Post-translational Modification of H-Ras Is Required for Activation of, but Not for Association with, B-Raf*

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B-Raf is regulated by Ras protein and acts as a mitogen-activated protein (MAP) kinase kinase kinase in PC12 cells and brain. Ras protein undergoes a series of post-translational modifications on its C-terminal CAAX motif, and the modifications are critical for its function. To elucidate the role of the post-translational modifications in interaction with, and activation of, B-Raf, we have analyzed a direct association between H-Ras and B-Raf, and constructed an in vitro system for B-Raf activation by H-Ras. By using methods based on inhibition of yeast adenyl cyclase or RasGAP activity and by in vitro binding assays, we have shown that the segment of B-Raf corresponding to amino acid 1-326 binds directly to H-Ras with a dissociation constant (Kd) comparable to that of Raf-1 and that the binding is not significantly affected by the post-translational modifications. However, when the activity of B-Raf to stimulate MAP kinase was measured by using a cell-free system derived from rat brain cytosol, we observed that the unmodified form of H-Ras possesses an almost negligible activity to activate B-Raf in vitro compared to the fully modified form. H-Ras510,181,184 mutant, which was farnesylated but not palmitoylated, was equally active as the fully modified form. These results indicate that the post-translational modifications, especially farnesylation, are required for H-Ras to activate B-Raf even though they have no apparent effect on the binding properties of H-Ras to B-Raf.

Ras protein is a plasma membrane-associated guanine nucleotide-binding protein that cycles between a GTP-bound active form and a GDP-bound inactive form, and operates in key processes of intracellular signal transduction systems that are involved in regulation of cell growth and differentiation. In higher eukaryotes including Caenorhabditis elegans, Drosophila melanogaster, and vertebrates, Ras is a key regulator that mediates signal transduction from cell surface tyrosine kinase receptors to the nucleus via activation of the MAP kinase cascade (for reviews, see Refs. 1 and 2). Recent studies demonstrated that Ras makes a direct association with a serine/threonine kinase Raf-1, a product of the c-raf-1 proto-oncogene (3-8) and that this association leads to stimulation of the activity of Raf-1 to phosphorylate MAP kinase kinase (MEK) (for reviews, see Refs. 1 and 2). However, the precise mechanism of the Raf activation by active form of Ras remains to be clarified.

Braf gene was discovered as a transforming gene in NIH3T3 cell transfection assays with human Ewing sarcoma DNA (9), and its protein product consists of 765 amino acid residues that contain three distinct regions of conservation with Raf-1; CR1, CR2, and CR3 (2, 10). In contrast to the ubiquitous distribution of Raf-1 in a variety of mammalian organs, expression of B-Raf is confined to brain and testis (11). Another member of the Raf family, A-Raf, is expressed most abundantly in ovary and epididymis (11). Recent studies have shown that B-Raf, instead of Raf-1, is responsible for Ras-dependent activation of the MAP kinase pathway in PC12 cells and mammalian (rat and bovine) brain (12-15).

Ras proteins undergo a series of post-translational modifications on their unique C-terminal region called a CAAX motif (C, cysteine; A, aliphatic, and X, any amino acid) (for reviews, see Refs. 16-18). The first stage of the processing consists of three successive modifications of the CAAX motif: (i) farnesylation of the cysteine residue, (ii) proteolytic cleavage of the amino acids AAX, and (iii) methyl esterification of the new C-terminal cysteine. This first stage of modification converts the primary translation product into an intermediate form. In the case of H-Ras, it is further modified by acylation with palmitic acid on cysteine residues (Cys-181 and Cys-184) immediately upstream of the CAAX motif, finally yielding the post-translationally fully modified form. These modifications are essential for anchoring Ras proteins to the plasma membrane (19, 20) and for a number of biological activities of Ras: malignant transformation of NIH3T3 cells (19, 20), induction of neuronal differentiation of PC12 cells (21), and induction of germinal vesicle breakdown in Xenopus laevis oocytes (22) by activated Ras. The activity of H-Ras to activate Raf-1 in vivo was also reported to be dependent on the modifications (23). However, these in vivo experiments entail an inherent problem in separating the effect of the modifications on the activity of Ras from that on its membrane anchoring. Recently an in vitro pure reconstituted system was used to show that the post-translational modifications, especially farnesylation, are critical for activation of Saccharomyces cerevisiae adenyl cyclase which is an immediate downstream effector of Ras in this organism (24). This suggested that the post-translational modifications are required.

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The abbreviations used are: MAP, mitogen-activated protein; MEK, MAP kinase kinase; extracellular signal-regulated kinase kinase; CR, conserved region; ERK, extracellular signal-regulated kinase; MBP, maltose-binding protein; GST, glutathione S-transferase; GAP, GTPase-activating protein; MES, 2-(N-morpholino)ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; GDP(i)S, guanosine 5’-O-(3-thiotriphosphate); GTPγS, guanosine 5’-O-(2-thiotriphosphate); PAGE, polyacrylamide gel electrophoresis.
for activation of Ras effectors. Efficient activation of MAP kinases by Ras in crude cell-free extracts from X. laevis oocytes was also reported to depend on the modifications (25, 26). However, requirement of the modifications of Ras has not been examined in vitro for the Raf-1 activation because a cell-free system for the Raf-1 activation has not been established.

To analyze the molecular mechanism underlying the requirement of the post-translational modifications for the Raf activation, we have established a cell-free system derived from rat brain cytosol in which exogenously added H-Ras protein can activate MAP kinase/ERK2 through activation of B-Raf and MEK. We have also examined the effect of the modifications on direct association of H-Ras with B-Raf, and the result is compared with that obtained on the B-Raf activation.

EXPERIMENTAL PROCEDURES

Materials—The post-translationally modified and unmodified forms of human H-Ras protein were purified from the membrane and cytosol fractions of Sf9 cells, respectively, which had been infected with a recombinant baculovirus carrying the human H-ras cDNA, as described previously (24). Two H-ras mutants encoding the proteins defective for the post-translational modifications, H-ras<sup>ser-181,184</sup> and H-ras<sup>ser-186</sup>, were constructed by site-specific mutagenesis using appropriate mutagenic oligonucleotides as described elsewhere (24). The effector mutant H-ras<sup>ser-181,184</sup> was provided by Dr. S. Yokoyama (University of Tokyo, Tokyo, Japan). Recombinant baculoviruses carrying the mutant H-ras genes were prepared as described before (24). The farnesylated but not palmitoylated H-ras<sup>ser-181,184</sup> and the fully modified H-ras<sup>ser-186</sup> proteins were solubilized and purified from the membrane fractions of Sf9 cells infected with recombinant baculoviruses carrying the respective H-ras genes similarly as described before (24). The unprocessed H-ras<sup>ser-186</sup> protein was purified from the cytosol fraction of Sf9 cells infected with the corresponding baculovirus. A plasmid carrying the full-length human B-Raf cDNA, pS-L-Braf, was provided by Dr. D. Drabkin (Division of Clinical Moro Genetics, Germany). The HindIII-Apal and HindIII-Xhol fragments of the B-raf cDNA corresponding to amino acid 1-326 and 1-445, respectively, were cloned into the matching cleavage sites of pMAL-cRI (New England Biolabs Inc.) to produce pMAL-B-Raf(1–326) and pMAL-B-Raf(1–445), respectively. The MBP-B-Raf fusion proteins were purified from Escherichia coli harboring the corresponding pMAL-B-Raf plasmids by affinity chromatography on amylose resin (27). A segment of the human c-raf-1 cDNA corresponding to amino acid 1–206 of Raf-1 was amplified by a polymerase chain reaction with suitable primers and cloned into pMAL-cRI for expression as an MBP-fusion protein in E. coli. Plasmids for expressing GST-fusion protein of MEK (GST-MEK) or GST-fusion protein of a kinase-dead, C-terminal farnesylated mutant of ERK2 (GST-KNERK) in E. coli were obtained from Dr. A. Kikuchi (Hiroshima University, Hiroshima, Japan). A rat cDNA encoding the full-length ERK2 was cloned from a rat brain cDNA library using a polymerase chain reaction with suitable primers and cloned into pGEX-2T (Pharmacia Biotech Inc.) for production of GST-fusion protein of ERK2 (GST-ERK2) in E. coli. GST-MEK, GST-ERK2, and GST-KNERK proteins were purified by glutathione-agarose chromatography as described elsewhere (28). Purified recombinant RasGAP p120 produced in E. coli and covalently attached to amylose resin in the column buffer (20 mM Tris/HCl, pH 7.4, 0.2 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 1 mM NaN<sub>3</sub>) by continuous mixing for 2 hours on ice, and the resin was washed twice with the column buffer. Fifteen-µl aliquots of the resin with MBP-B-Raf(1–326), MBP-B-Raf(1–445), or MBP-B-Raf(1–456) attached were incubated with various amounts of H-Ras (10 pmol), which had been loaded with GDP or GTP-γ-S, for 30 min at 25°C and for 30 min at 4°C in a total volume of 100 µl of the Ras-binding buffer (20 mM Tris/HCl, pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, and 0.1% Lubrol PX). To evaluate the binding affinity between B-Raf and H-Ras, increasing concentrations of H-Ras were added. After washing three times with the Ras-binding buffer, MBP-fusion proteins with the bound H-Ras were eluted from the resin by 10 mM maltose and separated by SDS-PAGE (12% gel). Bound H-Ras was detected by Western immunoblotting with the anti-H-Ras monoclonal antibody F235. The upper half of the gel was stained with Coomassie Brilliant Blue to detect the MBP-fusion proteins.

Preparation and Fractionation of the Rat Brain Cytosol—All manipulations were carried out at 0–4°C. A rat (Wistar, 300 g of body weight, the Center of Japan Biological Chemistry Co. Ltd.) brain was homogenized in 100 mM HEPES/NaOH, pH 7.4, 1 mM EDTA, 1 mM DTT, 2.5 mM MgCl<sub>2</sub>, 0.3 mM sucrose, 10 mM Mg<sub>2</sub>ATP, 20 mM glycerol, and 1 mM PMSF with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 100,000 × g for 1 h, and the resulting supernatant (20 mg of protein) was applied to a Mono S HR5/5 column (5 × 5 cm) (Pharmacia) equilibrated with buffer B (20 mM HEPES/NaOH, pH 6.8, 2 mM EDTA, 1 mM DTT, 2.5 mM MgCl<sub>2</sub>, 10 mM Mg<sub>2</sub>ATP, and 10 mM 2-mercaptoethanol) and 1 mM PMSF. A linear gradient elution was performed between 15 ml each of 0 and 1 M NaCl in buffer B, and 1-ml fractions were collected.

Assay of Ras-dependant MAP Kinase Stimulation Activity—Ras-dependant MAP kinase stimulation activity was measured by phosphorylation of myelin basic protein in the presence of GTP-S-bound or GDP-bound H-Ras. The sample (15 µl) to be tested was preincubated for 10 min at 30°C in a final volume of 50 µl containing 20 mM Tris/HCl, pH 8.0, 0.6 mM PEPES/NaOH, pH 6.8, 1.5 mM EDTA, 1 mM DTT, 5 mM MgCl<sub>2</sub>, 120 µM ATP, 80 mM GTP-MEK, and varying amounts of GTP-S or GTP-bound H-Ras, followed by addition of 10 µl of 3 µM recombinant GST-ERK2 and another incubation for 10 min. The final phosphorylation reaction was initiated by addition of 20 µl of reaction mixture containing 20 µl of Tris/HCl, pH 8.0, 1.5 mM EDTA, 1 mM DTT, 5 mM MgCl<sub>2</sub>, 50 µM [γ<sup>32</sup>P]ATP (1,000 cpm/pmole), and 50 µM 2-mercaptoethanol. After rapid filtration of the reaction mixture onto a piece of phosphocellulose paper (Whatman P81). After washing with 75 mM phosphoric acid, the radioactivity on the paper was measured by liquid scintillation spectrometry. To measure Ras-dependant phosphorylation of ERK2, the assay was performed similarly except that the reaction mixture contained purified GST-KNERK in place of GST-ERK2 and myelin basic protein and that GST-KNERK was absorbed onto glutathione-agarose resin after the final phosphorylation reaction. The resin was briefly washed with 20 mM Tris/HCl, pH 8.0, 1.5 mM EDTA, 1 mM DTT, and 5 mM MgCl<sub>2</sub>, and subjected to SDS-PAGE (10% gel) and autoradiography.

Immunodepletion Study—A 150-µl aliquot of the active fraction (fraction 42) of the Mono S column chromatography was incubated with continuous mixing for 1 h at 4°C with 30 µl of protein A-Sepharose 4B, or 30 µl of the resin plus 15 µl of the anti-H-Ras monoclonal antibody or the anti-B-Raf polyclonal antibody was attached. After a brief centrifugation, 15 µl of the supernatant were assayed for the Ras-dependant MAP kinase stimulation activity as described above.
Measurement of H-Ras binding to MBP-B-Raf by adenylyl cyclase inhibition assay. A, adenylyl cyclase activity was measured in the presence of 1 pmol of GTPγS-bound form of H-Ras with the addition of various amounts of MBP-B-Raf(1–326) (●), MBP-B-Raf(1–445) (○), and MBP (●). Essentially similar inhibition assay by MBP-B-Raf(1–445) was carried out in the presence of 2.5 mM Mn2+ instead of Mg2+ and H-Ras (●). Values on the vertical axis represent percentages of the activities obtained in the presence of the MBP-fusion proteins compared with those obtained in their absence. B, adenylyl cyclase activities dependent on various concentrations of H-Ras were measured in the presence of various amounts of MBP-B-Raf(1–445) as follows: 0 (●), 2 (●), 4 (●), and 8 pmol (●). One unit of activity is defined as 1 pmol of cAMP formed in 1 min of incubation with 1 mg of protein at 30 °C under standard assay conditions. C, double-reciprocal plot analysis of the binding reaction between MBP-B-Raf(1–445) and H-Ras. The amounts of free and B-Raf-bound H-Ras were calculated as described in the text. The symbols correspond to those used in B.

RESULTS

Measurement of Direct Association between B-Raf and H-Ras by Adenylyl Cyclase Inhibition Assay—Although a previous study showed that B-Raf forms a complex with Ras and exists in an activated state in the complex (14), their direct association has not been rigorously demonstrated. We examined whether B-Raf could bind directly to H-Ras protein and thereby compete for it with yeast adenylyl cyclase. The competitor polypeptide bound directly to H-Ras protein and competitively sequestered it from adenylyl cyclase. The Km values of B-Raf(1–326) and B-Raf(1–445) for H-Ras were calculated from the points of intersection with the horizontal axis and determined to be about 7 nM (data not shown) and 5 nM (Fig. 1C), respectively. These values are comparable to those of Raf-1 for H-Ras, 3.5 nM, or of Byr2 for Ras2, 7 nM, or of Byr2 for Ras2, 1 nM (see Ref. 29 for the data and a detailed description on the kinetic analysis).

Measurement of B-Raf Association with H-Ras by Inhibition of RasGAP Activity—The affinity of B-Raf could not be determined for the post-translationally unmodified form of H-Ras by the adenylyl cyclase inhibition assay because the unmodified form of H-Ras possesses a negligible activity to stimulate adenylyl cyclase (32). Therefore, we used a method based on inhibition of the GTPase-stimulating activity of RasGAP p120, whose interaction with H-Ras had been shown to be unaffected by the modifications (32). We purified the unmodified form of H-Ras from the cytosol fraction of SF9 cells infected with the baculovirus carrying the H-Ras cDNA. The purified protein migrated slower than the fully modified form upon SDS-PAGE (see Fig. 3A). Previous studies employing [3H]mevalonate labeling confirmed that H-Ras purified from the cytosol fraction

Fig. 1. Measurement of H-Ras binding to MBP-B-Raf by adenylyl cyclase inhibition assay. A, adenylyl cyclase activity was measured in the presence of 1 pmol of GTPγS-bound form of H-Ras with the addition of various amounts of MBP-B-Raf(1–326) (●), MBP-B-Raf(1–445) (○), and MBP (●). Essentially similar inhibition assay by MBP-B-Raf(1–445) was carried out in the presence of 2.5 mM Mn2+ instead of Mg2+ and H-Ras (●). Values on the vertical axis represent percentages of the activities obtained in the presence of the MBP-fusion proteins compared with those obtained in their absence. B, adenylyl cyclase activities dependent on various concentrations of H-Ras were measured in the presence of various amounts of MBP-B-Raf(1–445) as follows: 0 (●), 2 (●), 4 (●), and 8 pmol (●). One unit of activity is defined as 1 pmol of cAMP formed in 1 min of incubation with 1 mg of protein at 30 °C under standard assay conditions. C, double-reciprocal plot analysis of the binding reaction between MBP-B-Raf(1–445) and H-Ras. The amounts of free and B-Raf-bound H-Ras were calculated as described in the text. The symbols correspond to those used in B.
The post-translational modification of Ras on B-Raf Activation

**Inhibition of RasGAP-stimulation of GTPase activities of the modified and unmodified forms of H-Ras**

A, the post-translationally modified and unmodified forms of H-Ras loaded with [γ-32P]GTP were incubated in the presence or absence of 40 fmol of RasGAP p120 for the indicated periods, and the radioactivities remaining bound to H-Ras were measured as described under "Experimental Procedures." The modified H-Ras incubated with (●) or without (○) RasGAP. The unmodified H-Ras incubated with (●) or without (○) RasGAP. B, increasing concentrations of MBP-B-Raf(1–326) were added to the RasGAP assay reaction mixture containing the modified (●) or unmodified (○) form of H-Ras. Percentages of inhibition of RasGAP activity are plotted against the added MBP-B-Raf(1–326) concentrations.

**In vitro Binding of B-Raf N Terminus to the Modified and Unmodified Forms of H-Ras**

The post-translationally modified and unmodified forms of H-Ras were loaded with GTPγS or GDPβS, and examined for binding to MBP-B-Raf(1–326) and MBP-Raf(1–206), which had been immobilized on amylose resin as described under "Experimental Procedures." Both of the MBP-fusion proteins bound efficiently to the GTPγS form but not to the GDPβS form of both post-translationally modified and unmodified forms of H-Ras (Fig. 3A). The binding was abolished by a substitution of asparagine for aspartic acid at position 38 in the effector loop of H-Ras. This mutation had been shown to abolish the Raf-1 binding (4). We next examined whether the post-translational modifications affected the binding affinity of H-Ras to B-Raf (Fig. 3B). When increasing concentrations of the modified and unmodified forms of H-Ras were used for binding reactions with MBP-B-Raf(1–326), the amounts of bound H-Ras increased and finally reached a plateau level. No difference in the binding pattern was observed between the modified and unmodified forms (Fig. 3B). From the data, we estimated an apparent Kd value of B-Raf to both forms of H-Ras as the concentration of H-Ras exhibiting a half maximal binding, and obtained a value of around 50 nM. This is a bit higher than that obtained by the adenyl cyclase inhibition assay. These results indicated that H-Ras binds to the B-Raf N terminus in a GTP-dependent manner presumably at its effector domain and that the post-translational modifications of H-Ras have no effect on the binding.

**Establishment of a Cell-free System for Ras-dependent B-Raf Activation**

It was shown that activation of the MAP kinase cascade by Ras protein is mediated by B-Raf, not by Raf-1, in a crude extract from rat brain (14). We established here a cell-free system derived from rat brain cytosol in which exogenously added H-Ras could activate protein kinases MEK and ERK2 in vitro. The cytosolic extract (20 mg of protein) was applied to a Mono S HR5/5 column equilibrated with buffer B, and washed with 20 ml of the same buffer. Subsequently a linear gradient elution was performed between 0 and 1 M NaCl in a total volume of 30 ml. One-ml fractions were collected and assayed for stimulation of the activity of MAP kinase to phosphorylate myelin basic protein in the presence and absence of the GTPγS-bound H-Ras as described under "Experimental Procedures." The MAP kinase activity stimulated by H-Ras was eluted as a single peak around fractions 41–44 (Fig. 4A). The peak fraction 42 was used for subsequent studies. The Ras-dependent activation was not observed by either the GDP-bound H-Ras or the GTPγS-bound H-Ras (Fig. 4B). The Ras-dependent phosphorylation of myelin basic protein also depended on the inclusion of recombinant GST-ERK2 in the reaction mixture (Fig. 4B), indicating that H-Ras induced the enhanced phosphorylation of myelin basic protein through the activation of ERK2. When GST-fusion protein of a kinase negative mutant of ERK2, GST-KNERK, was used as a substrate, the same fraction exhibited H-Ras-dependent stimulation of its phosphorylation, and this activity was dependent on the presence of recombinant MEK (Fig. 4C). Both the Ras-dependent phosphorylations of myelin basic protein and GST-KNERK were efficiently inhibited by the addition of MBP-B-Raf(1–326) (Fig. 4B). The Ras-dependent phosphorylation of myelin basic protein also depended on the inclusion of recombinant GST-ERK2 in the reaction mixture (Fig. 4B), indicating that H-Ras induced the enhanced phosphorylation of myelin basic protein through the activation of ERK2. When GST-fusion protein of a kinase negative mutant of ERK2, GST-KNERK, was used as a substrate, the same fraction exhibited H-Ras-dependent stimulation of its phosphorylation, and this activity was dependent on the presence of recombinant MEK (Fig. 4C). Both the Ras-dependent phosphorylations of myelin basic protein and GST-KNERK were efficiently inhibited by the addition of MBP-B-Raf(1–326) (Fig. 4B). The Ras-dependent phosphorylation of myelin basic protein also depended on the inclusion of recombinant GST-ERK2 in the reaction mixture (Fig. 4B), indicating that H-Ras induced the enhanced phosphorylation of myelin basic protein through the activation of ERK2.
via activation of B-Raf in this soluble cell-free system.

Effect of Post-translational Modifications of H-Ras on Its Ability to Stimulate B-Raf Activity in Vitro—We used the in vitro cell-free system for examination of the effect of the post-translational modifications of H-Ras on its ability to activate B-Raf. The GTPγS-bound forms of post-translationally modified and unmodified H-Ras were added to the reaction mixture containing the Mono S peak fraction for measuring stimulation of MAP kinase activity. As shown in Fig. 5, the modified H-Ras efficiently activated phosphorylation of myelin basic protein. A half maximal activation was observed at the H-Ras concentration of about 2 nM, which was in good coincidence with the Kd value of B-Raf N terminus for the modified H-Ras determined by the adenyl cyclase inhibition assay. On the other hand, the post-translationally unmodified H-Ras exhibited an almost undetectable activity of stimulating the phosphorylation compared to the modified form.

To examine which step in the process of modifications of H-Ras is critical for activation of B-Raf, we constructed and purified H-RasSer181,184, which was farnesylated but lacked the two cysteine residues to be palmitoylated, and H-RasSer186, which lacked the cysteine residue to be farnesylated and, therefore, was not modified at all. The activities of these mutants to stimulate B-Raf were examined similarly by using the in vitro system. As shown in Fig. 6A, H-RasSer181,184 activated phosphorylation of myelin basic protein as efficiently as the fully modified form of H-Ras at the concentration of 5 nM. In contrast, H-RasSer186 had an almost negligible activity at the same concentration (Fig. 1A) or even at 20 nM (data not shown). Essentially similar result was obtained when the activities of H-RasSer181,184 and H-RasSer186 to stimulate S. cerevisiae adenyl cyclase were examined (Fig. 6B). This result is consistent with our previous observation that farnesylation, not palmitoylation, of yeast Ras2 is essential for its ability to activate adenyl cyclase in vitro (24). These results indicated that the post-translational modifications of H-Ras, especially the farnesylation step, are critical for the activation of B-Raf as well as of yeast adenyl cyclase.

**DISCUSSION**

We have shown here that post-translational modifications of H-Ras are not required for association with one of its effector molecule, B-Raf, but are essential for activation of B-Raf. The farnesylation step of the modifications, not the palmitoylation, is shown to be responsible for this effect. B-Raf is a serine/threonine kinase which is expressed specifically in neuronal tissues and testis (11), while Raf-1 is ubiquitously expressed in all cell types. Although the association of Raf-1 with Ras has been a subject of extensive investigation, B-Raf is not well analyzed for its interaction with Ras protein. In this paper we have shown for the first time that the N-terminal segment of B-Raf makes a direct association with H-Ras in a GTP-dependent manner. No association is observed with the effector mutant H-RasΔCys513, suggesting that B-Raf binds to the effector

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**Fig. 3. In vitro association of the post-translationally modified and unmodified forms of H-Ras with the N-terminal segment of B-Raf.** A, the post-translationally modified forms of H-Ras (lanes 1, 2, 6, and 7) and H-RasSer181,184 (lanes 5 and 10), and the unmodified form of H-Ras (lanes 3, 4, 8, and 9) (10 pmol each) were loaded with GTPγS (T) or GDPγS (D), and incubated with MBP-B-Raf(1–326) (0.2 μg) or MBP-Raf-1(1–206) (0.5 μg) immobilized on amylose resin as described under "Experimental Procedures." MBP-fusion proteins with the bound H-Ras were eluted by 10 mM maltose and separated by SDS-PAGE (12% gel). MBP-B-Raf(1–326) and MBP-Raf-1(1–206) were detected by staining with Coomassie Brilliant Blue (shown by the arrows in the upper panel). H-Ras proteins were detected by immunoblotting with the anti-H-Ras monoclonal antibody. B, various concentrations; 25 nM (lanes 1 and 5), 50 nM (lanes 2 and 6), 100 nM (lanes 3 and 7), and 200 nM (lanes 4 and 8), of the post-translationally modified (lanes 1–5) and unmodified (lanes 6–10) forms of H-Ras were loaded with GTPγS, and incubated with the fixed amount (0.2 μg) of immobilized MBP-B-Raf(1–326). MBP-B-Raf(1–326) and the bound H-Ras was detected as described in A. The result shown is a representative of three independent experiments, which gave equivalent results.

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**FIG. 1.** Association of the post-translationally modified and unmodified forms of H-Ras with immobilized MBP-B-Raf(1–326). A, MBP-B-Raf(1–326) (0.2 μg) or MBP-Raf-1(1–206) (0.5 μg) immobilized on amylose resin as described under "Experimental Procedures." MBP-fusion proteins with the bound H-Ras were eluted by 10 mM maltose and separated by SDS-PAGE (12% gel). MBP-B-Raf(1–326) and MBP-Raf-1(1–206) were detected by staining with Coomassie Brilliant Blue (shown by the arrows in the upper panel). H-Ras proteins were detected by immunoblotting with the anti-H-Ras monoclonal antibody. B, various concentrations; 25 nM (lanes 1 and 5), 50 nM (lanes 2 and 6), 100 nM (lanes 3 and 7), and 200 nM (lanes 4 and 8), of the post-translationally modified (lanes 1–5) and unmodified (lanes 6–10) forms of H-Ras were loaded with GTPγS, and incubated with the fixed amount (0.2 μg) of immobilized MBP-B-Raf(1–326). MBP-B-Raf(1–326) and the bound H-Ras was detected as described in A. The result shown is a representative of three independent experiments, which gave equivalent results.
Further, we have determined the $K_d$ value of B-Raf N terminus for the post-translationally modified H-Ras by the yeast adenylyl cyclase inhibition assay. The value is comparable to those of other Ras-effector molecules for their homologous Ras proteins. There exists little difference in the estimated $K_d$ values of B-Raf segments containing CR1 only and both CR1 and CR2, suggesting that CR1 contains a major Ras-binding site(s) as observed for Raf-1 (6, 31).

It was shown that B-Raf, not Raf-1, mediates the nerve growth factor-induced activation of the MAP kinase cascade through interaction with Ras in PC12 cell (13). In rat brain the association of MEK1 with Ras is dependent on B-Raf, not on Raf-1 (14). These data prompted us to establish a cell-free system for Ras-dependent stimulation of MAP kinase activity through B-Raf activation using rat brain cytosol. While this work was in progress, Yamamori et al. (15) reported establishment of such a system from bovine brain cytosol and purification of a Ras-dependent MEK kinase, which turned out to be a complex of B-Raf and 14–3-3 proteins, although the possibility of presence of other minor components was not excluded completely. Here we also have constructed a similar in vitro system and employed it to quantitatively examine the effect of the post-translational modifications of Ras on its activity.

**FIG. 4. Partial purification and characterization of Ras-dependent MAP kinase stimulation activity.** A, rat brain cytosol was fractionated by column chromatography on a Mono S column. Solid and broken lines indicate NaCl concentration and absorbance at 280 nm, respectively. A 15-$\mu$l aliquot of each fraction was assayed for phosphorylation activity of myelin basic protein in the presence of GST-MEK and GST-ERK2 along with 2 pmol each of GTP-$\gamma$S-bound H-Ras (●) or GDP-bound H-Ras (○) as described under "Experimental Procedures." B, a 15-$\mu$l aliquot of the fraction 42 was assayed for the phosphorylation activity of myelin basic protein as described under "Experimental Procedures" with the addition or omission of following ingredients: 2 pmol each GDP- or GTP-$\gamma$S-bound H-Ras (columns 1 and 2), 2 pmol each GDP- or GTP-$\gamma$S-bound H-RasAsn-38 (columns 3 and 4), 2 pmol each GDP- or GTP-$\gamma$S-bound H-Ras with omission of GST-ERK2 (columns 5 and 6), and 2 pmol each GDP- or GTP-$\gamma$S-bound H-Ras with the addition of 5 pmol (columns 7 and 8) or 20 pmol (columns 9 and 10) of MBP-B-Raf(1–326). C, a 15-$\mu$l aliquot of the fraction 42 was assayed for phosphorylation of GST-KNERK as described under "Experimental Procedures" without H-Ras (lane 1), or with the addition of 2 pmol each of GDP-bound (lane 2) or GTP-$\gamma$S-bound H-Ras (lanes 3–5) except that recombinant MEK was omitted in lane 4 and that 20 pmol of MBP-B-Raf(1–326) was added in lane 5. The arrowhead indicates the position of phosphorylated GST-KNERK. D, the rat brain cytosol (3.5 mg of protein) (lane 1) and the fraction 42 (1.5 mg of protein) (lane 2) were separated by SDS-PAGE (10% gel), and immunoblotted with the anti-B-Raf antibody. The arrowhead indicates the position of the 95-kDa B-Raf. E, the fraction 42 was preincubated with protein A-Sepharose alone (columns 1 and 2) or that attached with 1.5 mg each of the anti-Raf-1 antibody (column 3) or anti-B-Raf antibody (column 4). After a brief centrifugation, 15-$\mu$l of the supernatant were assayed for phosphorylation of myelin basic protein in the presence of 2 pmol each of GDP- or GTP-$\gamma$S-bound H-Ras as described under "Experimental Procedures" except that 20 pmol of MBP-B-Raf(1–326) were added to the reaction mixture in column 2. Ras-dependent stimulation of the phosphorylation was calculated by subtracting the radioactivity incorporated into myelin basic protein in the presence of GDP-bound H-Ras from that in the presence of GTP-$\gamma$S-bound H-Ras. The values were presented as percentages of the activities obtained under preincubation with protein A-Sepharose only (column 1).
Post-translational Modification of Ras on B-Raf Activation

Fig. 5. Dose-dependent stimulation of MAP kinase activity by the post-translationally modified and unmodified forms of H-Ras. A 15-μl aliquot of the fraction 42 was assayed for phosphorylation activity of myelin basic protein in the presence of GST-MEK and GST-ERK2 along with varying concentrations of the modified (●) or unmodified (○) form of H-Ras as described under "Experimental Procedures." The results were expressed as the radioactivity incorporated into myelin basic protein obtained in the presence of GTPγS-bound H-Ras subtracted by that in the presence of the same concentration of GDP-bound H-Ras.

Fig. 6. Stimulation of B-Raf and adenylyl cyclase activities by C-terminal mutants of H-Ras. A, the activity of B-Raf to induce phosphorylation of myelin basic protein was measured in the presence of 0.5 pmol each of the various forms of H-Ras protein; the post-translationally fully modified and unmodified forms of wild-type H-Ras, H-RasSer-181,184 and H-RasSer-186. The results were shown similarly as described in Fig. 5. B, adenylyl cyclase activity was measured in the presence of 10 pmol of the various forms of H-Ras as described in A.

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REFERENCES
1. Avruch, J., Zhang, X.-F., and Kyriakis, J. M. (1994) Trends Biochem. Sci. 19, 279–283
2. Daum, G., Eisenmann-Tappe, I., Fries, H.-W., Troppmair, J., and Rapp, U. R. (1994) Trends Biochem. Sci. 19, 474–480
3. Moodie, S. A., Williams, B. M., Weber, M. J., and Wolfman, A. (1993) Science 260, 1658–1661
4. Zhang, X.-F., Settleman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. (1993) Nature 364, 308–313
5. Warne, P. H., Viciana, P. R., and Downward, J. (1993) Nature 364, 352–355
6. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205–214
7. Vaillancourt, R. R., Gardner, A. M., and Johnson, G. L. (1994) Mol. Cell. Biol. 14, 6522–6530
8. Jaiswal, R. K., Moodie, S. A., Wolfman, A., and Landreth, G. E. (1994) Mol. Cell. Biol. 14, 6522–6530
9. Ito, T., Kaibuchi, K., Masuda, T., Yamamoto, T., Matsuura, Y., Maeda, A., Shimizu, K., and Takai, Y. (1993) J. Biol. Chem. 268, 3025–3028
10. Sithanandam, G., Kolch, W., Duh, F.-M., and Rapp, U. R. (1990) Oncogene 5, 1775–1780
11. Storm, S. M., Cleveland, J. L., and Rapp, U. R. (1991) Oncogene 5, 345–351
12. Vojtech, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205–214
13. Case, P. J. (1994) Curr. Opin. Cell Biol. 6, 219–225
14. Williamsen, B. M., Norris, K., Papageorge, A. G., Hubbert, N. L., and Lowy, D. (1984) EMBO J. 3, 2581–2585
15. Hancock, J. F., Paterson, H., and Marshall, C. J. (1990) Cell 63, 133–139
16. Qui, M.-S., Pitts, A. F., Winters, T. R., and Green, S. H. (1991). Cell Biol. 115, 795–808
17. Schaffer, W. R., Kim, R., Sterne, R., Thorner, J., Kim, S.-H., and Rine, J. (1989) Science 245, 379–385
18. Kim, J., and Williams, L. T. (1994) J. Biol. Chem. 269, 20054–20059
19. Kuroda, Y., Suzuki, N., and Kataoka, T. (1993) Science 259, 583–586
20. Shibusawa, H., and Polverino, A. J., Chang, E., Wigler, M., and Ruderman, J. V. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9831–9835
21. Moody, S. A., Paris, M. J., Kolch, W., and Wolfman, A. (1994) Mol. Cell. Biol. 14, 7153–7162
22. Willumsen, B. M., Norris, K., Papageorge, A. G., Hubbert, N. L., and Lowy, D. (1984) EMBO J. 3, 2581–2585
23. Hancock, J. F., Paterson, H., and Marshall, C. J. (1990) Cell 63, 133–139
24. Qui, M.-S., Pitts, A. F., Winters, T. R., and Green, S. H. (1991). Cell Biol. 115, 795–808
25. Schaffer, W. R., Kim, R., Sterne, R., Thorner, J., Kim, S.-H., and Rine, J. (1989) Science 245, 379–385
26. Kim, J., and Williams, L. T. (1994) J. Biol. Chem. 269, 20054–20059
27. Kuroda, Y., Suzuki, N., and Kataoka, T. (1993) Science 259, 683–686
28. Smith, D. B., and Johnson, K. S. (1988) Science 260, 31–40
29. Minato, T., Wang, J., Akasaka, K., Okada, T., Suzuki, N., and Kataoka, T. (1994) J. Biol. Chem. 269, 3025–3028
30. Masuda, T., Kariya, K., Shinkai, M., Okada, T., and Kataoka, T. (1995) J. Biol. Chem. 270, 797–1002
31. Chuang, E., Barnard, D., Hettich, L., Zhang, X.-F., Avruch, J., and Marshall, M. S. (1994) Mol. Cell. Biol. 14, 5318–5325
32. Ito, T., Kaibuchi, K., Masuda, T., Yamamoto, T., Matsuura, Y., Maeda, A., Shimizu, K., and Takai, Y. (1993) J. Biol. Chem. 268, 3025–3028
33. Fujii, Y., Kuroda, Y., Kataoka, T., Kawa, K., Watanabe, R., and Kataoka, T. (1993) Science 260, 133–139
34. Fuji, M., and Kataoka, T. (1991) J. Biol. Chem. 266, 17026–17031
35. Marshall, M. S. (1993) Trends Biochem. Sci. 18, 250–254
36. Stokoe, D., Macdonald, S. G., Cadwallader, K. A., Symons, M., and Hancock, J. F. (1994) Science 264, 1463–1467
37. Leeser, S. J., Paterson, H. F., and Marshall, C. J. (1994) Nature 369, 411–414
38. Cadwallader, K. A., Paterson, H., Macdonald, S. G., and Hancock, J. F. (1994) Mol. Cell. Biol. 14, 4722–4730
39. Bhattacharya, S., Chen, L., Brod, J. R., and Powers, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2984–2988
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