Involvement of B-Raf in Ras-induced Raf-1 activation

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Abstract The mechanism of Ras-induced Raf-1 activation is not fully understood. Previously, we identified a 400-kDa protein complex as a Ras-dependent Raf-1 activator. In this study, we identified B-Raf as a component of this complex. B-Raf was concentrated during the purification of the activator. Immunodepletion of B-Raf abolished the effect of the activator on Raf-1. Furthermore, B-Raf and Ras-activated Raf-1 co-operatively, when co-transfected into human embryonic kidney 293 cells. On the other hand, Ras-dependent extracellular signal-regulated kinase/mitogen-activated protein kinase kinase stimulator (a complex of B-Raf and 14-3-3) failed to activate Raf-1 in our cell-free system. These results suggest that B-Raf is an essential component of the Ras-dependent Raf-1 activator. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Ras; Raf-1; B-Raf

1. Introduction

A low-molecular-weight GTP-binding protein Ras cycles between an active GTP-bound form and an inactive GDP-bound form, and acts as a molecular switch that regulates cellular proliferation, differentiation, and transformation [1–3]. Several groups, including ourselves, identified Raf-1 as the first target molecule of Ras [4–9]. Since then, extensive studies have revealed the existence of multiple downstream effectors of Ras, including Raf-1, phosphatidylinositol-3 kinase, and RalGEF [10].

Raf is a cytosolic serine/threonine kinase that regulates expression of various genes through activation of the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway. The Raf family consists of three isoforms, A-Raf, B-Raf and Raf-1, in mammalian cells [11,12]. It has been reported that Ras associates with B-Raf and that formation of the Ras–B-Raf complex directly leads to B-Raf activation [13]. On the other hand, although Ras-induced translocation of Raf-1 to the plasma membrane is important for activation of Raf-1 [14–16], their interaction by itself is not sufficient for the Raf-1 activation [9,17]. These observations have suggested that the activation mechanism of Raf-1 is different from that of B-Raf, and have raised the possibility that an additional molecule(s) might be involved in Ras-induced Raf-1 activation. In fact, using a cell-free system, we previously identified a 400-kDa protein complex in rat brain cytosol as a Ras-dependent Raf-1 activator [18]. In the present study, we further characterized this activator and found that B-Raf is an important component of the activator complex.

2. Materials and methods

2.1. Materials

Polyclonal antibodies raised against the carboxy termini of A-Raf (sc-165), B-Raf (sc-166), Raf-1 (sc-227g) and H-Ras (sc-520), and a monoclonal antibody against FLAG-epitope (M2) were purchased from Santa Cruz Biotechnology and Eastman Kodak, respectively. DEAE Sephacel, butyl Sepharose, and Superose 6 were obtained from Amersham Pharmacia Biotech. Full-length cDNA of human B-*raf* (a kind gift from T. Yamamoto, University of Tokyo, Japan) was subcloned into the *Mlu*I site of pCMV5 [19]. The expression vector for human Raf-1 with FLAG-epitope tag and six histidine residues in its carboxy terminus (RafFH) was kindly provided by M. McMahon (University of California, San Francisco, CA, USA). Recombinant histidine-tagged *Xenopus* MEK (His-MEK) and glutathione-*S*-transferase-fused kinase-deficient *Xenopus* ERK (GST-kdERK) were produced and purified as described [20,21]. Ras-dependent ERK/MEK stimulator (REKS) was purified from rat brain, and its kinase activity was measured as described in a previous report [13]. Lipid modified K-Ras[G12V] was prepared as described [17].

2.2. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 10% CO_2/90% air. For transient expression, HEK293 cells were transfected with suitable combinations of expression vectors using the calcium-phosphate co-precipitation technique.

2.3. Kinase assays

Raf-1 kinase assay was performed as described [18]. Raf-1 and RafFH were immunoprecipitated using anti-Raf-1 and anti-FLAG antibodies, respectively. The precipitate was then incubated with His-MEK, GST-kdERK, and [γ-^{32}P]ATP for 20 min at 30°C. The reaction was terminated by addition of the sample buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the sample was resolved by SDS–PAGE. The radioactivity incorporated into GST-kdERK was measured by image analyzer (BAS2000, Fuji).

Assay for Raf-1 activator was carried out as previously described [18]. Samples were incubated for 30 min at 16°C with the membrane

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Abbreviations: ERK, extracellular signal-regulated kinase; GST, glutathione-*S*-transferase; HEK, human embryonic kidney; MEK, mitogen-activated protein kinase/ERK kinase; RafFH, Raf-1 with FLAG tag and six histidine residues in its carboxy terminus; REKS, Ras-dependent ERK/MEK stimulator

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fraction of baculovirus-infected Sf9 cells expressing H-Ras[G12V] (Ras[G12V] membrane), and the partially purified cytosolic fraction of HEK293 cells that express RafFH (RafFH fraction). The reaction was terminated by addition of Triton X-100 at a final concentration of 0.5%. RafFH was then immunoprecipitated from solubilized fraction, and its activity was measured as described above.

2.4. Purification of a Ras-dependent Raf-1 activator

All manipulations were carried out at 0–4°C. Brains (55.6 g) of Wistar rats were homogenized with a Potter–Elvehjem homogenizer in buffer A (20 mM HEPES/NaOH, pH 7.4, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol, 10 mM NaF, and 25 mM β-glycerophosphate) supplemented with 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 10 μg/ml pepstatin A. The homogenate was centrifuged at 100 000×*g* for 1 h, and the supernatant (1500 mg of protein) was absorbed to a DEAE Sephacel column (2.5×16.5 cm). After washing with buffer A, proteins were eluted with buffer A containing 0.25 M NH_4Cl, and the fractions (600 mg of protein) that exhibited Raf-1 activator activity were collected. Proteins in the collected fractions were precipitated with 40% saturated ammonium sulfate in the presence of 10% (v/v) glycerol. The pellet was dissolved in buffer A containing 0.7 M ammonium sulfate, and the soluble proteins (240 mg) were applied to a butyl Sepharose column (1.5×19 cm). The column was washed with buffer A containing 0.7 M ammonium sulfate, and proteins were eluted with a linear gradient between buffer A containing 0.7 M ammonium sulfate and buffer A. The fractions (18 mg) containing Raf-1 activator activity were collected, concentrated by precipitation with 50% saturated ammonium sulfate, and subjected to a Superose 6 column (1.0×30 cm). After elution with buffer A containing 0.1 M NH_4Cl, the activator-containing fractions (0.59 mg) were saved.

3. Results

To purify a Ras-dependent Raf-1 activator, we have established a cell-free system in which an activated mutant of H-Ras (Ras[G12V]) activates Raf-1 [17,18]. In this system, incubation of the membrane fraction containing Ras[G12V] (Ras[G12V] membrane) with the partially purified cytosolic fraction containing RafFH (RafFH fraction) leads to activation of RafFH, but only in the presence of the activator. Using this system, we purified the activator of Raf-1 from the cytosol of rat brain through a series of chromatographies (Table 1), and searched for a protein that was enriched in the process of purification. Consequently, we found that the concentration of B-Raf in the activator fraction increased as purification proceeded (Fig. 1A). Furthermore, the elution peak of B-Raf showed a good correspondence with that of the activator in the hydrophobic chromatography with butyl Sepharose (Fig. 1B) and the gel filtration chromatography with Superose 6 (data not shown). These observations raised the possibility that B-Raf might contribute to the activation of Raf-1 by Ras.

To examine whether B-Raf in the activator fraction is involved in the Ras-induced Raf-1 activation, B-Raf was removed from the activator fraction with the antibody (sc-166) raised against the last 19 carboxy-terminal amino acids

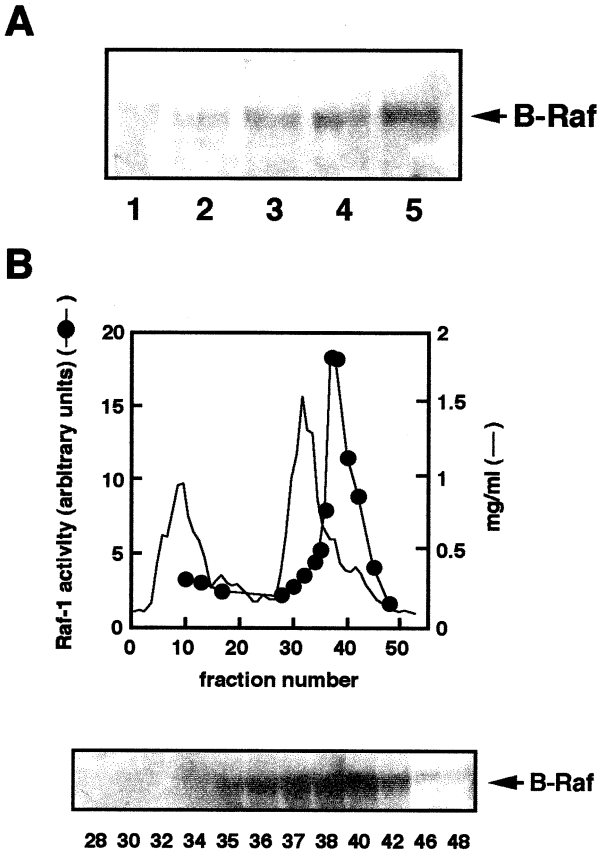

Fig. 1. Enrichment of B-Raf in the activator fraction. A: Comparison of the amount of B-Raf protein in the activator fraction. A portion (1 μg of protein) of the activator fraction obtained from each chromatography was subjected to Western blotting analysis with anti-B-Raf antibody. Lane 1, rat brain cytosol; lane 2, DEAE Sephacel; lane 3, ammonium sulfate precipitation (40% saturated); lane 4, butyl Sepharose; lane 5, Superose 6. B: Purification of the Ras-dependent Raf-1 activator with butyl Sepharose. Aliquots of each fraction were subjected to the activator assay (upper panel, closed circles) and Western blotting analysis with anti-B-Raf antibody (lower panel). The solid line in the upper panel indicates protein concentration.

of B-Raf (Fig. 2A). Since Raf isoforms (A-Raf, B-Raf and Raf-1) differ from each other at their carboxy termini, this antibody is specific for B-Raf among the three isoforms. When the activator fraction was incubated with the Ras[G12V] membrane and the RafFH fraction, RafFH was activated (Fig. 2B, bar 4). In contrast, when B-Raf was depleted from the activator fraction, the activation of RafFH was abolished (Fig. 2B, bar 6). When the same experiment was performed with anti-A-Raf antibody, the activator fraction did not lose its Raf-1-activating ability (Fig. 2B, bar 8),

Table 1
Purification of Ras-dependent Raf-1 activator

Fraction	Protein (mg)	Activity[a] (units)	Specific activity (units/mg)	Fold	Yield (%)
Rat brain cytosol	1500	263	0.18	1	100
DEAE Sephacel	600	439	0.73	4.1	167
Ammonium sulfate	240	368	1.53	8.5	140
Butyl Sepharose	18	92.1	5.12	28.4	35.0
Superose 6	0.59	5.4	9.15	50.8	2.1

[a]One unit is defined as an activity that stimulates RafFH to incorporate 1 pmol of [^{32}P]inorganic phosphate from ATP to GST-kdERK per 20 min at 30°C.

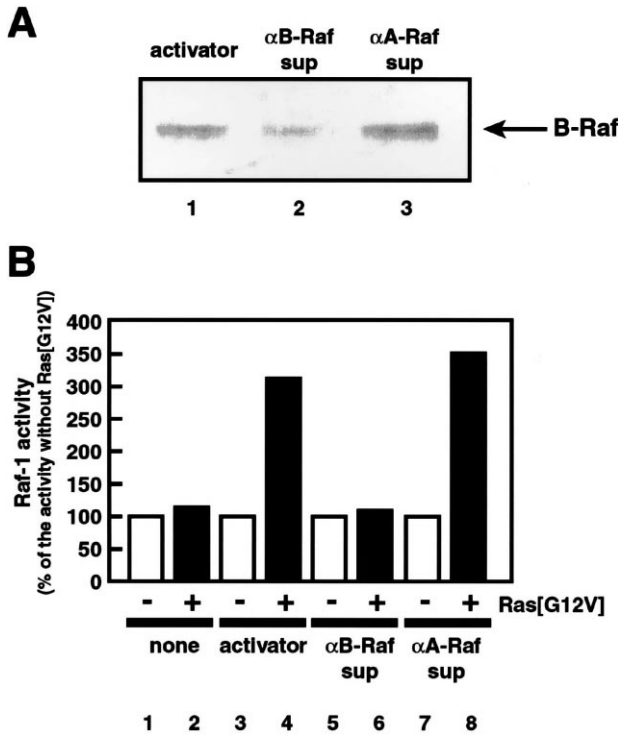

Fig. 2. B-Raf is an essential component of the activator. A: Depletion of B-Raf from the activator fraction. The activator fraction obtained from the ammonium sulfate precipitation (40%) (lane 1) was incubated with either anti-B-Raf or anti-A-Raf antibody, and the supernatant was saved as an immunodepleted fraction (lane 2 or 3, respectively). A portion (10 μg of protein) of the each fraction was subjected to Western blotting analysis with anti-B-Raf antibody. B: Activation of Raf-1 in the cell-free system. The RafFH fraction was incubated with or without the Ras[G12V] membrane in the presence or absence of the activator fraction, either depleted or non-depleted with antibodies. After immunoprecipitation with anti-FLAG antibody, the activity of RafFH was measured as described in Section 2. The Raf-1 activity obtained in the absence of the Ras[G12V] membrane was set to 100%.

indicating that the observed decrease of the activator activity was due to the depletion of B-Raf. These data suggest that B-Raf is an indispensable subunit of the activator.

If B-Raf is a component of the activator, B-Raf and Ras should activate Raf-1 co-operatively. To test this possibility, HEK293 cells were transfected with B-Raf and/or Ras[G12V], and the kinase activity of Raf-1 was measured. While Ras apparently increased the activity of Raf-1, we observed weak, if any, activation of Raf-1 by B-Raf (Fig. 3A). A combination of Ras and B-Raf, however, resulted in greatly enhanced activation of Raf-1. These results indicate that B-Raf can activate Raf-1 in a co-operative manner with Ras, when expressed in intact cells.

Our previous data indicated that the molecular size of the activator is around 400 kDa [18], while that of B-Raf is about 90 kDa. Therefore, we examined whether B-Raf alone is sufficient for Ras-dependent Raf-1 activation. For this, REKS, a complex of B-Raf and 14-3-3 [13], was purified from rat brains, and its ability to activate Raf-1 was examined. As previously reported [13], REKS could induce activation of MEK in a Ras-dependent manner (Fig. 4A). On the other hand, REKS showed no effect on the kinase activity of RafFH (Fig. 4B), suggesting that B-Raf complexed with 14-3-3 is insufficient to stimulate the activation of Raf-1.

4. Discussion

The mechanism of Raf-1 activation by Ras is complicated, and several questions arise. For example, why is it required for Ras to be farnesylated? Is phosphorylation of Raf-1 necessary? What is a third factor essential for Raf-1 activation? To answer these questions, we previously established a cell-free system, and found that Ras needs to be farnesylated, since the farnesylation allows Ras to undergo dimerization, a crucial step for Ras-induced Raf-1 activation [17]. Using this system, we also found the existence of a Ras-dependent Raf-1 activator in the cytosolic fraction [18]. This finding raised the possibility that the activator might be a cytosolic protein, although the activator has been speculated to be localized at the plasma membrane [14–16].

To explore this possibility, in the present study, we set out to further characterize the Ras-dependent Raf-1 activator in the cytosolic fraction. Since our previous observations suggest that the activator may be a complex consisting of several subunits [18], we searched for a protein that becomes concentrated during the purification of the activator. As a result, we found that a cytosolic protein kinase, B-Raf, increased its concentration in the activator fraction as the purification proceeded. To confirm involvement of B-Raf in the Ras-stimulated Raf-1 activation, B-Raf was depleted from the activator fraction, which abolished almost all the activity of the activator. Furthermore, it was found that B-Raf can enhance

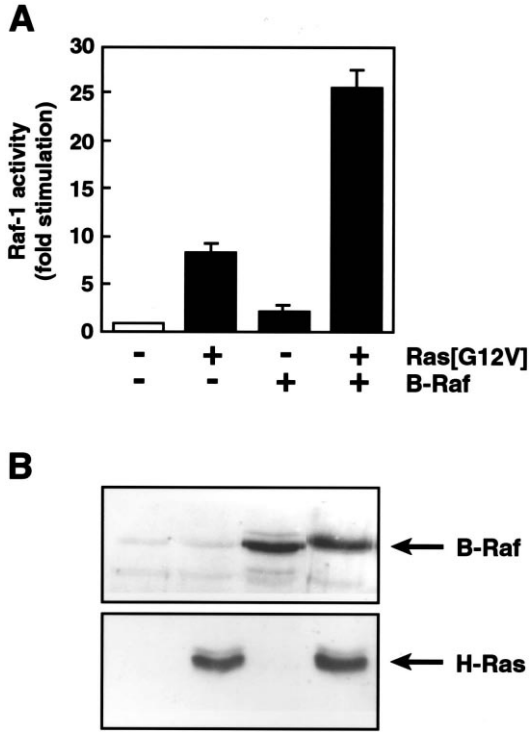

Fig. 3. Co-operative activation of Raf-1 by Ras and B-Raf. A: HEK293 cells were transiently transfected with H-Ras[G12V] and/or B-Raf. After 1 day serum starvation, the cells were harvested, lysed, and the kinase activity of endogenous Raf-1 in the lysate was measured as described in Section 2. The Raf-1 activity obtained in the absence of H-Ras[G12V] and B-Raf was set to 1.0. Values shown represent the mean and standard deviation ($n = 3$). B: Expression level of H-Ras[G12V] and B-Raf. A portion (16 μg of protein) of the lysate was subjected to immunoblotting with antibodies against B-Raf (upper panel) and H-Ras (lower panel).

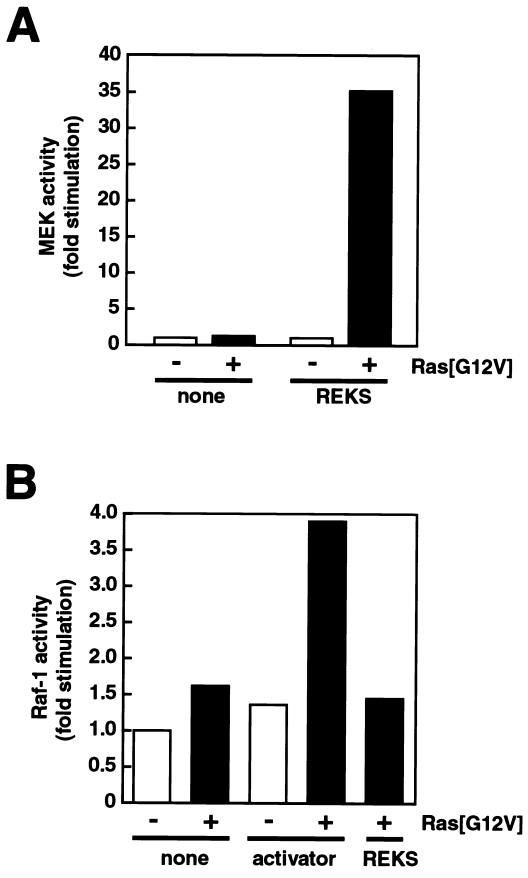

Fig. 4. B-Raf is insufficient for Ras-induced Raf-1 activation. A: Ras-dependent activation of MEK by REKS. REKS was incubated with His-MEK, GST-kdERK, and [γ-^{32}P]ATP in the presence or absence of lipid-modified K-Ras[G12V]. The samples were resolved by SDS–PAGE, and the radioactivity incorporated into GST-kdERK was measured. The activity obtained in the absence of K-Ras[G12V] was set to 1.0. B: REKS cannot activate Raf-1. The RafFH fraction was incubated with REKS or the activator fraction from DEAE Sephacel in the presence or absence of the Ras[G12V] membrane, and the activity of RafFH was measured. The Raf-1 activity obtained in the absence of the activator fraction and the Ras[G12V] membrane was set to 1.0.

Ras-induced activation of Raf-1 in vivo. These results strongly suggest that B-Raf plays an essential role in the activation of Raf-1 by Ras. On the other hand, B-Raf alone is likely to be insufficient for the Raf-1 activation, since partially purified B-Raf (i.e. REKS) failed to activate Raf-1 even in the presence of Ras. Taken together, our results indicate that B-Raf is an indispensable subunit of the Ras-dependent Raf-1 activator.

Though the present study demonstrated the involvement of B-Raf in Ras-induced Raf-1 activation, its mechanism remains unclear. Since Ras forms a homodimer at the plasma membrane [17], it is possible that Raf-1 and B-Raf form a heterodimer on the dimeric Ras. Indeed, Weber et al. have recently reported that an activated form of Ras induces formation of a Raf-1–B-Raf dimer [22]. Upon formation of the complex, B-Raf becomes activated by association with Ras [13], and in turn may facilitate the increase of Raf-1 kinase activity by an unknown mechanism. In this activation step, another subunit(s) of the activator, besides B-Raf, must be involved, since B-Raf alone is unable to activate Raf-1. It has been reported that artificial homodimerization results in activation of Raf-1 through transphosphorylation [23,24]. Therefore, it is possible that B-Raf directly phosphorylates and activates Raf-1 in the presence of the other subunit of the activator. An alternate possibility is that B-Raf phosphorylates, not Raf-1, but the other subunit, which somehow leads to Raf-1 activation. Identification of the other subunit will be necessary to clarify the mechanism of Raf-1 activation by Ras and B-Raf.

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