Association of MEK1 with p21^{ras} · GMPPNP Is Dependent on B-Raf

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We have previously reported that immobilized p21^{ras} forms a GMPPNP-dependent complex with a MEK activity. Furthermore, the association of the MEK activity was found to be independent of the presence of Raf-1. We have extended those observations to show that MEK1 is the MEK activity previously described to associate with immobilized p21^{ras} · GMPPNP. The association between MEK1 and immobilized p21^{ras} · GMPPNP increased its specific activity towards p42^{MAPK}. We detected the specific association of B-Raf with immobilized p21^{ras} · GMPPNP. In contrast to Raf-1-immunodepleted lysates, preclearance of the cytosolic B-Raf significantly reduced, by 96%, the amount of MEK1 activity associated with immobilized p21^{ras} · GMPPNP. The decrease in MEK1 activity correlated with complete loss in the binding of both B-Raf and MEK1 proteins with immobilized p21^{ras} · GMPPNP. These data suggest that the p21^{ras} · GMPPNP-dependent activation of MEK1 in brain extracts is dependent on the presence of the B-Raf protein kinase.

The link between activation of tyrosine kinase receptors and the propagation of mitogenic signals via the small guanine nucleotide-binding protein p21^{ras} is well documented. In quiescent cells, p21^{ras} is predominantly found in an inactive state bound to GDP. Active p21^{ras}, bound to GTP, accumulates in response to a large number of extracellular factors. These ligands include phorbol esters and receptor agonists in T lymphocytes, nerve growth factor (NGF) and epidermal growth factor (EGF) in PC12 cells, and platelet-derived growth factor, EGF, and insulin in various fibroblast cell lines (5). Activation of receptors with their respective ligand results in the phosphorylation of specific tyrosine residues on the carboxyl-terminal cytoplasmic domain of the receptor. This induces the formation of a signaling complex between the tyrosyl-phosphorylated residue and several SH2/SH3-containing proteins, including the adaptor protein GRB2. The formation of an activated receptor-adaptor protein complex results in the translocation of guanine nucleotide exchange factors (mSOS1 and -2) to the plasma membrane through their interactions with the adaptor protein's SH3 domains (3, 6, 11, 30, 44, 52). The juxtaposition of p21^{ras} and mSOS results in the rapid formation of p21^{ras} · GTP, thereby generating an appropriate cellular response, whether that be proliferation or differentiation.

Some of the events which lie downstream of signal propagation following the formation of active p21^{ras} · GTP have been identified. Microinjection of an inactivating p21^{ras} antibody, Y13-259, into quiescent serum-starved NIH 3T3 cells inhibits their serum-stimulated proliferation response (34). This inhibitory action was not observed in cells transformed by either of the serine-threonine protein kinases Raf or Mos. Src-transformed cells, however, were effectively blocked by Y13-259 microinjection (53). These data indicated that p21^{ras} lies downstream of tyrosine kinases but upstream or on a separate pathway from the cytoplasmic serine-threonine protein kinases.

Introduction of active p21^{ras} into whole cells or crude lysates results in the activation of a number of soluble protein kinases,

specifically Raf-1, MEK (mitogen-activation protein [MAP] kinase kinase, the protein responsible for the activation of MAP kinases), and members of the MAP kinase family. Expression of the dominant inhibitory mutant of p21^{ras}, p21^{Asn-17}, inhibits the growth factor-dependent activation of these cytosolic protein kinases (1, 4, 9, 10, 16, 29, 40, 42, 49, 57, 64). The observation that active Raf-1 protein activates inactive MEK (MAP kinase kinase) forged another link between tyrosine kinase receptor signaling and activation of cytoplasmic MAP kinase(s) (8, 17, 25). Expression of a dominant inhibitory Raf-1 mutant blocks transformation of cultured NIH 3T3 cells by activated p21^{ras} (23, 46) and inhibited p21^{ras}-dependent MAP kinase activation (46). These studies suggest that these cytosolic enzymes function as part of a downstream kinase cascade activated by p21^{ras} · GTP and support the contention that Raf-1 might be the immediate p21^{ras} effector protein.

Much of the research on the activation of Raf-1 and the MAP kinase cascade has been performed in mitogen-activated whole cells. The MAP kinase signaling cascade also appears to be required in the process of differentiation. Earlier studies on NGF-stimulated differentiation in rat PC12 pheochromocytoma cells implicated the involvement of the p21^{ras}-Raf-1-MAP kinase cascade (56, 57, 63, 64). In agreement with the activation of receptor tyrosine kinases, activation of the *trk* receptor by NGF stimulates the production of p21^{ras} · GTP (35, 36, 41). Overexpression of the dominant inhibitory mutant, p21^{Asn-17}, blocked NGF-stimulated differentiation, whereas introduction of oncogenic p21^{ras} mimicked neuronal differentiation (37, 45, 56). p21^{ras} also functions as a transducer of NGF-induced differentiation in rat PC12 cells. It is possible that both mitogenic and differentiating signals from active p21^{ras} · GTP converge on a similar signaling pathway involving Raf-1 and MAP kinases.

The components of this signaling pathway, however, may not always be coupled to each other. There are several signaling pathways which result in the activation of MAP kinases independent of Raf-1 function. A Raf-1-independent MAP kinase signaling pathway was recently identified in rat fibroblasts stimulated by the addition of either EGF or transforming growth factor β (21). The oncogenic form of either Ras or Raf-1 did not constitutively activate the MAP kinase pathway in Rat 1a fibroblasts (15). These studies suggest there are several mechanistically distinct pathways, all of which result in

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the activation of MAP kinase(s). Previous studies from this laboratory indicated the existence of a $p21^{ras}$ · GTP-dependent, Raf-1-independent MAP kinase pathway (33).

We designed experiments to directly identify the protein(s) involved in the $p21^{ras}$ · GTP-dependent activation of MAP kinase. Here we report that in rat brain cytosolic extracts, B-Raf is the protein kinase responsible for the association and activation of MEK1 by immobilized $p21^{ras}$ · GMPPNP.

MATERIALS AND METHODS

Reagents. Activated silica matrix (*N*-hydroxysuccinimide) was purchased from Affinity Technology (New Brunswick, N.J.), liquid nitrogen-frozen 6-week-old rat brains were purchased from Harlan Bioproducts for Science (Indianapolis, Ind.), and nonhydrolyzable GTP analogs were purchased from Boehringer Mannheim. All other reagents were purchased from standard vendors.

Preparation of $p21^{ras}$ and rat brain extracts. Recombinant $p21^{ras}$ was purified from soluble *Escherichia coli* extracts as previously described (58). Rat brain cytosolic extracts were prepared as previously described (62).

Preparation of immobilized $p21^{ras}$. A 1-g aliquot of silica matrix was washed twice with 10 ml of ice-cold water and twice with ice-cold 1× p21 buffer (20 mM morpholinepropanesulfonic acid [MOPS; pH 7.4], 200 mM sucrose, 4 mM $MgCl_2$, 0.1 mM EDTA); 10 mg of either recombinant $p21^{c-Ha-ras}$ (referred to as $p21^{ras}$ throughout this report) or $p21^{Ile-40}$ (5 to 10 mg/ml) was added to the prepared matrix. The coupling reaction proceeded overnight at 4°C on a labquake. The immobilized $p21^{ras}$ was washed three times with 1× p21 buffer containing 1 mM dithiothreitol.

Assay of immobilized $p21^{ras}$. Ten microliters of immobilized $p21^{ras}$ was added to 45 μ l of 1× p21 buffer containing 100 μ M unlabeled GTP, 2.5 μ Ci of [α - 32 P]GTP (400 Ci/mmol), and 10 mM EDTA. After a 30-min incubation at room temperature, Mg^{2+} was added to a final concentration of 20 mM. The immobilized $p21^{ras}$ was washed three times with 1× p21 buffer. The amount of immobilized $p21^{ras}$ was calculated from the specific activity of the [α - 32 P]GTP in the reaction mixture and the amount of [α - 32 P]GTP bound to the immobilized $p21^{ras}$. The total amount of $p21^{ras}$ bound to the activated silica matrix was calculated after each coupling reaction. Coupling efficiency varied from between 40 and 60%. The immobilized $p21^{ras}$ is stored at 4°C with a minimal loss of activity over a 2-week period. The amount of immobilized $p21^{ras}$ used throughout the study always reflect measurements made by GTP binding assays and not by protein determination.

Formation of active immobilized $p21^{ras}$ · GMPPNP. Immobilized apo- $p21^{ras}$ was prepared as previously described (13, 20). Briefly, immobilized $p21^{ras}$ was washed three times with 200 mM ammonium sulfate–20 mM MOPS (pH 8.0). Three units of calf intestine alkaline phosphatase per mg of immobilized $p21^{ras}$ was added to the washed pellets. The immobilized $p21^{ras}$ was incubated at room temperature for at least 1 h with constant mixing and washed four times with 1× p21 buffer; 1 mM GMPPNP and 10 mM $MgCl_2$ were added to the pellet. This procedure hydrolyzes all of the bound GDP to unbound guanosine, which was subsequently removed by the washes. The immobilized $p21^{ras}$ was quantitatively loaded with GMP-PNP as determined by fast protein liquid chromatography analysis (data not shown). The three-dimensional crystal structure of $p21^{ras}$ · GMPPNP is identical to that of $p21^{ras}$ · GTP (47).

Preparation of rabbit polyclonal antibody against B-Raf. The B-Raf antibody is a rabbit polyclonal antiserum directed

against a synthetic peptide corresponding to the carboxyl-terminal 12 amino acid residues with an additional cysteine residue (CIQAGGYGAFPVH). The peptide was coupled to keyhole limpet hemocyanin, and the antiserum was generated by standard immunization protocol of chinchilla rabbits.

Affinity precipitation of proteins specifically associating with $p21^{ras}$. Twenty-five micrograms of immobilized $p21^{ras}$ · GDP or GMPPNP was incubated with 250 μ l of rat brain cytosol (20 mg/ml) for 60 min at 4°C with constant mixing. The rat brain extracts containing the immobilized $p21^{ras}$ were layered onto 5× p21 buffer (1 M sucrose). The immobilized $p21^{ras}$ was allowed to settle, and the remaining supernatant was removed to the 1 M sucrose interface. The 1 M sucrose was overlaid with 2 M urea to wash the walls of the microcentrifuge tubes of nonspecifically bound proteins. The entire supernatant was removed, and the immobilized $p21^{ras}$ was washed three times with 1× p21 buffer containing 0.02% Nonidet (NP-40) and 1 mM dithiothreitol and three times with 1× p21 buffer alone. The washed immobilized $p21^{ras}$ was treated as described below.

Immunoblot analysis. Laemmli sample buffer (2×) was added to the washed immobilized $p21^{ras}$; the samples were heated at 110°C for 5 min and resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (8 or 10% gel), electrotransferred to Immobilon-P (Millipore), and immunoblotted as follows. The Immobilon-P was blocked for at least 1 h at room temperature with 1× Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% NP-40) containing calf serum (1%) and dry milk (5%). The Immobilon-P was washed three times with 1× TBS and incubated for 2 to 3 h at room temperature with the following antibodies: anti-p95^{B-Raf}, anti-MEK kinase and anti-MEK1 (Transduction Laboratories, Lexington, Ky.), anti-p74^{Raf-1} (gift from R. Schatzman, Syntex), anti-MEK1 and anti-MEK2 (gifts from M. J. Weber, University of Virginia), or anti-neurofibromin 1 (gift from N. Ratner, University of Cincinnati) at dilutions of between 1:2,500 and 1:5,000. The Immobilon-P was washed three times with TBS (15 min per wash), then incubated with horseradish peroxidase-linked donkey anti-rabbit or mouse secondary antibody (1:5,000 dilution) for 60 min at room temperature, and washed extensively with 1× TBS, and the bands were visualized by enhanced chemiluminescence as recommended by the manufacturer (Amersham).

Preparation of soluble MEK supernatants from immobilized $p21^{ras}$. Samples prepared with immobilized $p21^{ras}$ and washed as described above were incubated for 20 min at 37°C with 10 μ M ATP and the phosphatase inhibitors β -glycerophosphate, *O*-phospho-L-serine, *O*-phospho-L-threonine, *O*-phospho-L-tyrosine, *p*-nitrophenylphosphate (Tris salt), and sodium vanadate (10 μ M each) in a total volume of 50 μ l of 1× p21 buffer. The ATP-eluted supernatants (referred to hereafter as the soluble MEK supernatant) from these reactions were separated from the immobilized $p21^{ras}$ pellet and assayed for MEK activity as described below.

Assay for MEK. All kinase assays were performed within the linear range of 32 P incorporation into the relevant substrates. The soluble MEK supernatant was incubated at 37°C for 20 min in the presence of 0.1 μ g of wild-type recombinant inactive $p42^{MAPK}$, 25 μ M myelin basic protein (MBP), 10 μ M [γ - 32 P]ATP (10 μ Ci, >4,500 Ci/mmol), and 10 μ M phosphatase inhibitors in a total volume of 50 μ l of 1× p21 buffer. The kinase reaction was terminated by the addition of 120 μ l of 10% phosphoric acid. The reaction mixtures were spotted onto P81 phosphocellulose paper (Whatman), washed five times with 0.5% phosphoric acid and once with acetone, and then allowed to dry at room temperature before determination

of the amount of ³²P incorporated into MBP by liquid scintillation counting.

Assay for MEK1. The soluble MEK supernatants were incubated with 50 μ l of protein A-Sepharose coupled to either 1 μ l of a rabbit polyclonal anti-MEK1 antibody (a gift from R. Davis) or preimmune serum for 1 h at 4°C with constant mixing. The antibody-protein A-Sepharose complex was pelleted by centrifugation. The supernatants were removed and incubated with fresh antisera and protein A-Sepharose for an additional 60 min at 4°C. This procedure was repeated for a total of three sequential immunoprecipitations. The immunoprecipitates were washed three times with 5 $\times$ TBS-NP-40 and three times with 1 $\times$ p21 buffer. The washed immunoprecipitates and the postimmunoprecipitation soluble MEK supernatants were assayed for the presence of active MEK as described above.

Assay of the ability of B-Raf and Raf-1 to activate recombinant MEK1 in vitro. The activation of wild-type inactive recombinant MEK1 was examined as follows. One milliliter of crude rat brain cytosolic extract (11 mg/ml) was incubated for 2 h at 4°C with 50 μ l of protein A-Sepharose either alone or with 3 μ l of the following rabbit polyclonal antisera: nonimmune, anti-B-Raf, or anti-Raf-1. The immunoprecipitates were washed three times with 5 $\times$ TBS-NP-40 and three times with 1 $\times$ p21 buffer. The washed immunoprecipitates were incubated with 2 μ g of recombinant MEK1 (a gift from G. Landreth, Case Western Reserve University), 50 μ M [γ -³²P] ATP (20 μ Ci), 50 μ M phosphatase inhibitors, 0.4 μ g of recombinant wild-type inactive p42^{MAPK}, 50 μ M MBP, and 10 mM Mg²⁺ in a total volume of 100 μ l of 1 $\times$ p21 buffer for 20 min at 37°C. The soluble phosphorylated MBP was separated from the pellet, resolved by SDS-PAGE (12% gel), visualized, and quantitated by a Molecular Dynamics PhosphorImager.

Immunodepletion studies. Five hundred microliters of crude rat brain cytosolic extract (12 mg/ml) was incubated with constant mixing in the presence of 50 μ l of protein A-Sepharose alone or with 4 μ l of either rabbit nonimmune or rabbit anti-B-Raf polyclonal antiserum for 60 min at 4°C. The antibody-protein A-Sepharose complex was pelleted by centrifugation. The cytosolic extracts were removed and incubated with fresh antisera and protein A-Sepharose for an additional 60 min at 4°C. This procedure was repeated for a total of five sequential immunoprecipitations. Samples were prepared in duplicate as follows: 250 μ l of each of the immunodepleted cytosolic extracts was incubated with either 25 μ g of immobilized p21^{ras} · GDP or GMPPNP, washed, and assayed for MEK activity or immunoblotted to detect the proteins associating with the immobilized p21^{ras} incubated with the various pre-treated cytosolic extracts as previously described above.

Protein determination and SDS-PAGE. Protein assays were performed by either the Bradford or modified Lowry method (2, 39). SDS-PAGE was performed as described by Laemmli (27).

RESULTS

MEK1 association with immobilized p21^{ras} · GMPPNP. Previous studies from this laboratory indicated the presence of a MEK activity associating specifically with active forms of immobilized p21^{ras} (33). To identify which members of the recently described MEK family bound to immobilized p21^{ras}, we carried out immunoblot analysis of the proteins associating with immobilized p21^{ras} with specific rabbit polyclonal antibodies generated against the N-terminal amino acid sequence of either MEK1 or MEK2 (43). We detected the specific association of MEK1 with immobilized p21^{ras} · GMPPNP (Fig. 1A).

No binding of MEK1 was detected with immobilized p21^{ras} · GDP. MEK2 was not detected in the crude rat brain cytosolic extracts by immunoblot analysis (data not shown).

To examine whether MEK1 was the MEK responsible for the activity previously shown to associate with immobilized p21^{ras} · GMPPNP, the activity of the MEK1 binding with immobilized p21^{ras} · GMPPNP was studied by two different in vitro approaches. First, activation of MEK1 was monitored by the appearance of a more slowly migrating form of the protein upon analysis by SDS-PAGE. Incubation of the washed immobilized p21^{ras} pellets with unlabeled ATP resulted in a slight retardation in the mobility of the MEK1 associated with the immobilized p21^{ras} · GMPPNP compared with untreated samples (Fig. 1B, lanes 4 and 5). Immunoblot analysis showed that MEK1 was released from the immobilized p21^{ras} · GMPPNP after incubation with ATP (Fig. 1B, lane 6). This pool of soluble MEK1 was also found to migrate at an apparent higher molecular weight compared with the untreated MEK1. Furthermore, the release of MEK1 from immobilized p21^{ras} · GMPPNP was not detected when samples were incubated with AMPPNP, a nonhydrolyzable analog of ATP (data not shown). This finding suggests that MEK1 is released from immobilized p21^{ras} · GMPPNP in a phosphorylation-dependent manner. It is important to consider that band shifts do not necessarily correlate with enzymatic activation, since feedback phosphorylation of MEK1 by MAP kinase has been documented (32). We examined whether the more slowly migrating MEK1 protein released from immobilized p21^{ras} · GMPPNP was active. Soluble MEK supernatants prepared from both immobilized p21^{ras} · GDP and GMPPNP were immunoprecipitated with a specific rabbit anti-MEK1 antibody, and the immunoprecipitates were examined for the ability to activate exogenously added recombinant wild-type p42^{MAPK} (Fig. 1C). Activation of the p42^{MAPK} was detected in the presence of MEK1 immunoprecipitates from soluble MEK supernatants of immobilized p21^{ras} · GMPPNP. However, the soluble MEK supernatants were cleared of the endogenous MEK1 activity by three sequential immunoprecipitations, as shown by the decrease of ³²P incorporated into MBP. No activation was detected by anti-MEK1 immunoprecipitates prepared from identical immobilized p21^{ras} · GDP samples (Fig. 1C). No MEK activity, as measured by the lack of activation of recombinant wild-type p42^{MAPK}, remained in the soluble MEK supernatants following sequential immunoprecipitations with the MEK1-specific antibodies (Fig. 1D). Activation of p42^{MAPK} was detected, however, in the soluble MEK supernatants of immobilized p21^{ras} · GMPPNP immunoprecipitated with the rabbit preimmune serum.

These results suggest that MEK1 is the only active MEK associated and released from immobilized p21^{ras} · GMPPNP.

Specific activity of MEK1. To address the possibility that the MEK1 present in the brain cytosolic extracts is active prior to incubation with immobilized p21^{ras} · GMPPNP, studies were undertaken to determine whether the association of MEK1 with immobilized p21^{ras} · GMPPNP increased its kinase activity toward recombinant wild-type p42^{MAPK}.

We examined the activation of MEK1 immunoprecipitated either from crude rat brain cytosolic extracts before incubation with immobilized p21^{ras} or from soluble MEK supernatants prepared from immobilized p21^{ras} · GDP or GMPPNP (Fig. 2A). Duplicate samples were prepared and immunoblotted for the presence of MEK1 protein associating with the immunoprecipitates (Fig. 2B). The immunoprecipitated MEK1 from the crude cytosolic extracts possessed significant activity toward p42^{MAPK}. Only a 1.4-fold difference in the amount of ³²P incorporated into MBP was observed between samples immu-

FIG. 1. Specific association of MEK1 with immobilized p21^{ras} · GMPPNP. (A) A 250- μ l aliquot of crude rat brain cytosolic extract (20 mg/ml) was incubated with 25 μ g of either immobilized p21^{ras} · GDP (lane 2) or p21^{ras} · GMPPNP (lane 3) for 1 h at 4°C. The pellets were washed, 2 $\times$ Laemmli sample buffer was added, and the mixture was heated and analyzed on an SDS–8% polyacrylamide gel. Immunoprecipitation of equivalent amounts of lysate with an N-terminal 15-amino-acid-specific rabbit anti-MEK1 antibody (generous gift from R. Davis, Howard Hughes Institute) detected a single band of 44 kDa (lane 5), whereas the preimmune serum did not react with this protein (lane 4). Cytosolic extract (25 μ g; lane 1) was also analyzed for the presence of MEK1 by immunoblot analysis as described in Materials and Methods. (B) A 250- μ l aliquot of crude rat brain cytosolic extract (20 mg/ml) was incubated with 25 μ g of either immobilized p21^{ras} · GDP (lanes 1 to 3) or p21^{ras} · GMPPNP (lanes 4 to 6) for 1 h at 4°C. The pellets were washed and incubated with 10 μ M ATP and 10 μ M phosphatase inhibitors in a total volume of 50 μ l of 1 $\times$ p21 buffer for 20 min at 37°C. The proteins released into the soluble ATP supernatants (lanes 3 and 6) and the proteins that associate with the immobilized p21^{ras} before (lanes 1 and 4) and after (lanes 2 and 5) ATP incubation were analyzed by immunoblot analysis. The position of phosphorylated MEK1 is indicated by an arrow. Sizes are indicated in kilodaltons. (C) Soluble MEK supernatants prepared, as described in Materials and Methods, from immobilized p21^{ras} · GDP ($\square$) or p21^{ras} · GMPPNP ($\blacksquare$) were immunoprecipitated with a rabbit polyclonal antibody against MEK1 for 1 h at 4°C. The antibody-protein A-Sepharose complex was pelleted by centrifugation. The soluble MEK supernatants were removed and incubated with fresh antisera

and protein A-Sepharose for an additional 60 min at 4°C. This procedure was repeated for a total of three times. The washed immunoprecipitates (IP1, IP2, and IP3) were assayed for the presence of active MEK1 by the addition of 0.1 μ g of recombinant wild-type inactive p42^{MAPK} in the presence of 10 μ M [γ -³²P]ATP (10 μ Ci), 10 μ M phosphatase inhibitors, and 25 μ M MBP in a total volume of 50 μ l of 1 $\times$ p21 buffer for 20 min at 37°C. The amount of phosphorylated MBP was determined as described in Materials and Methods. The rabbit preimmune control immunoprecipitation of the soluble MEK supernatants possessed background levels of ³²P incorporated into MBP (data not shown). (D) The soluble MEK supernatants remaining from panel C after sequential immunoprecipitation with either rabbit preimmune serum ($\blacksquare$) or rabbit anti-MEK1 antibody ($\square$) prepared from immobilized p21^{ras} · GDP (lane 1) or p21^{ras} · GMPPNP (lane 2) were assayed for the presence of active MEK as described in Materials and Methods.

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FIG. 2. Increase in MEK1 activity upon association with immobilized p21^{ras} · GMPPNP. (A) A 250- μ l aliquot of crude rat brain cytosolic extract (13.5 mg/ml) was incubated with 25 μ g of either immobilized p21^{ras} · GDP (lane 1) or GMPPNP (lane 2) for 1 h at 4°C. The immobilized p21^{ras} was washed and incubated for 20 min at 37°C with 50 μ l of 1 $\times$ p21 buffer containing 10 μ M ATP and 10 μ M phosphatase inhibitors. The soluble MEK supernatants were separated from the immobilized p21^{ras} and immunoprecipitated with a rabbit anti-MEK1 antibody. A 250- μ l aliquot of cytosolic extracts was immunoprecipitated with the specific rabbit anti-MEK1 antibody (lane 4) or preimmune serum (lane 3) as described in Materials and Methods. The amounts of immunoprecipitated MEK1 from soluble MEK supernatants prepared from immobilized p21^{ras} pellets and from the cytosolic extracts were assayed for MEK activity as described in Materials and Methods. The MEK1 antibody used did not inhibit the MEK1 activity (data not shown). (B) As in panel A except that both the MEK1 immunoprecipitated from soluble MEK supernatants prepared from immobilized p21^{ras} and that associated with the preimmune and anti-MEK1 immunoprecipitates were resolved on an SDS-8% gel and immunoblotted as described in Materials and Methods. The immunoprecipitated MEK1 from soluble MEK1 supernatants prepared from immobilized p21^{ras} · GMPPNP is shown by an arrow. The position of the molecular weight marker is shown at the left in kilodaltons. The results are representative of at least three separate determinations.

not shown). Multiple bands of approximately 65 to 70 and 95 to 105 kDa were detected in immunoprecipitates of the cytosolic extracts, using a rabbit polyclonal antibody generated against the C-terminal 12 amino acids of the B-Raf protein (Fig. 3A). Inclusion of an excess of antigen with the primary antibody solution completely blocked the observed signal with all of these proteins. The multiple B-Raf bands immunoprecipitated were also detected by immunoblotting with a mouse monoclonal antibody raised against the conserved Raf catalytic domain (24) (data not shown). Furthermore, the rabbit polyclonal B-Raf antibody did not cross react with Raf-1, as indicated by the failure of the antibody to recognize a 74-kDa protein associating with immunoprecipitates prepared with a specific antibody generated against the C-terminal 12 amino acids of Raf-1 (data not shown). These data, therefore, suggest that all of these proteins (65 to 70 and 95 to 105 kDa) belong to the B-Raf family. Immunoblot analysis detected significant amounts of B-Raf protein associating with immobilized p21^{ras} · GMPPNP (Fig. 3B). In agreement with the interaction between Raf-1 and p21^{ras}, we were unable to detect the association of B-Raf with immobilized p21^{ras} · GDP.

FIG. 3. Specific association of B-Raf with immobilized p21^{ras} · GMPPNP. (A) A 250- μ l aliquot of crude brain cytosolic extract (20 mg/ml) was incubated with either rabbit nonimmune serum (lane 1) or rabbit anti B-Raf antibody (lane 2) coupled to 50 μ l of protein A-Sepharose for 2 h with constant mixing. The immunoprecipitated pellets were washed three times with 5 $\times$ TBS-NP-40 and three times with 1 $\times$ p21 buffer. The B-Raf protein was resolved by SDS-PAGE (8% gel) and immunoblotted with a primary antibody solution of the anti B-Raf antibody alone or in combination with an excess of peptide antigen used to generate the antibody [+ Peptide (CIQAGGYGAFVH)]. Arrows denote the multiple B-Raf proteins observed with the molecular masses of 65 to 70 and 95 to 105 kDa. (B) A 250- μ l aliquot of crude cytosolic extracts (20 mg/ml) was incubated with 25 μ g of either immobilized p21^{ras} · GDP (lanes 1 to 5) or p21^{ras} · GMPPNP (lanes 6 to 10) for 60 min at 4°C with constant mixing. The immobilized p21^{ras} pellets were washed, and either 2 $\times$ Laemmli sample buffer was added (lanes 1 and 6) or the pellets were incubated with 10 μ M ATP and 10 μ M phosphatase inhibitors in a total volume of 50 μ l of 1 $\times$ p21 buffer for 20 min at 37°C. The soluble supernatant (lanes 4, 5, 9, and 10) and the proteins associating with the immobilized p21^{ras} pellets after ATP incubation (lanes 2, 3, 7, and 8) were resolved by SDS-PAGE (8% gel) and immunoblotted with anti B-Raf antibodies as described in Materials and Methods. Positions of molecular weight markers are shown at the right in kilodaltons.

Several studies have demonstrated that the mobility of B-Raf in SDS-PAGE is retarded upon activation of PC12 cells with NGF, EGF, and fibroblast growth factor. This shift in apparent molecular weight was phosphorylation dependent (38, 54). Incubation of the B-Raf bound to immobilized p21^{ras} · GMPPNP with ATP induced a mobility shift of the B-Raf protein similar to that reported by others (Fig. 3B). In contrast to MEK1, incubation with ATP did not result in the dissociation of B-Raf from the immobilized p21^{ras} · GMPPNP. This finding suggests that the release mechanism of B-Raf is mechanistically distinct from that of MEK1 and occurs in an ATP-independent manner. In contrast to the observed mobility shifts of both B-Raf and MEK1, we could not detect an ATP-induced mobility shift of the Raf-1 protein associating with immobilized p21^{ras} · GMPPNP (data not shown).

In summary, brain cytosolic extracts contain multiple molecular mass species of B-Raf proteins, 65 to 70 and 95 to 105 kDa, which are recognized by two different antibodies raised against distinct regions of the B-Raf protein, one raised against the conserved catalytic domain of Raf proteins and the second generated against the C-terminal 12 amino acids of B-Raf. Immunoblot analysis indicated that all possess the ability to interact with immobilized p21^{ras} in a guanine nucleotide-dependent manner.

Identification of the site of interaction of B-Raf and MEK1 with immobilized p21^{ras} · GMPPNP. Previous studies showed that an intact effector binding domain (amino acids 30 to 42) is required for the interaction between p21^{ras} · GTP and Raf-1 (22, 33, 59–61, 67). We examined the ability of an effector binding domain mutant of p21^{ras}, p21^{Ile-40}, to bind to B-Raf and activate MEK1.

FIG. 4. Identification of the site of interaction between B-Raf and MEK1 with immobilized p21^{ras} · GMPPNP. (A) A 250- μ l aliquot of crude rat brain cytosolic extract (20 mg/ml) was incubated with 25 μ g of immobilized p21^{Hle-40} · GDP (lane 1), p21^{Hle-40} · GMPPNP (lane 2), p21^{ras} · GDP (lane 3), or p21^{ras} · GMPPNP (lane 4) for 1 h at 4°C. The immobilized p21^{ras} pellets were washed, and 2 $\times$ Laemmli sample buffer was added. The proteins were analyzed by SDS-PAGE (8% gel) and immunoblotted with rabbit polyclonal antibodies to B-Raf (top) and MEK1 (bottom) as described in Materials and Methods. Positions of molecular weight markers are shown on the right in kilodaltons. (B) Samples were prepared as described for panel A except that the washed immobilized p21^{ras} pellets were incubated with 10 μ M ATP and 10 μ M phosphatase inhibitors in a total volume of 50 μ l of 1 $\times$ p21 buffer for 20 min at 37°C. The soluble MEK supernatants were assayed for MEK activity as described in Materials and Methods.

Immobilized p21^{Hle-40}, bound to either GDP or GMPPNP, failed to bind detectable levels of either B-Raf or MEK1 protein (Fig. 4A), and no MEK activity was detected associated with the immobilized p21^{Hle-40} · GMPPNP (Fig. 4B).

As for the interaction between Raf-1 and p21^{ras} · GTP, an intact effector binding domain is required for the association of B-Raf and MEK1 with immobilized p21^{ras} · GMPPNP.

Activation of recombinant wild-type MEK1 by B-Raf in vitro. We examined whether B-Raf from brain cytosolic extracts possessed an ability to activate exogenously added wild-type recombinant MEK1 similar to that reported for Raf-1 (18, 26, 31).

Addition of recombinant wild-type MEK1 to immunoprecipitated B-Raf increased the amount of ³²P incorporated into MBP by 20-fold compared with the rabbit nonimmune and protein A-Sepharose-alone controls (Fig. 5). The recombinant MEK1 protein by itself possessed minimal basal activity. In this assay, MAPK activity, as measured by the phosphorylation of MBP, increased in a dose-dependent manner relative to the amount of exogenously added recombinant MEK1 (data not shown). Consistent with previous reports, immunoprecipitated Raf-1 also activated recombinant MEK1 (a 12-fold activation).

Therefore, rat brain-derived B-Raf and Raf-1 proteins possess the ability to phosphorylate and activate recombinant MEK1 in vitro, indicating that both can act as MEK activators.

FIG. 5. Abilities of both B-Raf and Raf-1 to act as MEK activators in vitro. One milliliter of crude rat brain cytosolic extract (11 mg/ml) was incubated with 50 μ l of protein A-Sepharose either alone (lane 1) or with 3 μ l of nonimmune (lane 2), Raf-1 (lane 3), and B-Raf (lane 4) rabbit polyclonal antisera for 2 h at 4°C with constant mixing. The pellets were extensively washed as described in Materials and Methods and incubated with 2 μ g of recombinant wild-type inactive MEK1, 0.4 μ g of recombinant wild-type inactive p42^{MAPK}, 50 μ M MBP, 50 μ M [γ -³²P]ATP (20 μ Ci), 50 μ M phosphatase inhibitors, and 10 mM Mg²⁺ in a total volume of 100 μ l of 1 $\times$ p21 buffer for 20 min at 37°C. The phosphorylated MBP was separated from the pellets, resolved by SDS-PAGE (12% gel), and quantitated by a Molecular Dynamics PhosphorImager. The results are representative of at least five separate determinations.

Association of MEK1 with immobilized p21^{ras} · GMPPNP is dependent on the presence of B-Raf. Raf-1 was shown to interact directly with MEK1 in in vitro reconstitution experiments using purified recombinant proteins. This result supported observations made with the yeast two-hybrid system, which determined that the point of interaction between MEK1 and Raf-1 was located within the C-terminal domain of the Raf-1 protein. We previously showed that the association of MEK1 activity with immobilized p21^{ras} · GMPPNP was independent of Raf-1 (33). We examined the requirement of B-Raf for the association and activation of MEK1 with immobilized p21^{ras} · GMPPNP. To test this, we examined the association of MEK1 to immobilized p21^{ras} · GMPPNP incubated with cytosolic extracts immunodepleted of B-Raf.

B-Raf-depleted extracts prepared as described in Materials and Methods were incubated with equivalent amounts of immobilized p21^{ras} · GDP or p21^{ras} · GMPPNP. Soluble MEK supernatants prepared by incubating the washed immobilized p21^{ras} with ATP were assayed for the ability to activate p42^{MAPK}. The soluble MEK supernatants prepared by incubation of immobilized p21^{ras} · GMPPNP with B-Raf-immunodepleted extracts lacked the ability to activate recombinant p42^{MAPK}. There was a 96% reduction in the phosphorylation of MBP compared with the control cytosolic extracts (Fig. 6A). The amount of B-Raf protein associating with duplicate samples was measured by immunoblot analysis using the primary rabbit polyclonal B-Raf solution alone or in the presence of an excess of antigen peptide to control for the presence of nonspecific bands (Fig. 6B). This was necessary because a nonspecific band similar in molecular weight to B-Raf was detected in the samples incubated only with the cytosolic extracts repeatedly immunoprecipitated with the rabbit polyclonal B-Raf antisera. This band bound nonspecifically to the silica matrix, and the signal was not abolished by the addition of an excess of antigen peptide during the primary antibody

FIG. 6. Association and activation of MEK1 with immobilized p21^{ras} · GMPPNP is dependent on B-Raf and not Raf-1. (A) Rat brain cytosolic extracts were prepared as described in Materials and Methods for the immunodepletion studies. A 250- μ l aliquot of cytosolic extract (12 mg/ml) precleared with protein A-Sepharose either alone (lanes 1 and 4) or with rabbit nonimmune (columns 2 and 5) or B-Raf (columns 3 and 6) serum was incubated with either 25 μ g of immobilized p21^{ras} · GDP (columns 1 to 3) or p21^{ras} · GMPPNP (columns 4 to 6) for 60 min at 4°C. The pellets were washed and incubated with 10 μ M ATP and 10 μ M phosphatase inhibitors in a total volume of 50 μ l of 1 $\times$ p21 buffer for 20 min at 37°C. The soluble MEK supernatants were assayed as described in Materials and Methods. The decrease in MEK activity associating with p21^{ras} · GMPPNP compared with the control samples, protein A-Sepharose (0%; lane 4), was 27% with nonimmune serum (column 5) and 96% with B-Raf serum (column 6). The results are representative of five separate determinations with the decreases in MEK activity of 5% $\pm$ 12% (mean $\pm$ standard error) for nonimmune serum and 96% $\pm$ 1.4% for B-Raf serum. Minimal MEK activity was associated with immobilized p21^{ras} · GDP. (B) A 250- μ l aliquot of cytosolic extract (12 mg/ml) pretreated as described for panel A was incubated with 25 μ g of either immobilized p21^{ras} · GDP or GMPPNP for 60 min at 4°C with constant mixing. The pellets were washed, 2 $\times$ Laemmli sample buffer was added, and the proteins associating with the immobilized p21^{ras} were resolved by SDS-PAGE (8% gel), transferred to Immobilon-P, and immunoblotted with a primary antibody solution of the rabbit B-Raf antiserum alone (B-Raf; left) or in the presence of an excess of antigen peptide used to generate the antibody [B-Raf + Peptide (CIQAGGYGAFPVH); right] as described in Materials and Methods. (C) As in panel B except that duplicate immobilized p21^{ras} samples were immunoblotted with rabbit polyclonal antibodies raised against neurofibromin-1 (top), Raf-1 (middle), and MEK1 (bottom). Positions of molecular weight markers are shown at the right in kilodaltons.

incubation (Fig. 6B; compare lanes 3 and 6 of both panels). In contrast to this nonspecific band, excess antigen effectively blocked the detection of the specific 65- to 70- and 95- to 105-kDa B-Raf proteins associating with immobilized p21^{ras} · GMPPNP (Fig. 6B; compare lanes 4 and 5 of both panels). In addition to the absence of B-Raf protein binding, we were unable to detect MEK1 protein associating with immobilized p21^{ras} · GMPPNP in the samples prepared with B-Raf-immunodepleted cytosolic extracts (Fig. 6C, lane 6). In agreement with the earlier observations that the association of the MEK1 was a Raf-1 independent event (33), the amount of Raf-1 protein associating with immobilized p21^{ras} · GMPPNP remained constant regardless of the presence of B-Raf protein (Fig. 6C, lanes 4 to 6). To ensure that the lack of MEK1 protein binding was a B-Raf-specific event, we examined the association of neurofibromin-1 in samples treated as in Fig. 6B. Removal of B-Raf from the cytosolic extracts did not alter the amount of neurofibromin-1 associated with immobilized p21^{ras} · GMPPNP, indicating that the binding of neurofibromin-1 to immobilized p21^{ras} · GMPPNP is independent of B-Raf (Fig. 6C). Furthermore, failure to detect MEK1 association with immobilized p21^{ras} · GMPPNP in the absence of B-Raf suggests a direct protein interaction between B-Raf and MEK1.

In summary, the decrease in activation of exogenously added wild-type p42^{MAPK} by soluble MEK supernatants of immobilized p21^{ras} · GMPPNP prepared from B-Raf-depleted extracts correlated with the loss of both MEK1 and B-Raf protein binding. We conclude, therefore, that the association of MEK1 with immobilized p21^{ras} · GMPPNP is dependent on B-Raf and not Raf-1.

In conclusion, our data suggest that (i) MEK1 associates with immobilized p21^{ras} · GMPPNP and (ii) activation of MEK1 is dependent on the presence and association of B-Raf with immobilized p21^{ras} · GMPPNP.

DISCUSSION

Previous studies with mammalian cultured cells revealed the existence of a MAP kinase-activating activity in growth factor-stimulated cells (7, 48, 50). Purification, cloning, and characterization of this activity, now identified as a MAP kinase kinase or MAP kinase/ERK-activating kinase (MEK), demonstrated that this enzyme acts as a dual-specificity kinase; that is, it phosphorylates MAP kinases on both threonine and tyrosine residues which are required for the activation of this protein. The existence of multiple MEKs (MEK1, MEK2, and MEK3) was recently described. MEK3 is an alternative spliced product of MEK1, whereas MEK2 is a distinct gene product which has approximately 81% sequence homology to MEK1 (65, 66). Both recombinant MEK1 and MEK2 possess the ability to activate both p42^{MAPK} and p44^{MAPK} in vitro, whereas MEK3 shows no MAP kinase kinase activity. Immunoblot analysis of the proteins binding to immobilized p21^{ras} detected the specific interaction between MEK1 and p21^{ras} · GMPPNP. In vitro kinase assays of soluble MEK1 released from immobilized p21^{ras} · GMPPNP revealed a substantial increase in the ability of the MEK1 to activate recombinant wild-type inactive p42^{MAPK}. This activation is the result of phosphorylation of the MEK1 protein, reflected by an apparent shift in mobility on SDS-PAGE and ³²P incorporation into MEK1 in labeled ATP-eluted supernatants (data not shown).

MEK is activated during mitogen-induced signal transduction, though the mechanism of MEK activation is not completely understood. Active p74^{Raf-1}, immunoprecipitated from serum-stimulated cells, or the activated truncated v-Raf-1

phosphorylates and activates inactive MEK in vitro (8, 17, 25). In addition, Raf-1 and MEK1 were shown to form a stable complex when examined either in vitro with purified recombinant proteins or by the yeast two-hybrid method (18, 31, 59–61, 67). However, previous studies from this laboratory identified the existence of a Raf-1-independent MAP kinase signaling mechanism in crude rat brain cytosolic extracts. Addition of immobilized p21^{ras}·GMPPNP to cytosolic extracts immunodepleted of endogenous Raf-1 did not decrease the activation of the p21^{ras}·GMPPNP-associated MEK activity (33). In this study, we examined the involvement of additional proteins which are involved in the p21^{ras}-dependent activation of MAP kinases.

p74^{Raf-1} belongs to a family of proteins which also includes p68^{A-Raf} and p95^{B-Raf}. These protein kinases exhibit more than 70% amino acid sequence homology within the kinase domain. mRNA transcripts for these genes show strikingly dissimilar patterns of expression. In contrast to the ubiquitously expressed Raf-1, A-Raf mRNA levels are highest in the kidney, ovaries, and epididymis, whereas B-Raf transcripts are present at highest levels in the brain (55). The expression of both A-Raf and B-Raf in nonproliferating reproductive and brain tissues suggest that these protein kinases may be involved in maintaining or regulating the differentiated state. Earlier studies indicated the possible involvement of B-Raf in the signaling pathways activated by NGF stimulation of PC12 cells. Following NGF stimulation, B-Raf was observed to be retarded in electrophoretic mobility and possessed an increased kinase activity toward casein, a nonphysiological substrate (38). Two molecular mass forms of B-Raf, 65 and a 95 kDa, were observed in both rat PC12 pheochromocytoma cells and crude mouse brain lysates (38, 51). We also detected multiple B-Raf bands within these two molecular mass ranges, all of which were effectively competed for by the inclusion of excess peptide antigen. These results indicate that both the 65- to 70- and 95- to 105-kDa bands possess regions identical or highly homologous to the B-Raf C-terminal peptide antigen. The 65- to 70-kDa bands were also detected in B-Raf immunoprecipitates immunoblotted with a mouse monoclonal antibody raised against the Raf catalytic domain (24) (data not shown). The intact Raf catalytic domain, interaction with a B-Raf C-terminal antibody, and its ability to form a stable complex with immobilized p21^{ras}·GMPPNP (presumably through its N-terminal p21^{ras} binding site) suggest that the 65- to 70-kDa proteins are more likely alternative spliced products of the B-Raf transcript than proteolytic products of p95^{B-Raf}. Similar alternative spliced products of the B-Raf gene have previously been reported (12). Two proteins of 93.5 and 95 kDa were found to be expressed at high levels in neural tissue. These alternative spliced proteins were found to contain the conserved cysteine-rich domain and surrounding amino acids and the serine/threonine-rich CR2 domain of Raf proteins. The major structural difference was found to reside within the amino-terminal region of the proteins.

Earlier studies from this laboratory showed that the complex formation between immobilized p21^{ras}·GMPPNP and the MEK activity was independent of the presence of Raf-1 (33). The data presented in this report indicate that B-Raf is the protein responsible for the association of MEK1 with immobilized p21^{ras}·GMPPNP. This was clearly demonstrated by the lack of MEK1 protein and MEK activity, as measured by the activation of recombinant p42^{MAPK}, associating with immobilized p21^{ras}·GMPPNP when cytosolic extracts immunodepleted of B-Raf were used. Further experiments designed to test the specificity of the interaction between B-Raf and MEK1 with the immobilized p21^{ras} suggested that sequential incuba-

tions of the cytosolic extract with fresh immobilized p21^{ras}·GMPPNP depleted the cytosolic extracts of total soluble B-Raf protein without significantly altering the total amount of MEK1 protein (data not shown). The association of MEK1 with immobilized p21^{ras}·GMPPNP is dependent on the presence of B-Raf, and the amount of B-Raf protein present in the cytosolic extracts is the limiting factor. Future experiments will address the question of whether the residual MEK1 is able to associate with immobilized p21^{ras}·GMPPNP by the addition of exogenously added recombinant full-length B-Raf to these depleted cytosolic extracts.

The existence of preformed complexes between signaling proteins is becoming more widely observed. Recently, the adaptor protein GRB2 was found to interact with the guanine nucleotide exchange factor mSOS1. The existence of this complex was independent of growth state (6, 52). Raf-1, in the absence of active p21^{ras}, also forms a stable complex with MEK1 (18, 31). Experiments examining the ability of immunoprecipitated B-Raf to activate wild-type inactive p42^{MAPK} in the absence of exogenously added recombinant MEK1 protein in vitro, as measured by the amount of ³²P incorporated into MBP, detected significant phosphorylation of MBP (data not shown). This finding suggests that a small proportion of the B-Raf coexists as a preformed complex with a MEK. However, we were unable to detect the presence of MEK1 coimmunoprecipitating with the B-Raf in immunoblot analysis utilizing enhanced chemiluminescence. It appears that the interaction between immobilized p21^{ras}·GMPPNP increase the affinity of B-Raf for MEK1. We have not yet determined whether the association between immobilized p21^{ras}·GMPPNP with B-Raf is sufficient to increase its specific activity. Addition of soluble p21^{ras} loaded with the nonhydrolyzable analog of GTP, GMP-PNP, to B-Raf immunoprecipitates from cytosolic extracts did not increase the kinase activity of B-Raf toward recombinant MEK1 (data not shown). It is possible that the interaction between B-Raf and immobilized p21^{ras}·GMPPNP mediates the association of additional signaling proteins which might in turn activate B-Raf or facilitate the interaction between B-Raf and MEK1. We have been unable to eliminate the possibility that another kinase associated with the B-Raf is responsible for the association and activation of MEK1 with immobilized p21^{ras}·GMPPNP. We are currently developing a recombinant expression system to produce full-length recombinant B-Raf in order to test the ability of B-Raf to directly associate and activate MEK1.

In a recent report, B-Raf rather than Raf-1 was shown to be responsible for the NGF-dependent phosphorylation and activation of MEK1 in PC12 cells (19). This report, as shown here, also demonstrated the GTP-dependent association of B-Raf with p21^{ras}. The association of B-Raf with p21^{ras} was independent of receptor tyrosine kinase function; therefore, the limiting factor in the association of B-Raf with p21^{ras} is the production of Ras·GTP. Taken together, these findings and those presented in this study clearly indicate that differentiating signals converge on a p21^{ras}·GTP, B-Raf, and MEK1 signaling cascade.

In conclusion, it is clear that the effect of a growth factor on a cytoplasmic signaling cascade may depend on the cell type being studied and on the growth factor used to activate the intracellular events. The activation of a particular pathway may be dependent on the presence or absence of mediators or inhibitors. The existence of multiple family members of p21^{ras}, Raf, MEK, and MAP kinase will allow specificity in the signal generated which may function to produce different biological endpoints through subtle changes in these signaling systems.

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