Activation of Brain B-Raf Protein Kinase by Rap1B Small GTP-binding Protein*

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Rap1 small GTP-binding protein has the same amino acid sequence at its effector domain as that of Ras. Rap1 has been shown to antagonize the Ras functions, such as the Ras-induced transformation of NIH 3T3 cells and the Ras-induced activation of the c-Raf-1 protein kinase-dependent mitogen-activated protein (MAP) kinase cascade in Rat-1 cells, whereas we have shown that Rap1 as well as Ras stimulates DNA synthesis in Swiss 3T3 cells.

We have established a cell-free assay system in which Ras activates bovine brain B-Raf protein kinase. Here we have used this assay system and examined the effect of Rap1 on the B-Raf activity to phosphorylate recombiant MAP kinase kinase (MEK). Recombinant Rap1B stimulated the activity of B-Raf, which was partially purified from bovine brain and immunoprecipitated by an anti-B-Raf antibody. The GTP-bound form was active, but the GDP-bound form was inactive. The fully posttranslationally lipid-modified form was active, but the unmodified form was nearly inactive. The maximum B-Raf activity stimulated by Rap1B was nearly the same as that stimulated by Ki-Ras. Rap1B enhanced the Ki-Ras-stimulated B-Raf activity in an additive manner. These results indicate that not only Ras but also Rap1 is involved in the activation of the B-Raf-dependent MAP kinase cascade.

The Rap1 family consists of two highly homologous members, Rap1A and Rap1B, and belongs to the small GTP-binding protein superfamily (1–3). Rap1 has the same amino acid sequence at its effector domain as that of Ras, suggesting that Rap1 shows the functions similar and/or antagonistic to those of Ras. Consistently, Rap1A and/or Rap1B have been shown to antagonize the Ras functions, such as the Ki-Ras-induced transformation of NIH 3T3 cells (3), the Ha-Ras-induced germinal vesicle breakdown in Xenopus oocytes (4), the N-Ras-inhibited muscarinic K+ channel activity (5), the Ki-Ras-induced activation of the c-fos promoter/enhancer in NIH 3T3 cells (6), the proliferation of middle T antigen-transformed Rat-2 cells (7), and the Ha-Ras-induced activation of the c-Raf-1 protein kinase-dependent MAP kinase cascade in Rat-1 cells (8), whereas we have shown that Rap1B as well as Ki-Ras stimulates DNA synthesis in Swiss 3T3 cells (9). In addition to these functions, Rap1A and/or Rap1B have been reported to regulate plasma membrane Ca2+ transport in human platelets (10), to enhance the protein kinase C activity in a cell-free system (11), and to inhibit the oxidative burst of Epstein-Barr virus-transformed human B lymphocytes (12). Rap1A and/or Rap1B have been shown to be associated with the cytoskeleton in activated human platelets (13) and with the Golgi complex in Rat-1 cells (14) and to be up-regulated by platelet-derived growth factor in smooth muscle cells (15).

Rap1A and/or Rap1B have been shown to be phosphorylated by protein kinase A in both intact cells and cell-free systems (16–21), by Ca2+/calmodulin-dependent protein kinase Gr in a cell-free system (22), and by protein kinase G in a cell-free system (23). The protein kinase A-catalyzed phosphorylation sites of Rap1A and Rap1B are Ser180 and Ser179, respectively, in their C-terminal regions (21, 24). This phosphorylation of Rap1B lowers its membrane binding activity and induces its translocation from the membrane to the cytosol (18, 24, 25). The phosphorylation of Rap1B makes it sensitive to the action of Smg GDS to stimulate its GDP/GTP exchange reaction (24). These observations suggest that Rap1 shows multiple functions, but the mode of action of Rap1 at a molecular level remains to be clarified.

The mode of action of Ras has recently been intensively investigated, and one of its direct downstream target molecules has been identified to be c-Raf-1, which induces the activation of the MAP kinase/ERK through MEK (for a review, see Ref. 26). Although GTP-Ras has been shown to interact directly with c-Raf-1 (27, 28), evidence has not been obtained that GTP-Ras activates c-Raf-1 in a cell-free assay system. We have developed a cell-free assay system in which GTP-Ras activates MEK and identified a Ras-dependent MEK kinase in Xenopus oocytes and eggs (29, 30). Furthermore, we have purified a Ras-dependent MEK kinase from bovine brain cytosol and identified it as B-Raf, which was tightly complexed with 14-3-3 proteins (31). This result is consistent with the earlier observations that MAP kinase is activated by B-Raf, but not by c-Raf-1, in intact PC12 cells in response to nerve growth factor (32), that the association of MEK with immobilized Ha-Ras is dependent on B-Raf, but not on c-Raf-1, in rat brain (33), and that the MEK-activating activity is accompanied with B-Raf, but not with c-Raf-1, in bovine brain and bovine adrenal chromaffin cells (34).

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Ras has a Cys-A-A-X structure (where A is aliphatic amino acid and X is any amino acid) that undergoes post-translational modifications; the cysteine residue is first farnesylated followed by prototactylic removal of the A-A-X portion and the carboxyl methylation of the exposed cysteine residue (for a review, see Ref. 35). Rap1B has a Cys-A-A-Leu structure, which similarly undergoes post-translational modifications except that it is geranylgeranylated (36). These modifications of Ras are essential for its biological activities (35). We have also shown that they are biochemically important for its activation by its regulators, such as Smg GDS (37), mCdc25 (38), and mSos (39), and for its functions, such as activation of yeast adenylate cyclase (40), Xenopus oocyte MEK kinase (41), and bovine brain B-Raf (31). The lipid modifications of Rap1 are also essential for its activities to inhibit the Ki-Ras-induced transformation of NIH 3T3 cells (42) and the Ki-Ras-induced activation of the c-fos promoter/enhancer in NIH 3T3 cells (6) and for its activation by Smg GDS (37).

It has recently been shown that Rap1 as well as Ras interacts with c-Raf-1 by use of a yeast two-hybrid system (27). More recently, the structure of a complex of Rap1A with the Ras-binding domain of c-Raf-1 has been determined by x-ray crystal structure analysis (43). However, no evidence has thus far been obtained that Rap1 directly affects the c-Raf-1 activity or the Rap-stimulated c-Raf-1 activity. In the present study, by use of our cell-free assay system, we have attempted to clarify whether Rap1B stimulates the B-Raf activity or whether Rap1B antagonizes the Ki-Ras-stimulated B-Raf activity.

**EXPERIMENTAL PROCEDURES**

Materials and Chemicals—Post-translationally lipid-modified Rap1B, lipid-unmodified Rap1B, and lipid-modified Ki-Ras were prepared as described (27). GST-MEK and GST-ERK 2 were prepared as described (30). GTP-γ-S-Rap1B, GDP-Rap1B, GTP-γ-S-Ki-Ras, and GDP-Ki-Ras were prepared as described (37). An anti-B-Raf antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Assay for the Ras-stimulated B-Raf Activity—The B-Raf activity was assayed by measuring the phosphorylation of MBP by recombinant GST-ERK2 in the presence of recombinant GST-MEK as described with a slight modification (31). Briefly, 5 µl of various combinations of GTP-γ-S-Rap1B, GDP-Rap1B, GTP-γ-S-Ki-Ras, and GDP-Ki-Ras were mixed with 15 µl of B-Raf. The concentrations of these compounds used were indicated in each experiment. Fifteen µl of a reaction mixture containing 120 µM ATP was added, and the mixture was incubated for 5 min at 30°C. After the incubation, 15 µl of GST-MEK was added and the incubation was continued for 10 min at 30°C. After further incubation, 10 µl of GST-ERK2 was added and the incubation was continued for 20 min at 30°C. Then, 20 µl of a reaction mixture containing 100 µM [γ-32P]ATP (300 cpn/mmol) and 220 µM MBP was added. After further incubation for 10 min at 30°C, 30% of the mixture was spotted onto a phosphocellulose paper sheet. The sheet was washed with 75 mM phosphanic acid, and the radioactivity was measured by liquid scintillation spectrometry.

Purification of B-Raf—B-Raf was purified from bovine brain as described (31). Briefly, bovine brain cytosol was subjected to Mono S column chromatography and the active fractions were collected. They were subjected to Mono Q column chromatography and the active fractions were collected. These fractions, containing B-Raf complexed with 14-3-3 proteins, were used.

Assay for MEK Phosphorylation—Five µl of GTP-γ-S-Rap1B, GDP-Rap1B, GTP-γ-S-Ki-Ras, or GDP-Ki-Ras was mixed with 15 µl of B-Raf partially purified from bovine brain and 5 µl of Buffer A containing 20 mM Tris/HEC at pH 7.5, 18 mM EGTA, 25 µM MgCl₂, and 100 µM ATP. The mixture was incubated for 5 min at 30°C. After 45 µl of GTP-MEK (0.3 µg of protein) and 5 µl of Buffer B containing 20 mM Tris/HEC at pH 7.5, 18 mM EGTA, 75 mM MgCl₂, and 200 µM [γ-32P]ATP (10,000 cpm/µmol) were added, the incubation was continued for a further 30 min at 30°C. The reaction was terminated by the addition of Laemmli’s sample buffer and subjected to SDS-PAGE (10% polyacrylamide gel). The radioactivity of 32p incorporated into MEK was detected by bioimaging analyzer BAS 2000. Other Procedures—SDS-PAGE was performed by the method of Laemmli (44). Protein concentrations were determined with bovine serum albumin as a standard protein by the method of Bradford (45).

**RESULTS**

GTP-γ-S-Rap1B as well as GTP-γ-S-Ki-Ras stimulated the B-Raf activity in a dose-dependent manner, whereas GDP-Rap1B or GDP-Ki-Ras did not (Fig. 1A). The maximum B-Raf activity stimulated by GTP-γ-S-Ki-Ras was nearly the same as that stimulated by GTP-γ-S-Ki-Ras. To confirm that MBP phosphorylation indeed resulted from the B-Raf-induced activation of exogenously added GST-MEK, we examined the phosphorylation of GST-MEK by B-Raf in the presence of GTP-γ-S-Rap1B, GDP-Rap1B, GTP-γ-S-Ki-Ras, or GDP-Ki-Ras. GST-MEK was indeed phosphorylated in the presence of GTP-γ-S-Ki-Ras but was not phosphorylated in the presence of their GDP-bound form (Fig. 1B). The maximum level of the Rap1-induced phosphorylation of GST-MEK was nearly the same as that of the Ki-Ras-induced one. To further confirm that Ras and Rap1B directly act on B-Raf, we examined the phos-
phorylation of GST-MEK by B-Raf immunoprecipitated by its antibody. Immunoprecipitated B-Raf indeed phosphorylated GST-MEK in GTP\(\gamma\)S-Ki-Ras- and GTP\(\gamma\)S-Rap1B-dependent manners (Fig. 1C). The maximum level of the Rap1B-induced phosphorylation of GST-MEK was slightly lower than that of the Ki-Ras-induced one.\(^2\)

We have shown that the lipid modifications of Ki-Ras are important for the B-Raf activity (31). Similarly, lipid-modified GTP\(\gamma\)S-Rap1B stimulated the B-Raf activity, whereas the lipid-unmodified one was nearly inactive (Fig. 2).

Rap1 has been shown to antagonize various Ras functions (3–8). The effect of GTP\(\gamma\)S-Rap1B on the Ki-Ras-stimulated B-Raf activity was examined. GTP\(\gamma\)S-Rap1B enhanced the Ki-Ras-stimulated B-Raf activity in an additive manner (Fig. 3).

We have previously shown that Rap1B is phosphorylated by protein kinase A at Ser\(^{179}\) in the C-terminal region (24). The effect of this protein kinase A-catalyzed phosphorylation of Rap1B on the B-Raf activity was examined. The protein kinase A-catalyzed phosphorylation of Rap1B itself did not affect the B-Raf activity nor the Ki-Ras-stimulated B-Raf activity (data not shown).

**DISCUSSION**

Rap1 has been shown to interact with c-Raf-1 in a yeast two-hybrid system (27) and to affect the c-Raf-1 activity in intact cells (8). Recently, Rap1A has been co-crystallized with the Ras-binding domain of c-Raf-1 (43). However, the relationship between Rap1 and B-Raf has not thus far been studied. We have shown here in our cell-free assay system that GTP\(\gamma\)S-Rap1B as well as GTP\(\gamma\)S-Ki-Ras stimulates the B-Raf activity and that GTP\(\gamma\)S-Rap1B enhances the Ki-Ras-stimulated B-Raf activity in an additive manner. These results indicate that both Ras and Rap1 interact with B-Raf and induce its activation in a similar manner and suggest that Rap1 as well as Ras induces the activation of the B-Raf-dependent MAP kinase cascade and does not antagonize the Ras-induced activation of the B-Raf-dependent MAP kinase cascade in intact cells.

It has been shown in many systems that Rap1 antagonizes various Ras functions, some of which have been considered to be mediated by the action of c-Raf-1 (3–8). c-Raf-1 has been shown to be expressed in most tissues, but B-Raf has been shown to be expressed in brain, spinal cord, and testis (46). Our analysis\(^3\) has, however, revealed that B-Raf is also expressed in rat spleen and heart, suggesting that both c-Raf-1 and B-Raf are expressed in most tissues. The reason Rap1B does not antagonize the Ki-Ras-stimulated B-Raf activity is not known, but it is possible that Rap1 as well as Ras activates B-Raf whereas Rap1 does not activate c-Raf-1 and antagonizes the Ras-induced activation of c-Raf-1. It is important to establish the cell-free assay system in which Ras stimulates the c-Raf-1 activity and to examine the effect of Rap1 on the c-Raf-1 activity and the Ras-stimulated c-Raf-1 activity.

We obtained the result that the protein kinase A-catalyzed phosphorylation of Rap1B itself did not affect the B-Raf activity or the Ki-Ras-stimulated B-Raf activity (data not shown). Rap1 has been shown to be phosphorylated by protein kinase A (16–21), and this phosphorylation of Rap1 has been suggested to induce the activation of Rap1 (24, 47). Therefore, the present results together with these earlier observations suggest that the protein kinase A-catalyzed phosphorylation of Rap1 is important for its activation but not for its function, such as the activation of B-Raf.

It has been shown that the cyclic AMP-protein kinase A system induces the activation of the MAP kinase cascade in intact PC12 cells (48) and intact COS7 cells (49). In contrast, it has been shown that the cyclic AMP-protein kinase A system inhibits the B-Raf activity in intact PC12 cells (50) and the Ras-dependent MAP kinase activity in intact NIH 3T3 cells and Rat-1 cells (51), in Rat-1 cells overexpressing human epidermal growth factor receptor (52), and in a cell-free assay system (53). However, it has recently been reported that the B-Raf activity is not inhibited by cyclic AMP in PC12 cells maintained in the serum-containing medium but is inhibited in serum-starved PC12 cells (54). This result, together with the present result that the protein kinase A-catalyzed phosphorylation of Rap1B did not antagonize the Ki-Ras-stimulated B-Raf activity, suggests that the apparent discrepancies observed between cell-based and in vitro protein kinase A studies reported might reflect different regulatory mechanisms of B-Raf, which are affected by growth conditions and cell type-specific differences of B-Raf expression. Further studies are necessary to clarify these discrepancies.

It may be noted that the lipid modifications of Rap1B are important for the B-Raf activity. We have previously shown

\(^2\)The exact reason for the small difference between their maximum levels is not known, but one explanation is that, since GTP\(\gamma\)S-Rap1B has less affinity with B-Raf than GTP\(\gamma\)S-Ki-Ras, some GTP\(\gamma\)S-Rap1B might dissociate from the GTP\(\gamma\)S-Rap1B-B-Raf complex and the B-Raf activity might be decreased during the preparation of the immunoprecipitate.

\(^3\)Rat spleen and heart cytosol extracts were subjected to Mono S column chromatography. Immunoblot analysis of each fraction with an anti-B-Raf antibody identified B-Raf with a M\(_r\) value of 95 kDa.
that the lipid modifications of Rap1B are important for the Smg GDS-induced activation of Rap1B (37). Thus, the lipid modifications of Rap1B are important for its activation and function. This property of Rap1B is similar to that of Ras of which lipid modifications are important for both its activation and function (31, 35, 37–41). However, it still remains to be clarified how the lipid modifications are important for the interaction of Ras and Rap1 with their regulators and target molecules.

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