Different Effects of Various Phospholipids on Ki-Ras-, Ha-Ras-, and Rap1B-induced B-Raf Activation*

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We have recently purified a Ki-Ras- and Ha-Ras-dependent extracellular signal-regulated kinase from bovine brain and identified it as B-Raf protein kinase complexed with 14-3-3 proteins (Yamamori, B., Kuroda, S., Shimizu, K., Fukui, K., Ohtsuka, T., and Takai, Y. (1995) J. Biol. Chem. 270, 11723–11726). Moreover, we found that Rap1B as well as Ki-Ras and Ha-Ras stimulate the B-Raf activity. Since B-Raf contains a cysteine-rich domain originally found in protein kinase C as a domain responsible for interaction with phosphatidyserine (PS) and diacylglycerol or 12-O-tetradecanoylphorbol-13-acetate, we have examined here the effect of these compounds on the Ki-Ras-, Ha-Ras-, and Rap1B-induced activation of bovine brain B-Raf. Bovine brain PS enhanced Ki-Ras-stimulated B-Raf activity. Phosphatidic acid was slightly active, but other phospholipids, such as phosphatidylinositol (PI), PI-4-monophosphate, PI-4,5-bisphosphate, and PI-3,4,5-trisphosphate, were inactive. However, none of the above phospholipids affected the Ha-Ras-stimulated B-Raf activity, whereas PI, PS, phospatidylethanolamine, and phosphatidic acid inhibited the Rap1B-stimulated B-Raf activity. Phosphatidylinositol or PI-4-monophosphate did not show any effect on the Rap1B-stimulated B-Raf activity. Synthetic PS with two unsaturated fatty acids, such as 1,2-dioleoyl-PS or 1,2-dilinoleoyl-PS, showed the same effect toward the Ki-Ras- and Rap1B-stimulated B-Raf activities, but synthetic PS with two saturated fatty acids, such as 1,2-distearoyl-PS, was inactive. 12-O-Tetradecanoylphorbol-13-acetate did not affect the stimulatory or inhibitory effect of PS on the Ki-Ras- and Rap1B-stimulated B-Raf activities, respectively. PS did not affect the Ki-Ras-, Ha-Ras-, or Rap1B-independent basal B-Raf activity or the mitogen-activated protein kinase kinase or extracellular signal-regulated kinase activity. These results indicate that various phospholipids differentially affect Ki-Ras-, Ha-Ras, and Rap1B-induced B-Raf activation.

A variety of extracellular signals activate mitogen-activated protein kinase/ERK1 through the Ras-dependent MEK activation (for reviews, see Refs. 1–4). However, the molecular mechanism of Ras-dependent MEK activation is not understood fully. Recent investigations have revealed that c-Raf-1 (5, 6) and B-Raf (7–9) are responsible for Ras-dependent MEK activation, and that they directly interact with Ras (10–15), but no evidence has demonstrated that Ras directly activates c-Raf-1 or B-Raf in cell-free assay systems. We have developed a cell-free assay system in which GTP-Ras activates MEK and have identified a protein factor necessary for Ras-dependent MEK activation in Xenopus oocytes and eggs (16, 17). Furthermore, we have purified a Ras-dependent MEK kinase from bovine brain cytosol and identified it as B-Raf complexed with 14-3-3 proteins (18).

On the other hand, it has recently been shown that Rap1, belonging to the small GTP-binding protein superfamily (19–21), interacts with c-Raf-1 in a yeast two-hybrid system (12), and 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 (22). However, it is not clear whether Rap1 affects Ras-dependent Raf activation. We have found that Rap1B as well as Ki-Ras and Ha-Ras stimulates the B-Raf activity and that Rap1B does not antagonize the Ras-stimulated B-Raf activity (23).

The Raf protein kinase family consists of c-Raf-1, A-Raf, and B-Raf in mammals (4). Raf is also evolutionally highly conserved in Drosophila (24, 25) and Camnorhabditis elegans (26). Structurally, Raf displays significant similarity to the PKC superfamily (27). The PKC superfamily is classified into three families, the conventional PKC, new PKC, and atypical PKC families (for a review, see Ref. 28). The conventional PKC family contains two cysteine-rich domains and one Ca2+-dependent phospholipid-binding domain; the former is responsible for its activation by diacylglycerol or tumor-promoting phorbol ester, such as 12-O-tetradecanoylphorbol-13-acetate, in the presence of anionic phospholipid. The latter is responsible for its activation by Ca2+ in the presence of anionic phospholipid, such as PS. The new PKC family contains two cysteine-rich domains responsible for its activation by diacylglycerol or tumor-promoting phorbol ester in the presence of anionic phospholipid (28). In contrast, the atypical PKC family contains only one cysteine-rich domain, but its activity is not affected by diacylglycerol or phorbol ester (28). PKCδ, a member of the

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atypical PKC family, has been shown to be enhanced by PS and PI-3,4,5-trisphosphate (29). Structurally, the Raf family resembles the atypical PKC family; it contains only one cysteine-rich domain in the amino-terminal region and a catalytic domain in the carboxyl-terminal region and lacks the Ca^{2+}-dependent phospholipid-binding domain. Recently, it has been shown that c-Raf-1 binds to PS liposomes, although it does not bind to phosphor ester (30), and that membrane phospholipid enhances the recombinant c-Raf-1 activity (31). However, since a cell-free assay system for the Ras-or Rap1-dependent Raf activation was not available, phospholipid effect on the Ras- or Rap1-dependent Raf activation has not been studied.

In this study, we have examined phospholipid effects on Ras- and Rap1-dependent B-Raf activation and found that PS further enhances Ki-Ras-stimulated, but not Ha-Ras-stimulated, B-Raf activation, and that PI, PS, and PE inhibit Rap1B-stimulated activation.

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals—**Post-translationally lipid-modified Ki-Ras, Ha-Ras, and Rap1B were prepared as described (32). GST-MEK and GST-ERK2 were prepared as described (17, 33, 34). GTP-S-Ki-Ras, GDP-Ki-Ras, GTP-S-Ha-Ras, GDP-Ha-Ras, GTP-S-Rap1B, and GDP-Rap1B were prepared as described (32). An anti-B-Raf polyclonal antibody was purchased from Santa Cruz Biotechnology Santa Cruz, CA). 12-O-Tetradecanoylphorbol-13-acetate and myelin basic protein were purchased from Sigma.

Phospholipid Preparation—PS, PI-4-monophosphate, and PI-4,5-bisphosphate were purchased from Sigma. PA, phosphatidyicholine, PE, and PI were purchased from Serdary Research Laboratories (Ontario, Canada). PI-3,4,5-trisphosphate was kindly provided by Dr. T. Takenawa (Tokyo University, Tokyo, Japan). 1,2-Dioleoyl-PS, 1,2-dilinoleoyl-PS, and 1,2-distearyl-PS were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). The purity of the respective lipids was confirmed by thin-layer chromatography as described (35, 36). The required amounts of phospholipids in chloroform were dried under a stream of N, suspended in 20 mM Tris/HCl at pH 7.5 by vortexing, and sonicated for 30 s four times.

Assay for Ras-dependent B-Raf Activation—The B-Raf activity was assayed by measuring the phosphorylation of myelin basic protein by recombinant GST-ERK2 in the presence of recombinant GST-MEK as described (17, 18, 34). Briefly, 5 μl of GTP-S-Ki-Ras, GTP-S-Ha-Ras, or GTP-S-Rap1B, 15 μl of B-Raf, and 5 μl of phospholipid to be tested were mixed in this order. The concentrations of these compounds used were indicated in each experiment. After 15 μl of GST-MEK and 10 μl of a reaction mixture containing 120 μM ATP were added, incubation was continued for 10 min at 30 °C. After the incubation, 10 μl of GST-ERK2 was added and the incubation was continued for another 20 min at 30 °C. Then 20 μl of a reaction mixture containing 100 μM [γ-32P]ATP (300 cpm/pmol) and 220 μM myelin basic protein was added. After further incubation for 10 min at 30 °C, 30 μl of the mixture were spotted onto a phosphocellulose paper sheet. The sheet was washed with 75 mM phosphoric acid, and the radioactivity was measured by liquid scintillation spectrometry.

Purification of B-Raf—Bovine brain B-Raf was purified as described (18). Briefly, bovine brain cytosol was subjected to Mono Q 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.

**Determination of Protein Concentrations—**Protein concentrations were determined with bovine serum albumin as a standard protein by the method of Bradford (37).

**RESULTS**

The effect of various phospholipids on GTP-S-Ki-Ras-, GTP-S-Ha-Ras-, and GTP-S-Rap1B-dependent B-Raf activation was first examined. In the presence of GTP-S-Ki-Ras, PS synergistically stimulated B-Raf activity (Fig. 1A). PA slightly stimulated it, but other phospholipids, such as phosphatidyicholine, PE, PI, and PI-4-monophosphate, did not. The K, value for PS in the presence of GTP-S-Ki-Ras, giving a half-maximal activation, was about 12.5 μM. In the absence of GTP-S-Ki-Ras, PS was not active. The other phospholipids were also inactive. PI-4,5-bisphosphate and PI-3,4,5-trisphosphate were inactive irrespective of the presence and absence of GTP-S-Ki-Ras (data not shown). When similar experiments were performed with GTP-S-Ha-Ras, none of the phospholipids examined affected the B-Raf activity (Fig. 1B). When similar experiments were done with GTP-S-Rap1B, PI inhibited the Rap1B-stimulated B-Raf activity (Fig. 1C). PS and PE also inhibited it, but they were slightly less active than PI. PA slightly inhibited it. The other phospholipids were inactive. 12-O-Tetradecanoylphorbol-13-acetate did not affect the B-Raf activity irrespective of the presence and absence of GTP-S-Ki-Ras, GTP-S-Ha-Ras, or GTP-S-Rap1B. Solid line, in the presence of GTP-S-Ki-Ras, GTP-S-Ha-Ras, or GTP-S-Rap1B; dashed line, in the absence of GTP-S-Ki-Ras, GTP-S-Ha-Ras, or GTP-S-Rap1B. , with PS; , with PA; , with phosphatidyicholine; , with PE; , with PI; , with PI-4-monophosphate. The results shown are representative of three independent experiments.

**FIG. 1. Effect of various phospholipids on K, Ras-, Ha-Ras-, and Rap1B-dependent B-Raf activation.** The B-Raf activity was measured in the presence of various combinations of 50 nM GTP-S-Ki-Ras, 50 nM GTP-S-Ha-Ras, 50 nM GTP-S-Rap1B, and various phospholipids, A, in the presence or absence of GTP-S-Ki-Ras. B, in the presence or absence of GTP-S-Ha-Ras. C, in the presence or absence of GTP-S-Rap1B. Solid line, in the presence of GTP-S-Ki-Ras, GTP-S-Ha-Ras, or GTP-S-Rap1B; dashed line, in the absence of GTP-S-Ki-Ras, GTP-S-Ha-Ras, or GTP-S-Rap1B. , with PS; , with PA; , with phosphatidyicholine; , with PE; , with PI; , with PI-4-monophosphate. The results shown are representative of three independent experiments.
Phospholipid Effect on Ki-Ras- and Rap1B-dependent B-Raf Activation

**Fig. 2. Effect of PS on the B-Raf activity stimulated by various concentrations of GTPγS-Ki-Ras or GTPγS-Rap1B.** The B-Raf activity was measured in the presence of various concentrations of GTPγS-Ki-Ras or GTPγS-Rap1B with or without PS (25 μg/ml). A, in the presence of GTPγS-Ki-Ras; B, in the presence of GTPγS-Rap1B. ●, with PS; ○, without PS. The results shown are representative of three independent experiments.

**Fig. 3. Effect of various synthetic PSs on GTPγS-Ki-Ras and GTPγS-Rap1B-dependent B-Raf activation.** The B-Raf activity was measured in the presence or absence of 50 nM GTPγS-Ki-Ras or GTPγS-Rap1B with various synthetic PSs. A, in the presence of GTPγS-Ki-Ras; B, in the presence of GTPγS-Rap1B. Solid line, in the presence of GTPγS-Ki-Ras or GTPγS-Rap1B; dashed line, in the absence of GTPγS-Ki-Ras or GTPγS-Rap1B. ○, with PS purified from bovine brain; ●, with 1,2-dioleoyl-PS; ■, with 1,2-dilinoleoyl-PS; ▲, with 1,2-distearoyl-PS. The results shown are representative of three independent experiments.

leoyl-PS, inhibited GTPγS-Rap1B-dependent B-Raf activation (Fig. 3B). Synthetic PS with two saturated fatty acids, such as 1,2-distearoyl-PS, was inactive.

In the absence of B-Raf, PS did not stimulate the GST-MEK or GST-ERK2 activity irrespective of the presence or absence of GTPγS-Ki-Ras (Table I). The basal activity in the B-Raf fraction may not be derived from any contaminating kinase but possibly from B-Raf itself, because the activity was eliminated by immunodepletion of B-Raf by an anti-B-Raf antibody (data not shown). Moreover, PS did not affect the phosphorylation of myelin basic protein in the absence of GST-MEK.

**TABLE I**

| MBP phosphorylation | cpm |
|---------------------|-----|
| B-Raf + MEK + ERK   | 16,650 38,850 16,250 69,950 |
| MEK + ERK           | 930 1,050 990 1,100 |
| B-Raf + ERK         | 3,270 3,450 3,330 3,770 |

*Indicates −PS or +PS.

We have shown that, of the various phospholipids examined, PS, PI, PA, and PE showed different effects on Ki-Ras-, Ha-Ras-, and Rap1B-dependent B-Raf activation. For instance, PS stimulated Ki-Ras-dependent B-Raf activation, whereas PS inhibited the Rap1B-dependent B-Raf activation and showed no effect on Ha-Ras-dependent B-Raf activation.

**DISCUSSION**

We have shown recently that no phospholipid, including PS, affects the basal activity of B-Raf in the absence of small GTP-binding proteins. Moreover, we have found that PS does not affect the MEK or ERK activity. Therefore, these results suggest that the effective phospholipids do not affect the downstream molecules of B-Raf and that they do not simply interact with B-Raf alone to affect its enzymatic activity. It is possible that the effective phospholipids interact with Ki-Ras or Rap1B alone or their complex with B-Raf so that the small GTP-binding protein-stimulated B-Raf activity is modulated. It is currently unknown which is the case. We have, moreover, shown here that synthetic PS with two unsaturated fatty acids, such as 1,2-dioleoyl-PS or 1,2-dilinoleoyl-PS, affects Ki-Ras- and Rap1B-dependent B-Raf activation, but synthetic PS with two saturated fatty acids, such as 1,2-distearoyl-PS, is inactive. These results suggest that not only the polar head groups of phospholipids but also their acyl moieties are important for affecting Ki-Ras and Rap1B-dependent B-Raf activation. However, the precise mechanism of how the effective phospholipids affect Ki-Ras- and Rap1B-dependent B-Raf activation remains to be clarified.

Ki-Ras, Ha-Ras, and Rap1B are bound to membrane phospholipids through their C-terminal regions (for a review, see Ref. 38). In the case of Ki-Ras, the farnesyl moiety and the clustered basic amino acids are important for Ki-Ras to interact with the plasma membrane. Ha-Ras interacts with the plasma membrane through the farnesyl and palmitoyl moieties, and Rap1B interacts with the plasma membrane through the geranylgeranyl moiety and the clustered basic amino acids. The GDP-bound form of Ki-Ras and Ha-Ras is activated by conversion to the GDP-bound form on the plasma membrane by the action of GDP/GTP exchange proteins such as SOS and Cdc25 (for a review, see Ref. 39). SOS is activated by Grb2, which interacts with the tyrosine kinase-type membrane receptors, such as platelet-derived growth factor and epidermal growth factor receptors (39), whereas Cdc25 is activated by the Ca2+-calmodulin complex in cortical neurons (40). The GDP-bound form of Rap1B has recently been shown to be converted to the GDP-bound form on the plasma membrane by the action of C3G, which is activated by the action of Crk (41, 42). Crk is shown to be tyrosine-phosphorylated by c-Abl, BCR/Abl, and insulin-like growth factor I receptor (43-45). It has been
shown, on the other hand, that, when Ras is activated by membrane receptors, c-Raf-1 is translocated from the cytosol to the plasma membrane and that the artificial attachment of the CAAX box to c-Raf-1, which makes c-Raf-1 undergo lipid modifications, causes its translocation from the cytosol to the plasma membrane and its subsequent activation by some membrane factors even in the absence of Ras activation (46, 47). On the basis of these observations, it has been proposed that the GTP-bound form of Ras produced on the plasma membrane plays a role just in recruiting c-Raf-1 from the cytosol to the plasma membrane, where it is activated by some factors in the plasma membrane different from Ras. However, we have previously shown that Ki-Ras, Ha-Ras, and Rap1B activate purified B-Raf even in the absence of any membrane components (16,18,23,48), and we have shown here that this activation is differently affected by membrane phospholipids and that any membrane phospholipids alone thus far studied do not affect the basal B-Raf activity in the absence of small GTP-binding proteins. It is not known whether B-Raf as well as c-Raf-1 is translocated from the cytosol to the plasma membrane, when Ras is activated by membrane receptors. It has not been shown either that purified c-Raf-1 is activated by Ras or Rap1 in a cell-free system free from any membrane components. However, our earlier and present results suggest that, when Ras or Rap1 is activated on the plasma membrane, c-Raf-1 or B-Raf is recruited to the plasma membrane, where they are activated by the GTP-bound form of Ras or Rap1, and that this activation is modulated differently by various phospholipids of the plasma membrane. Further studies are necessary to establish whether phospholipids play this role in intact cells.

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