Synergistic Activation by Ras and 14-3-3 Protein of a Mitogen-activated Protein Kinase Kinase Kinase Named Ras-dependent Extracellular Signal-regulated Kinase Kinase Stimulator*

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We have identified, in Xenopus oocyte cytosol, a protein kinase named REKS (Ras-dependent extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase kinase (MEK) stimulator), which phosphorylates and activates recombinant ERK2 through recombinant MEK in a recombinant GTPyS (guanosine 5'-O-(3-thio)triphosphate)-Ras-dependent manner. We show here that this REKS activity is synergistically enhanced by a combination of mammalian recombinant GTPyS-Ki-Ras and 14-3-3 protein purified from rat brain. 14-3-3 protein is known to activate tyrosine and tryptophan hydroxylases, to modulate the protein kinase C activity, to stimulate secretion, and to show phospholipase A$_2$ activity per se. 14-3-3 protein did not affect the MEK activity. 14-3-3 protein neither interacted with Ki-Ras nor affected the neurofibromin activity to stimulate the GTPase activity of Ki-Ras under the conditions where the recombinant N-terminal fragment of c-Raf-1 inhibited it. These results suggest that 14-3-3 protein has an additional function in the regulation of the Ras-MEK-ERK cascade pathway through the activation of REKS.

The 14-3-3 family of proteins (14-3-3 protein) was originally isolated by systematic analysis of brain proteins as a series of acidic proteins that have molecular masses of around 30 kDa and an isoelectric point of around 5 (for reviews, see Refs. 1 and 2). Subsequent studies have shown that the proteins of this family exist in dimeric form (3, 4). Seven isoforms have been identified, and these members are expressed in most tissues and particularly abundant in brain (2). 14-3-3 protein possesses multiple functions: an activator protein of tyrosine and tryptophan hydroxylases (4), a potent inhibitor or activator of protein kinase C (5, 6), a stimulatory regulator (Exol) for Ca$^{2+}$-dependent exocytosis in permeabilized adrenal chromaffin cells (7), and phospholipase A$_2$ per se (8). 14-3-3 protein has also been identified in yeast and implicated in growth (9).

Ras is a key regulator of growth and differentiation of higher eukaryotes. Ras is activated by the conversion from the GDP-bound inactive form to the GTP-bound active form in response to various extracellular signals (for reviews, see Refs. 10 and 11). However, no target molecule of Ras has been identified except adenylate cyclase in Saccharomyces cerevisiae (12). Recent studies indicate that Ras acts upstream of the MAP kinase/extracellular signal-regulated kinase (ERK) kinase (MEK) cascade in Xenopus oocytes and mammalian tissues (13–17). In this signal cascade, MEK is phosphorylated and activated by its kinase (MEK kinase) (18–24). Activated MEK then phosphorylates and activates ERK. c-Raf-1 is one of the MEK kinases (22–24). Moreover, c-Raf-1 directly binds to GTP-Ras in a cell-free system (25–28) and to wild-type Ras and dominant active Ras in a yeast two-hybrid system (29, 30). However, no direct evidence has been obtained that GTP-Ras directly activates c-Raf-1 in a cell-free system. To identify the target molecule of Ras, we have developed the cell-free system in which mammalian recombinant GTP-Ras activates MEK (31, 32). We have succeeded in identifying and partially purifying a protein molecule, named REKS, from the cytosol fraction of Xenopus oocytes, which phosphorylates and activates MEK in a GTP-Ras-dependent manner.

Matsumoto’s group has recently isolated one gene as an enhancer of c-Raf-1 in S. cerevisiae by use of yeast genetics. The structural analysis has revealed that this gene encodes yeast 14-3-3 protein. On the basis of these observations, we have examined here whether rat brain 14-3-3 protein is active on REKS.

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1 The abbreviations used are: 14-3-3 protein, the 14-3-3 family of proteins; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MEK, MAP kinase kinase/ERK kinase; MAL, maltose-binding protein; GTPyS, guanosine 5'-O-(3-thio)triphosphate; NF-1, neurofibromin; GST, glutathione S-transferase; MBP, myelin basic protein; REKS, (Ras-dependent extracellular signal-regulated kinase (ERK/mitogen-activated protein kinase kinase (MEK) stimulator).


FIG. 1. Mono S column chromatography of the cytosol. The cytosol fraction of eggs in the interphase stage (24 mg of protein, 6 ml) was applied to a Mono S column (0.5 x 5 cm) equilibrated with Buffer A (20 mM HEPES/NaOH at pH 7.0, 1 mM dithiothreitol, 10 mM EGTA, 5 mM MgCl₂, and 10 μM (p-aminophenyl) methanesulfonyl fluoride). After the column was washed with 24 ml of Buffer A, elution was performed with a 30-ml linear gradient of NaCl (0–1.0 M) in the presence of both 80 nM GST-MEK and 500 nM GST-ERK2. A, with Ras and 14-3-3 protein; B, with Ras alone; C, with 14-3-3 protein alone; D, NaCl concentration; E, absorbance at 280 nm. B, immunoblot analysis with an anti-MEK polyclonal antibody. C, immunoblot analysis with an anti-ERK polyclonal antibody. D, immunoblot analysis with an anti-14-3-3 protein. E, immunoblot analysis with an anti-c-Raf-1 monoclonal antibody. The results were representative of three independent experiments.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Eggs were obtained from fully mature Xenopus laevis females as described (31, 33). Eggs, dejellied with cysteine and washed with Modified modified Ringer's solution (0.1 mM NaCl, 2.0 mM KCl, 1.0 mM MgSO₄, 2.0 mM CaCl₂, 5.0 mM HEPES/NaOH at pH 7.4, and 0.1 mM EDTA), were activated by electric shock as described (34) to make them enter into interphase to inactivate endogenous ERK activity (35, 36). The activated eggs were homogenized with an equal volume of homogenizing buffer (20 mM Tris/HCl at pH 8.0, 1 mM dithiothreitol, 10 mM EGTA, 5 mM MgCl₂, 10 μM (p-aminophenyl)methanesulfonyl fluoride, 10 μg/ml leupeptin, and 20 μg/ml aprotinin). The cytosol fraction was obtained by centrifugation at 300.000 g for 30 min twice as described (31). 14-3-3 protein was purified from rat brain cytosol by several column chromatographies as described (4). The purified protein was over 95% pure as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. An expression plasmid, pMAL-14-3-3, was constructed (37), and 14-3-3 protein fused to maltose-binding protein (MAL) was purified from overexpressing Escherichia coli (38). Recombinant Ki-Ras was purified from the membrane fraction of overexpressing insect cells (39). GTPyS-Ki-Ras was prepared as described (38). A mouse MEK cDNA was cloned from the D9 cDNA library using a polymerase chain reaction (40). Recombinant MEK, the GTPase-activating protein-related domain of neurofibromin (NF-1) and Ha-Ras fused to glutathione S-transferase (GST) protein were purified from overexpressing E. coli (41). The expression plasmid, pMAL-c-Raf-1 (1-257), encoding the N-terminal fragment of c-Raf-1 fused to MAL (MAL-c-Raf-1), was kindly provided by T. Kataoka (Kobe University, Kobe, Japan), and MAL-c-Raf-1 was purified from overexpressing E. coli (41). An anti-Xenopus c-Raf-1 monoclonal antibody was kindly provided by L. T. Williams (University of California, San Francisco, CA). An anti-Xenopus ERK polyclonal antibody was kindly provided by E. Nishida (Kyoto University, Kyoto, Japan). An anti-MEK polyclonal antibody was generated against a 16-mer peptide corresponding to the partial amino acid sequence of Xenopus MEK (His-Arg-Asp-Val-Lys-Pro-Ser-Asn-Ile-Leu-Val-Aln-Ser-Arg-Gly-Glu) (40). Myelin basic protein (MBP) was purchased from Sigma.

Assays for ERK, MEK, and REKS Activities—REKS activity was detected by the phosphorylation of MBP as a model substrate for ERK with recombinant GST-MEK and GST-ERK2 in the presence or absence of GTPγS-Ki-Ras. The sample to be assayed was incubated for 10 min at 30 °C in a final volume of 50 μl containing 20 mM Tris/HCl at pH 8.0, 10 mM MgCl₂, 6 mM EGTA, 120 μM ATP, 80 μM recombinant GST-MEK, and 100 nM GTPγS-Ki-Ras. After the 10-min incubation, 10 μl of 3 μM recombinant GST-ERK2 was added. The reaction mixture was incubated for additional 20 min at 30 °C. Then, 20 μl of a reaction mixture (20 mM Tris/HCl at pH 8.0, 6 mM EGTA, 100 μM [γ-32P]ATP (600 cpm/pmol), 10 mM MgCl₂, and 220 μM MBP) was added. Incubation was continued for another 10 min at 30 °C, after which 30 μl of the reaction mixture was spotted onto a phosphocellulose paper sheet. The sheet was washed with 75 mM phosducic acid, and the radioactivity was measured by liquid scintillation spectrometry (42).

RESULTS AND DISCUSSION

The cytosol fraction of Xenopus eggs was subjected to Mono S column chromatography and each fraction was assayed for the REKS activity in the presence of various combinations of GTPγS-Ki-Ras and 14-3-3 protein purified from rat brain. In the absence of Ki-Ras and 14-3-3 protein, two peaks (Peaks 1 and 2) were detected (Fig. 1A). The activity of Peak 1 was not affected by Ki-Ras, but the activity of Peak 2 was enhanced by...
Effect of 14-3-3 protein and MAL-c-Raf-1 on the NF-1 activity. Forty nM [γ-32P]GTP-Ki-Ras was incubated for 1 min at 24 °C with various combinations of MAL-c-Raf-1 and 14-3-3 protein in the presence and absence of 68 nM GST-NF-1. The reaction was stopped, followed by rapid filtration on a nitrocellulose filter. The radioactivity on the filter was determined. A, effects with various doses of MAL-c-Raf-1. B, effects with various doses of 14-3-3 protein in the presence and absence of 25 nM MAL-c-Raf-1 (○), without GST-NF-1; □, with GST-NF-1; ■, with GST-NF-1 and MAL-c-Raf-1. The results were representative of three independent experiments.

was detected in fractions 3–9 (Fig. 1D). It is also possible that the basal REKS activity observed in the absence of Ki-Ras is a real basal activity of REKS or due to the artificial activation of REKS during its preparation. If the former is the case, 14-3-3 protein could interact directly with REKS even in the absence of Ki-Ras, but if the latter is the case, 14-3-3 protein might interact with the REKS preactivated by Ras. Thus, two possible mechanisms for the activation of REKS by 14-3-3 protein are plausible; one is that 14-3-3 protein only acts on the REKS preactivated by Ras and enhances its maximum activity, and the other is that 14-3-3 protein makes REKS more sensitive to Ki-Ras for its maximum activation. Further studies are necessary to distinguish these two possible mechanisms.

Evidence is accumulating that c-Raf-1 may be a direct target molecule for Ras (25–30), but it has not yet been shown that purified GTP-Ras directly activates purified c-Raf-1 in a cell-free system. In the last set of experiments, the effect of 14-3-3 protein on c-Raf-1 was examined. The immunoreactivity against c-Raf-1 was mainly detected in the fractions other than Peaks 1 and 2, but it was slightly detected in Peak 2 (Fig. 1E). This immunoreactivity is most likely to be derived from oocyte c-Raf-1. REKS in Peak 2 was further purified by Ha-Ras-linked agarose affinity column chromatography to remove contaminating c-Raf-1.2 The immunoreactivity against c-Raf-1 was not detected in this highly purified REKS preparation and the activity of this REKS preparation was similarly enhanced by 14-3-3 protein (data not shown). Under these conditions, the oocyte c-Raf-1 activity to stimulate the MEK activity was not affected by 14-3-3 protein, irrespective of the presence and absence of GTPyS-Ki-Ras (Fig. 1A). However, 14-3-3 protein

It is possible that the stimulation of the REKS activity by GTPyS-Ki-Ras in the absence of 14-3-3 protein is just due to the presence of endogenous oocyte 14-3-3 protein in the REKS fraction. However, this was not the case, because the immunoreactivity against 14-3-3 protein was not detected in Peak 2, but it. The immunoreactivities against both MEK and ERK were detected in Peak 1 but not in Peak 2 (Fig. 1, B and C). The immunoreactivity against MEK was detected as a doublet. The fast migrating protein might be a proteolytic fragment of MEK. The activity of Peak 2 was not detected in the absence of GST-MEK (data not shown). These results indicate that Peak 1 is MEK and ERK, whereas Peak 2 is REKS, which we have identified previously (31, 32).

The activity of Peak 2 in the absence of GTPyS-Ki-Ras was slightly enhanced by 14-3-3 protein, whereas that in the presence of GTPyS-Ki-Ras was markedly enhanced by 14-3-3 protein. In contrast, the activity of Peak 1 was not affected by 14-3-3 protein irrespective of the presence and absence of Ki-Ras. The same results were obtained when recombinant 14-3-3 protein fused to MAL was used (data not shown). These results indicate that 14-3-3 protein and Ki-Ras synergistically stimulate the REKS activity.

14-3-3 protein stimulated the REKS activity in a dose-dependent manner in the presence and absence of GTPyS-Ki-Ras (Fig. 2). In the absence of Ki-Ras, 14-3-3 protein slightly stimulated the REKS activity, whereas in the presence of Ki-Ras, 14-3-3 protein synergistically stimulated it. In these experiments, 100 nM Ki-Ras was used. This concentration of Ki-Ras was sufficient for the stimulation of the REKS activity in the presence and absence of 14-3-3 protein, indicating that 14-3-3 protein enhances the REKS activity maximally stimulated by Ki-Ras.

14-3-3 protein neither phosphorylated MBP nor affected GST-ERK or GST-MEK activity, irrespective of the presence and absence of GTPyS-Ki-Ras under the conditions where it stimulated the REKS activity (Table I). Moreover, 14-3-3 protein did not stimulate the active MEK activity, which was partially purified from mature oocytes. These results indicate that 14-3-3 protein acts on either Ki-Ras or REKS and does not affect the molecules downstream of REKS, including MEK and ERK.

14-3-3 protein did not inhibit the NF-1 activity under the conditions where MAL-c-Raf-1 inhibited it in a dose-dependent manner (Fig. 3). 14-3-3 protein did not affect this inhibitory effect of MAL-c-Raf-1 on the NF-1 activity. Moreover, 14-3-3 protein did not directly interact with GTPyS-GST-Ha-Ras linked to the agarose beads (data not shown). These results suggest that 14-3-3 protein does not interact with Ras but interacts with REKS. However, it remains to be clarified whether 14-3-3 protein indeed directly interacts with REKS, because the REKS preparation used in these experiments had not been purified.

It is possible that the stimulation of the REKS activity by GTPyS-Ki-Ras in the absence of 14-3-3 protein is just due to the presence of endogenous oocyte 14-3-3 protein in the REKS fraction. However, this was not the case, because the immunoreactivity against 14-3-3 protein was not detected in Peak 2, but...
bound to the MAL-c-Raf-1 linked to the amylose beads (data not shown). Consistently, Matsumoto's group has obtained the result by use of a yeast two-hybrid system that 14-3-3 protein binds to c-Raf-1. 3 The exact reason for the failure of 14-3-3 protein to stimulate the oocyte c-Raf-1 activity is not known, but it is possible that another member of 14-3-3 protein binds and activates it, or that another factor or some modification of c-Raf-1, such as phosphorylation, may be necessary for the activation of c-Raf-1 by 14-3-3 protein and Ras. Further studies are necessary for understanding the relationship between 14-3-3 protein and c-Raf-1. In conclusion, 14-3-3 protein and Ki-Ras synergistically activate REKS in a cell-free system. This result suggests that activation of e-Raf-1 by 14-3-3 protein and Ras. Further studies are necessary for understanding the relationship between 14-3-3 protein and c-Raf-1.

REFERENCES