorylated and was essentially inactive. In marked contrast, B-Raf, which displays substantial activity in both cytosols and membranes, did not increase in activity following transformation by Ras. We conclude that tyrosine phosphorylation of Raf-1 is necessary for its activation by Ras, even in the absence of an overexpressed tyrosine kinase, and that Ras transformation is not sufficient to enzymatically activate B-Raf.

MATERIALS AND METHODS

Cell culture and virus propagation. NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. L61 cells were created by transfection of NIH 3T3 cells with an expression plasmid encoding Ha-RasL61 (gift from C. Der, Chapel Hill, N.C.). G418 selection, and isolation of clones. One clone with a highly transformed morphology was chosen for further study and was grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and transferred by pipetting medium to detach cells from dishes.

Phosphorylation and dephosphorylation studies. Cytosolic and pellet fractions were prepared by centrifuging at 100,000 × g for 15 min at 4°C. Cytosolic fractions were washed twice in a buffer containing 250 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 0.1 mM diethiothreitol, 1% (vol/vol) Triton X-100, 0.5% (wt/vol) n-octyl-β-D-glucopyranoside, 0.05% (wt/vol) sodium dodecyl sulfate (SDS), and 0.03% (wt/vol) sodium deoxycholate, supplemented with protease and phosphatase inhibitors as described above. Lysates were cleared of chromatin by centrifugation at 100,000 × g for 30 min.

Plasma membranes were partially purified as described previously (10) and homogenized in a solution containing 250 mM Tris (pH 7.9), 0.5% (vol/vol) Nonidet P-40, 0.05% (wt/vol) sodium deoxycholate, 0.025% (wt/vol) SDS, 0.5 mM EDTA, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA), 10% (vol/vol) glycerol, 0.05% (vol/vol) 2-mercaptoethanol, and protease and phosphatase inhibitors as described above.

Raf-1 preparations were prepared by homogenization of cells in p21 buffer with protease and phosphatase inhibitors and centrifugation at 100,000 × g for 30 min, as described previously (24).

Detection of Raf proteins. Raf-1 preparations were assayed for purity by SDS-polyacrylamide gel electrophoresis (PAGE) and silver staining.

Raf-1 and B-Raf were immunoblotted with primary antibodies purchased from Santa Cruz (C-12 for Raf-1 and C-19 for B-Raf) and secondary anti-rabbit immunoglobulin G antibodies conjugated to horseradish peroxidase. For analysis of Raf-1 phosphotyrosine content, blots were probed with anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc.). Chemiluminescence was used to visualize bands.

Immunoprecipitation of Raf proteins from fibroblast lysates. To immunoprecipitate Raf proteins from lysates, 20 μg of C-12 anti-Raf-1 antibody (Santa Cruz) or C-19 anti-B-Raf antibody (Santa Cruz) and 100 μl of protein A-agarose were incubated for 1 h at 4°C. Beads were washed four times in lysis buffer and twice in kinase buffer, with vigorous mixing at each step. Raf-1 was immunoprecipitated from solubilized cell membranes (100 μg of protein) by incubation with 50 μg of asagere-conjugated C-12 anti-Raf-1 antibody (Santa Cruz) for 3 h at 4°C. Beads were incubated with 600 μg of antigenic peptide for 2 h at 4°C and then washed prior to immunoprecipitation. Beads were allowed to settle and were washed three times with membrane homogenization buffer; twice with a solution containing 25 mM Tris (pH 7.9), 10% (vol/vol) glycerol, 0.03% (vol/vol) Nonidet P-40, 0.1 mM EDTA, 0.1 mM EGTA, and 0.1% (vol/vol) 2-mercaptoethanol, with protease and phosphatase inhibitors; and twice in kinase buffer.

Inactivation of Raf-1 with PTP-1B. Raf-1 bound to silica-Ras/GMPPPNP was washed twice in phosphate buffer (25 mM Tris [pH 7.6], 10% (vol/vol) glycerol, 1 mM diethiothreitol, 0.1 mM EDTA, 0.01% [vol/vol] Nonidet P-40) and incubated with 0.5 μg of GST-PTP-1B in phosphate buffer. In certain experiments, 1 μg of 14-3-3 or Hsp90 was added to the reaction mixtures prior to the addition of phosphatase. Samples were mixed and then incubated at 35°C for 30 min. Phosphatase activity was monitored in a parallel, colorimetric reaction by hydrolysis of 4-nitrophenylphosphate.

Inactivation of Raf-1 immunoprecipitated from fibroblast membranes was done with 1 μg of PTP-1B for 45 min at 30°C.

Assay of Raf-1 and B-Raf kinase activity. Immobilized Raf-1 bound to silica-GST-Ras/GMPPPNP was washed in kinase buffer (50 mM N-2-hydroxyethyl-2-piperazine-N’-2-ethanesulfonic acid [HEPES] [pH 7.5], 50 mM NaCl, 10 mM MgCl2, 1 mM diethiothreitol) and incubated with 1 μg (per sample) of bacterially expressed, His-6-tagged MEK-1 in kinase buffer (2,500 cpm/pmol) at 30°C for 10 min. For assays of MEK activation, 200 ng of MEK-1 was incubated with Raf-1 in kinase buffer–100 mM ATP for 15 min. At this time, 2 μg of kinase-inactive MAP kinase (K52R) and [γ-32P]ATP (final specific activity, 2,500 cpm/pmol) was added per sample for an additional 15 min. Excess 5′-excess terminated by the addition of SDS electrophoresis sample buffer.
RESULTS

Purification of Raf-1 from infected SF9 cells. To facilitate the analysis of Raf-1 in isolation from its many associated proteins, we developed a one-step purification strategy, based on the intrinsic affinity of Raf-1 for Ras-GTP, to isolate Raf-1 from infected SF9 cells. We used GST-Ras conjugated to silica beads and loaded with GMPPNP as an affinity ligand for Raf-1 purification. Raf-1 purified in this manner was essentially devoid of other proteins and free of endogenous MEK activity (data not shown). On the basis of estimated concentrations, Raf-1 activities against recombinant MEK-1 were approximately 1 nmol/min/mg for Raf-1 purified from singly infected SF9 cells (referred to here as Raf-1[alone]) and 40 nmol/min/mg for Raf-1[Ras]. Raf-1[Ras] showed a slightly variable activity, depending on the preparation, but the activity was always intermediate between those of Raf-1[alone] and Raf-1[Ras/Src] (approximately fivefold higher than that of Raf-1[alone]).

Inactivation of Raf-1 by PTP-1B. The action of tyrosine kinases in cells is often thought to be upstream of Ras. Were this the case, activation of Raf-1 by Ras might not require tyrosine phosphorylation. Alternatively, the enhanced activity of Raf-1[Ras/Src] compared with that of Raf-1[Ras] might be due to an increased efficiency of the same basic activation mechanism and Raf-1[Ras] activity would also depend on tyrosine phosphorylation. We have previously shown that Raf-1[Ras/Src] can be inactivated by the tyrosine-specific protein phosphatase PTP-1B and that this inactivation can be blocked by the Hsp90 and 14-3-3 proteins, which are known to bind to Raf-1 (9). If Raf-1[Ras] becomes activated by tyrosine phosphorylation, then it should also be possible to inactivate it with PTP-1B. When purified Raf-1[Ras] (Fig. 1A, lane 6) or Raf-1[Ras/Src] (lane 10) was preincubated with PTP-1B, kinase activity towards recombinant MEK-1 decreased approximately 60% (compare lanes 6 and 10 with 5 and 9). Inclusion of 14-3-3 or Hsp90 protein in the phosphatase reactions protected both Raf-1 preparations from inactivation, although Hsp90 was slightly less effective than 14-3-3 at blocking inactivation (Fig. 1A, lanes 7, 8, 11, and 12). Inactivation of both Raf-1[Ras] and Raf-1[Ras/Src] preparations by PTP-1B, and protection from inactivation by the same mechanisms, suggests that both became activated by a mechanism involving tyrosine phosphorylation.

To confirm that Raf-1[Ras] was inactivated by dephosphorylation of tyrosine residues, duplicate Raf-1[Ras] samples were immunoblotted with antiphosphotyrosine antibody. As shown in Fig. 1B, incubation of Raf-1[Ras] with PTP-1B caused a reduction in phosphotyrosine content (compare lanes 6 and 5), while inclusion of Hsp90 or 14-3-3 blocked dephosphorylation (lanes 7 and 8). A slight acceleration in the mobility of Raf-1 is visible in Fig. 1B, lane 6, but we do not believe that tyrosine phosphorylation accounts for the frequently seen (and more prominent) mobility shift of Raf-1, which does not correlate with its activity and which we can completely reverse by treatment with serine/threonine phosphatases (data not shown). Raf-1 immunoblotting detected equal amounts of Raf-1 in all four lanes (data not shown). These data indicate that the activity of Raf-1[Ras] is dependent on tyrosine phosphorylation.

Raf-1 activity and phosphotyrosine colocalize to the plasma membranes of Ras-transformed cells. Raf-1 activity in NIH 3T3 cells was previously determined to be modest relative to that of B-Raf (43), with all detectable Raf-1 activity localized to cell membranes (43, 47, 49). Since Raf-1 is known to bind Ras, we analyzed Raf-1 activity in NIH 3T3 cells and in a cell line transformed by Ras[L61] (Leu61 cells) to determine whether Raf-1 activity was enhanced by Ras transformation. We also sought to assess whether tyrosine phosphorylation was associated with Raf-1 activity under these circumstances.

To study Raf-1 in plasma membranes, we enriched and solubilized membranes from both cell lines and immunoprecipi-

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FIG. 1. Inactivation of purified Raf-1 by PTP-1B. (A) Immobilized (approximately 10 ng) Raf-1[alone] (lanes 1 to 4), Raf-1[Ras] (lanes 5 to 8), or Raf-1[Ras/Src] (lanes 9 to 12) was washed twice in phosphate buffer and incubated in phosphate buffer with 0.5 μg of GST-PTP-1B (lanes 2 to 4, 6 to 8, and 10 to 12) or in phosphate buffer alone (lanes 1, 5, and 9) for 30 min at 35°C. One microgram of 14-3-3 (lanes 3, 7, and 11) or Hsp90 protein (lanes 4, 8, and 12) was added to immobilized Raf-1 prior to the addition of phosphatase. Samples were washed in kinase buffer and incubated in a solution containing kinase buffer, 1 mM Na3VO4, 1 μg of MEK-1 per sample, and 100 μM [γ-32P]ATP (2,500 cpm/pmol) for 15 min at 30°C. Samples were resolved by SDS-PAGE and detected by autoradiography. (B) Duplicate samples of Raf-1[Ras] treated with protein phosphatase as for panel A were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with antiphosphotyrosine antibody. The enhanced chemiluminescence detection system was used.
tated Raf-1. Raf-1 was associated with the plasma membranes of Leu61 cells (Fig. 2A, lane 4), while only a small amount was associated with the membranes of NIH 3T3 cells (lane 2). The amount of Raf-1 in NIH 3T3 cells was unaffected by blocking with antigenic peptide and was equal to the amount immunoprecipitated from Leu61 cells in the presence of antigenic peptide, suggesting that this level of Raf-1 is the threshold of specificity for peptide competition. A strong phosphotyrosine signal was detected in a protein exactly overlapping Raf-1 in immunoprecipitates of Leu61 cell membranes (Fig. 2B, lane 4), with only a trace visible when immunoprecipitations were performed in the presence of antigenic peptide (lane 3). No detectable phosphotyrosine was present in the small amount of Raf-1 in membranes from the untransformed NIH 3T3 cells (Fig. 2B, lanes 1 and 2). Precipitation of the antiphosphotyrosine antibody with monomeric phosphotyrosine abolished the Raf-1 signal (data not shown). This indicates that tyrosine-phosphorylated Raf-1 is specifically associated with the plasma membranes of Leu61 cells.

Assessment of the kinase activity of Raf-1 immunoprecipitations showed that Raf-1 activity was present in the Leu61 cell membrane preparation but essentially absent from the membranes of untransformed NIH 3T3 cells (Fig. 2C, lanes 4 and 2). Thus, the active and tyrosine-phosphorylated Raf-1 populations in Leu61 cells constitute a membrane-associated subpopulation.

Cell fractionation experiments indicated that nearly all of the Raf-1 in NIH 3T3 cells was cytosolic and that while a significant amount of Raf-1 translocated to the membrane in Leu61 cells, the majority was still cytosolic (see Fig. 5). Although Raf-1 can be easily detected in the cytosols of both cell lines, those Raf-1 molecules contain only vanishingly small amounts of phosphotyrosine and are virtually inactive (data not shown).

**Inactivation of Raf-1 from Ras-transformed cell membranes by PTP-1B.** To confirm that the tyrosine phosphorylation of the membrane-associated, active pool of Raf-1 controlled its activity, we immunoprecipitated Raf-1 from membranes and treated the immune complexes with PTP-1B. As shown in Fig. 3, treatment with PTP-1B diminishes the ability of Raf-1 to phosphorylate and activate MEK, while inclusion of 14-3-3 or sodium orthovanadate blocks Raf-1 inactivation. This result indicates that, as in Sf9 cells, Ras-induced Raf-1 activity in fibroblasts is dependent on tyrosine phosphorylation.

**Regulation of Raf-1 and B-Raf activities in Ras-transformed cells.** We compared the ability of Ras to regulate the activity of Raf-1 and with its ability to regulate B-Raf, since the latter composes a considerable proportion of the total MEK-activating activity in fibroblasts (43). Importantly, alignment of the sequences of Raf-1 and B-Raf indicates that tyrosines 340 and 341 of Raf-1, the previously identified sites of tyrosine phosphorylation (14), are replaced by two aspartic acid residues in B-Raf. These sites are approximately 15 residues upstream of the Gly-X-Gly-X-X-Gly motif signifying the start of the kinase domain, and the presence of negatively charged residues in B-Raf suggests that B-Raf should have an elevated basal activity but should not be further activated by the phosphotyrosine-dependent mechanism which activates Raf-1.

To assess the manner in which the activities of Raf-1 and B-Raf are regulated by Ras transformation, the two Raf proteins were separately immunoprecipitated from serum-deprived NIH 3T3 cells and Leu61 cells. The activities of the Rafs were measured by MEK-1 activation to confirm the specificity of MEK-1 phosphorylations. MEK-1 activity against kinase-inactive p42 MAP kinase (K52R) in the absence of a Raf immune complex was defined as the baseline and was subtracted from observed Raf activities (Fig. 4A, lanes 1 and 8).

**FIG. 2.** Activity and tyrosine phosphorylation of Raf-1 associated with the plasma membranes of normal and Ras-transformed NIH 3T3 fibroblasts. Raf-1 immunoprecipitates (minus or plus antigenic peptide) from enriched, homogenized plasma membranes were analyzed by anti-Raf-1 immunoblotting (A), antiphosphotyrosine blotting (B), or phosphorylation of MEK-1 in kinase buffer with 100 μM [γ−32P]ATP (5,000 cpm/pmol) for 15 min at 30°C, resolution by SDS-PAGE, and autoradiography (C).

**FIG. 3.** Inactivation of Raf-1 isolated from Ras-transformed NIH 3T3 fibroblast membranes by PTP-1B. To confirm that the tyrosine phosphorylation of the membrane-associated, active pool of Raf-1 controlled its activity, we immunoprecipitated Raf-1 from membranes and treated the immune complexes with PTP-1B. As shown in Fig. 3, treatment with PTP-1B diminishes the ability of Raf-1 to phosphorylate and activate MEK, while inclusion of 14-3-3 or sodium orthovanadate blocks Raf-1 inactivation. This result indicates that, as in Sf9 cells, Ras-induced Raf-1 activity in fibroblasts is dependent on tyrosine phosphorylation.
B-Raf was active in NIH 3T3 cells (Fig. 4A, lane 5) and Leu61 cells (lane 12), while total Raf-1 activity in NIH 3T3 cells represented only 3% of the total activity of B-Raf (lanes 7 and 5) but 82% of the total activity of B-Raf in Leu61 cells (lanes 14 and 12). In repetitions of this experiment, the ranges of values obtained for total Raf-1 activity (as percentages of total B-Raf activity) were 3 to 5% in NIH 3T3 cells and 65 to 82% in Leu61 cells. Raf immunoprecipitations were all devoid of MEK activity (Fig. 4A, lanes 4, 6, 11, and 13), and normal rabbit serum did not immunoprecipitate any MEK activators (lanes 3 and 10). These results indicate that Ras transformation could increase the total activity of Raf-1 in these cells to a level comparable to that of B-Raf. In contrast, the activity of B-Raf, which has negatively charged residues in place of the tyrosine phosphorylation sites of Raf-1, was not significantly affected by Ras transformation.

Previous reports have described active Raf-1 in association with plasma membranes, and our results confirm these reports. Cell fractionation experiments were carried out to determine the proportion of each Raf family member which was membrane localized in Leu61 cells. Following homogenization, unbroken cells were removed by brief centrifugation, and remaining homogenates were partitioned between particulate and cytosolic fractions. Negligible levels of either Raf protein were present in NIH 3T3 cell particulate fractions (data not shown), whereas in Leu61 cells, a significant amount of Raf-1 was in the membrane pellet (we estimate 20% [Fig. 5B, lane 3]). In contrast, B-Raf was not detectable in the membrane pellet (Fig. 5A, lane 3), suggesting that B-Raf is primarily cytosolic, in agreement with previous reports (5, 23, 43). These observations constitute further evidence suggesting differential regulation of Raf-1 and B-Raf.

**DISCUSSION**

**Regulation of Raf-1.** Although it is clear that members of the Raf family play a central role in signal transduction, the mechanism(s) by which their activities are regulated is incompletely understood and has been the subject of considerable controversy. Studies of Raf regulation have been hindered by the fact that only recently has it become possible to assay Rafs appropriately, with a physiologic substrate (MEKs) (8, 21, 30), and...
also because Rafs are not abundant cellular proteins. Thus, it has been difficult to reliably analyze either Raf activity or Raf phosphorylation.

The initial report linking tyrosine phosphorylation of Raf-1 to its enzymatic activation following platelet-derived growth factor treatment of BALB 3T3 cells (41) was followed by numerous reports that Raf-1 does not become detectably phosphorylated on tyrosine (1, 3, 4, 29). Tyrosine phosphorylation of Raf-1 is generally accepted in the case of Sf9 cells coexpressing high levels of Src or a related kinase (14, 55), and similar experiments have recently been reported for fibroblasts (33), but the relevance of these observations to normal signal transduction pathways has been uncertain. Only in the case of lymphoid-cell activation has tyrosine phosphorylation of Raf-1 been generally observed in cells which have not been engineered to overexpress a tyrosine kinase (reviewed in references 42 and 48).

An alternative approach to assessing whether tyrosine phosphorylation of Raf-1 is required for its activity is to determine whether it can be inactivated by phosphotyrosine-specific phosphatases. Inactivation of Raf-1 by tyrosine phosphatase CD45 has been reported (50), but the results of that study are not conclusive because MEK activation was not used to measure Raf activity, and it thus was uncertain whether Raf or an associated kinase was being assayed. Recently, we showed that highly purified Raf-1 \( \text{Ras/Src} \) could be inactivated by PTP-1B and that inactivation could be blocked by 14-3-3 and Hsp90 (9), thus providing direct biochemical evidence that tyrosine phosphorylation of Raf-1 is important for its activity in the triple-overexpression system. In the study reported here, we have used a similar method to analyze the mechanism of Raf-1 activation by Ras, in the absence of an overexpressed tyrosine kinase. Raf-1 \( \text{Ras/Src} \) was susceptible to inactivation by PTP-1B, constituting the first direct evidence that Ras-induced activation of Raf-1 is dependent on tyrosine phosphorylation.

We also demonstrated for the first time that the mechanism by which 14-3-3 and Hsp90 block Raf-1 inactivation by PTP-1B is by blocking dephosphorylation, presumably by masking the sites of tyrosine phosphorylation. Thus, we propose that these proteins can act as regulators of Raf-1 by inhibiting dephosphorylation rather than by directly activating Raf-1 or by maintaining Raf-1 in an active conformation independent of its phosphorylation state.

We suspect that prior failures to observe tyrosine phosphorylation of Raf-1 in fibroblasts stimulated with growth factors might have been a consequence of the fact that ligand stimulation of these cells leads to less than 20% Ras-GTP loading, constituting a stimulation of less than fivefold (6, 18, 56), and thus Ras-dependent changes in Raf-1 phosphorylation would be difficult to detect under these circumstances. We reasoned that, if it occurred at all, Raf-1 tyrosine phosphorylation should be evident in Ras-transformed cells, which display as much as a 100-fold increase in the levels of Ras-GTP (45). As predicted, no phosphotyrosine was detected on Raf-1 from normal NIH 3T3 cells, and nearly all the cellular Raf-1 protein was cytosolic and inactive. By contrast, in cells transformed by Ha-Ras \(^{61T} \), a small amount of Raf-1 protein was associated with plasma membranes, but importantly, all detectable Raf-1 activity as well as Raf-1 phosphotyrosine localized with plasma membranes. Therefore, while only a portion of the total Raf-1 protein is membrane associated, this population consists of those Raf-1 molecules which have become enzymatically activated and are sensitive to inactivation by PTP-1B. Therefore, the phosphotyrosine content of Raf-1 correlates with its activity, and tyrosine dephosphorylation inactivates Raf-1. Since Ras expression induces tyrosine phosphorylation of Raf-1 and tyrosine phosphatases inactivate Raf-1 which has been activated by Ras, we propose that tyrosine phosphorylation is an essential component of the mechanism by which Ras-dependent ligands activate Raf-1.

As described above, we believe that it has been difficult to detect tyrosine-phosphorylated and active Raf-1 in ligand-stimulated fibroblasts because only small amounts of Ras and Raf-1 become activated. In addition, the active Raf-1 is not readily solubilized from fibroblast membranes by standard lysis buffers (47). In lymphocytes, by comparison, the magnitude of Ras-GTP loading is up to 50% (13), a level comparable to that seen in Ras-transformed fibroblasts, and this could lead to a higher stoichiometry of Raf-1 tyrosine phosphorylation. In addition, Raf-1 in lymphocytes translocates to the cytoplasm following tyrosine phosphorylation and activation, which may have eased the extraction and analysis of the active Raf-1 pool (35, 36).

Regulation of B-Raf. The Raf family member B-Raf has been determined by biochemical assays to be the major MEK activator in neuronal tissue (5, 23) and, more recently, in quiescent or agonist-stimulated fibroblasts (43). Alignment of Raf-1 and B-Raf sequences shows that the amino acids homologous to tyrosines 340 and 341 of Raf-1 (also present in A-Raf), which are located 15 residues upstream of the Gly-X-Gly-X-Gly motif, are replaced by aspartic acid residues in B-Raf. It is worth noting that these positions are represented by glutamic acid residues in D-Raf and aspartic acid residues in Caenorhabditis elegans Raf, which are the two systems in which genetic evidence demonstrates the central role of Raf in signalling (12, 19, 28, 44).

Mutation of tyrosine 340 of Raf-1 to aspartic acid generates a protein with an elevated enzymatic activity and the ability to cause neoplastic transformation (14). B-Raf displays a high basal level of activity even in quiescent fibroblasts (43), which is consistent with the notion that a negative charge at this site enzymatically activates Rafs. However, this endogenous B-Raf activity is insufficient to transform cells, and MAP kinase and MEK are not constitutively active in cells expressing constitutively active B-Raf. Therefore, additional regulatory mechanisms must exist to couple enzymatically active Rafs with their effector molecules. In particular, it is possible that intracellular localization of the Ras as well as their enzymatic activity determines their MEK-activating and -transforming activities, since B-Raf is largely cytosolic whereas activated Raf-1 is membrane associated.

If a function of Ras is to recruit Raf-1 to a site of membrane-associated tyrosine phosphorylation, then the fact that B-Raf already possesses negative charges at these sites should render this isoform insensitive to Ras-induced activation. In fact, we found that while Raf-1 activity, membrane localization, and phosphotyrosine content were significantly elevated by Ras transformation, B-Raf was largely unaffected in serum-deprived cells. However, as it has previously been shown that B-Raf is able to interact with Ras in vitro (38, 43), we suspect that Ras plays a role in the regulation or function of B-Raf activity in vivo, but that role has not been elucidated in our studies. Furthermore, our measurements were of steady-state B-Raf activity, while others have studied transient B-Raf activation in response to agonists. It is possible that Ras overexpression sensitizes B-Raf to activation by other agonists or intracellular signals, and this is currently being investigated.

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