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A protein factor for *ras* p21-dependent activation of mitogen-activated protein (MAP) kinase through MAP kinase kinase

(extracellular signal-regulated kinase/mitogen-activated protein kinase kinase/*Xenopus* oocytes)

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ABSTRACT **To identify the direct target molecule of *ras* p21 in higher eukaryotes, we have recently developed the cell-free system in which *ras* p21 activates mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase (ERK). In this cell-free system, the guanosine 5′-[γ-thio]triphosphate-bound form of Ki-*ras* p21, but not the GDP-bound form, activates endogenous *Xenopus* MAP kinase as well as recombinant ERK2 in the presence of the cytosol fraction of *Xenopus* oocytes. We separated two protein factors from the cytosol fraction of *Xenopus* oocytes by column chromatography: one was the inactive form of MAP kinase kinase and the other was a factor tentatively named *ras* p21-dependent ERK-kinase stimulator (REKS). The former and latter showed M_r values of ≈45,000 and 150,000–200,000, respectively, as estimated by gel filtration. Both factors were necessary for Ki-*ras* p21-dependent activation of MAP kinase/ERK2. These results indicate that an additional protein factor (REKS) is essential for Ki-*ras* p21 to activate MAP kinase through MAP kinase kinase.**

ras p21 is a small GTP-binding protein (G protein) that exhibits both GDP/GTP-binding and GTPase activities (1). *ras* p21 has the GDP-bound inactive and GTP-bound active forms, which are interconvertible by GDP/GTP exchange and GTPase reactions (1). The GTPase reaction is regulated by *ras* p21 GTPase-activating protein (GAP) (2), whereas the GDP/GTP exchange reaction is regulated by GDP/GTP exchange protein (GEP) (3). *ras* p21 has been shown to be converted from the GDP-bound inactive form to the GTP-bound active form upon stimulation by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, and phorbol 12-myristate 13-acetate (4–7). It has also been shown that *ras* p21 GAP is phosphorylated by tyrosine kinases of the PDGF and EGF receptors (8, 9). These results, together with the accumulating evidence, indicate that *ras* p21 is a downstream molecule of the receptor tyrosine kinases and protein kinase C (10, 11). However, it has not been fully understood how *ras* p21 activity is regulated by these receptors and protein kinase C through its GEP or GAP, or how *ras* p21 transduces the signal to genes.

On the other hand, evidence is accumulating that a wide variety of extracellular signals such as PDGF, EGF, insulin, nerve growth factor, phorbol 12-myristate 13-acetate, and progesterone activate mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase (ERK) in various types of cells, including mouse fibroblasts, PC12 cells, and *Xenopus* oocytes, and that MAP kinase mediates at least a part of these signals (12–15). This MAP kinase activation is mediated by MAP kinase kinase, which is activated in mammalian cells and *Xenopus* oocytes upon stimulation by the extracellular signals (16–20). Accumulating evidence indicates that some of the extracellular signals activate MAP kinase through the action of *ras* p21 (21–26). However, it remains to be clarified how *ras* p21 regulates MAP kinase activity and what the direct target molecule of *ras* p21 is.

In light of these observations, we have recently established a cell-free system using the cytosol fraction of *Xenopus* oocytes, in which *ras* p21 activates *Xenopus* MAP kinase and recombinant MAP kinase (35). By use of this cell-free system, we have found and partially purified an additional protein factor that is necessary for *ras* p21 to activate MAP kinase through MAP kinase kinase.

MATERIALS AND METHODS

Materials. The cytosol fraction of *Xenopus laevis* oocytes was prepared as described with a slight modification (15). Immature oocytes (stage VI) were obtained surgically from hypothermically anesthetized females without collagenase treatment. Mature oocytes were obtained from females injected with gonadotropin (500 international units) 15 hr before use. The immature oocytes, washed with buffer A [20 mM Tris·HCl, pH 8.0/10 mM EGTA/5 mM $MgCl_2$/1 mM dithiothreitol/1 μM (*p*-amidinophenyl)methanesulfonyl fluoride/leupeptin (10 μg/ml)/aprotinin (20 μg/ml)], were homogenized with 1 vol of buffer A by a Teflon glass homogenizer. The homogenate was centrifuged at 5000 × *g* for 10 min at 4°C. The supernatant was then centrifuged twice at 300,000 × *g* for 30 min at 2°C. The supernatant was used as the cytosol fraction of immature oocytes. The mature oocytes, dejellied with cysteine (2%; pH 7.8) and washed with buffer B (buffer A containing 20 mM β-glycerophosphate/0.1 mM NaF/1 mM sodium orthovanadate), were homogenized with 1 vol of buffer B and centrifuged as described above. The supernatant was used as the cytosol fraction of mature oocytes. Recombinant ERK2 was purified from overexpressing *Escherichia coli* as a glutathione *S*-transferase (GST)–ERK2 fusion protein using a glutathione Sepharose 4B column as described (35). The GDP-bound and guanosine 5′-[γ-thio]triphosphate (GTP[γS])-bound forms of Ki-*ras* p21 were prepared as described (27). Phosphatase 2A was purified from human erythrocytes as described (28). An anti-MAP kinase polyclonal antibody was a generous gift from E. Nishida (University of Tokyo). Myelin basic protein (MBP) and aprotinin were purchased from Sigma.

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Abbreviations: G protein, GTP-binding protein; GAP, GTPase activating protein; GEP, GDP/GTP exchange protein; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; GTP[γS], guanosine 5′-[γ-thio]triphosphate; MBP, myelin basic protein.

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Assay for MAP Kinase. The MAP kinase activity was detected by the in-gel kinase assay as described (15). Briefly, the sample to be assayed was incubated for 10 min at 30°C in a final vol of 50 μl containing 20 mM Tris·HCl (pH 7.5), 100 μM ATP, 6 mM EGTA, 10 mM $MgCl_2$, and 300 nM Ki-*ras* p21 (GDP-bound or GTP[γS]-bound form). Where indicated, 10 μl of the inactive form of MAP kinase (4 μg of protein) or GST–ERK2 (500 ng of protein) was added, and the reaction mixture was incubated for an additional 20 min at 30°C. The reaction was terminated by addition of Laemmli's sampling buffer and boiled for 5 min at 100°C (29). The sample was subjected to SDS/PAGE using 10% gel containing 0.5 mg of MBP per ml. After renaturation of the sample, it was incubated with [γ-^{32}P]ATP to detect protein kinase activity toward MBP. The radioactivity of ^{32}P incorporated into MBP was detected by autoradiography or bioimaging analyzer BAS2000 (FUJIX, Tokyo). Details of the assay conditions will be described (35).

DEAE-Sephacel Column Chromatography of Cytosol Fractions. The cytosol fractions of immature and mature oocytes (0.5 ml each) were diluted with 4 vol of buffers A and B, respectively, and separately applied to DEAE-Sephacel columns (1 × 1.3 cm) equilibrated with each buffer. The flow-through fraction (2 ml) was collected and used as the unadsorbed fraction. After the column was washed with 5 column vol of each buffer containing 0.1 M NaCl, elution was performed with 2 ml of each buffer containing 0.2 M NaCl. The fraction eluted by 0.1–0.2 M NaCl was used as the adsorbed fraction. The adsorbed fractions from the cytosol fractions of immature and mature oocytes contained the inactive and active form of MAP kinase, respectively (see *Results*) (18).

Gel Filtration of Each Fraction from DEAE-Sephacel Column Chromatography. To partially purify *Xenopus* MAP kinase kinase, the unadsorbed fraction from the cytosol fraction of mature oocytes was concentrated 10:1 by Centricon 10 (300 μl; 2.4 mg of protein) and applied to a Superose 12 HR10/30 column equilibrated with buffer A. Elution was performed with the same buffer at a flow rate of 0.25 ml/min and 0.5-ml fractions were collected. The activity of MAP kinase kinase appeared in fractions 26–28 as a single peak (see Fig. 3*A*). These fractions were collected and used as the active form of MAP kinase kinase. The active form of MAP kinase kinase was detected by the MAP kinase assay. The unadsorbed fraction (2.4 mg of protein) and the adsorbed fraction (3.6 mg of protein) from the cytosol fraction of immature oocytes were subjected to the same columns, respectively, in a similar way (see Figs. 3*B* and 4).

Partial Purification of *Xenopus* MAP Kinase. The adsorbed fraction from the cytosol fraction (10 ml) of mature oocytes was dialyzed against buffer C [20 mM Tris·HCl, pH 8.0/2 mM EGTA/10 mM $MgCl_2$/1 mM dithiothreitol/1 μM (*p*-amidinophenyl)methanesulfonyl fluoride] and then applied to a Mono Q HR5/5 column equilibrated with buffer C. After the column was washed with the same buffer, elution was performed with a 50-ml linear gradient of NaCl (0–0.5 M) in buffer C and collected in 0.5-ml fractions. The active form of MAP kinase appeared in fractions 32–40 as a single peak. These fractions were collected and used as the active form of MAP kinase. The active form of MAP kinase was detected by the MAP kinase assay. The inactive form of MAP kinase was partially purified from the adsorbed fraction from the cytosol fraction of immature oocytes in a similar way. The inactive form of MAP kinase was detected by immunoblot analysis.

Phosphatase Treatment. The active form of MAP kinase kinase (10 μg of protein) was incubated for 10 min at 30°C with phosphatase 2A (50 ng of protein) in a final vol of 50 μl containing 20 mM Tris·HCl (pH 7.5), 9 mM EGTA, 1 mM EDTA, 4 mM $MgCl_2$, and 1 mM dithiothreitol. The reaction was terminated by addition of okadaic acid to a final concentration of 10 μM.

Other Procedures. The H1 histone kinase assay was carried out as described (15). Immunoblot analysis by use of the anti-MAP kinase antibody was carried out as described (30). SDS/PAGE was performed by the method of Laemmli (29). Protein concentrations were determined with bovine serum albumin as a standard protein (31).

RESULTS

Activation by Ki-*ras* p21 of MAP Kinase in the Cell-Free System. We have recently developed the cell-free system in which *ras* p21 activates endogenous *Xenopus* MAP kinase and GST–ERK2 in the presence of the cytosol fraction of immature oocytes (35). The *ras* p21-dependent activation of MAP kinase and GST–ERK2 is shown in Fig. 1. The GTP[γS]-bound form of Ki-*ras* p21 markedly activated MAP kinase and GST–ERK2 in the presence of the cytosol fraction of immature oocytes. The GDP-bound form of Ki-*ras* p21 was far less effective under the same conditions. Since MAP kinase is known to be activated by H1 histone kinase (15), we sought to determine whether *ras* p21 affected H1 histone kinase activity. The GTP[γS]-bound form of Ki-*ras* p21 did not affect H1 histone kinase activity (data not shown). On the other hand, the cytosol fraction of mature oocytes showed strong MAP kinase activity as described (15). This activity was not further activated by Ki-*ras* p21. Since GST–ERK2, with a M_r of ≈70,000 on SDS/PAGE, was easily distinguished from *Xenopus* MAP kinase, we used GST–ERK2 as the exogenous inactive form of MAP kinase in the following experiments.

Identification of a Protein Factor for Ki-*ras* p21 to Activate MAP Kinase Through MAP Kinase Kinase. It has recently been reported that MAP kinase is directly activated by its activator, MAP kinase kinase in response to various extracellular signals (16–20). Therefore, it is possible that Ki-*ras* p21 directly or indirectly stimulates MAP kinase kinase. To address this question, the cytosol fractions of mature and immature oocytes were fractionated by DEAE-Sephacel column chromatography into two fractions, the unadsorbed and adsorbed fractions. Immunoblot analysis by use of the

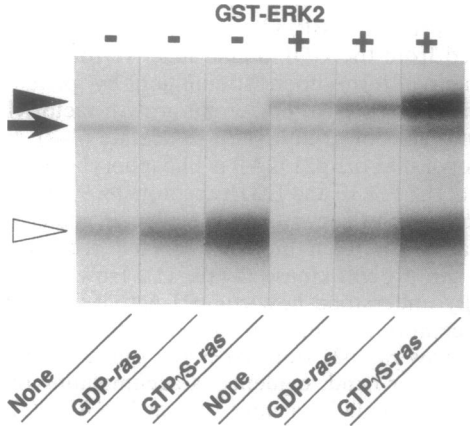

FIG. 1. Ki-*ras* p21-dependent activation of *Xenopus* MAP kinase and GST–ERK2 in the presence of the cytosol fraction of *Xenopus* oocytes. The cytosol fraction (200 μg of protein) of immature oocytes was incubated for 10 min at 30°C with the GDP-bound or GTP[γS]-bound form of Ki-*ras* p21 in the presence or absence of GST–ERK2 (500 ng of protein). The MAP kinase activity was detected by the in-gel kinase assay. Open and solid arrowheads indicate positions of *Xenopus* MAP kinase and GST–ERK2, respectively. Solid arrow indicates position of unidentified protein kinase with a M_r of ≈60,000. Results are representative of three independent experiments.

anti-MAP kinase antibody revealed that the adsorbed fractions from both cytosol fractions contained endogenous MAP kinase (data not shown). The unadsorbed fraction from the cytosol fraction of mature oocytes showed the MAP kinase kinase activity as described (18), but the unadsorbed or adsorbed fraction from the cytosol fraction of immature oocytes did not show this activity by itself (Fig. 2). The GTP[γS]-bound form of Ki-*ras* p21 activated endogenous MAP kinase and GST–ERK2 in the presence of both the unadsorbed and adsorbed fractions from the cytosol fraction of immature oocytes, but not in the presence of either one of them alone (Fig. 2). The GDP-bound form of Ki-*ras* p21 or each free nucleotide alone was almost inactive in this capacity.

When the unadsorbed fraction from the cytosol fraction of mature oocytes was subjected to gel filtration on a Superose 12 HR10/30 column, the activity of MAP kinase kinase appeared as a single peak with a M_r of ≈45,000 (Fig. 3*A*). This result is consistent with an earlier observation (18). Therefore, we regarded this activity as the active form of MAP kinase kinase. This active form of MAP kinase kinase was not further activated by addition of either the adsorbed fraction from the cytosol fraction of immature oocytes, the GTP[γS]-bound form of Ki-*ras* p21, or both (Fig. 3*A*). When the unadsorbed fraction from the cytosol fraction of immature oocytes was subjected to the same gel filtration, the activity to stimulate MAP kinase appeared as a single peak (Fig. 3*B*). This activity was dependent on the presence of both the adsorbed fraction and the GTP[γS]-bound form of Ki-*ras* p21, and depletion of either one of them decreased the activity. The elution position of this activity was apparently the same as that of the active form of MAP kinase kinase. Therefore, we regarded this activity as the inactive form of MAP kinase kinase and used it as the inactive form of MAP kinase kinase.

When the adsorbed fraction from the cytosol fraction of immature oocytes was subjected to the same gel filtration, the activity to stimulate MAP kinase appeared as a single peak with a M_r of 150,000–200,000 (Fig. 4). This activity was completely dependent on the presence of both the inactive form of MAP kinase kinase and the GTP[γS]-bound form of Ki-*ras* p21, and depletion of either one of them decreased the activity to near basal level. This activity was abolished by treatment with tryptic digestion or heat boiling for 5 min at 100°C (data not shown), indicating that this activity was most

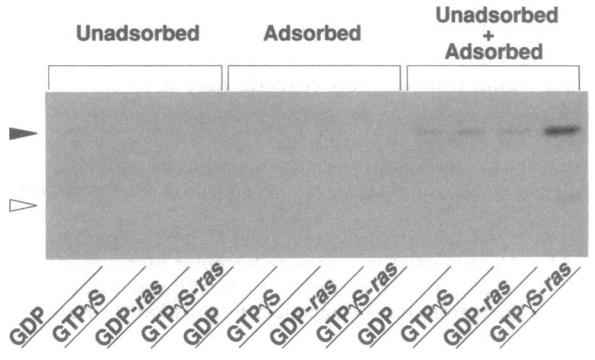

FIG. 2. Requirement of the unadsorbed and adsorbed fractions of DEAE-Sephacel column chromatography for Ki-*ras* p21 to activate *Xenopus* MAP kinase and GST–ERK2. The unadsorbed fraction (20 μg of protein) and/or the adsorbed fraction (15 μg of protein) from the cytosol fraction of immature oocytes were incubated for 10 min at 30°C with the GDP-bound or GTP[γS]-bound form of Ki-*ras* p21. GST–ERK2 (500 ng of protein) was then added and the reaction mixture was incubated for an additional 20 min at 30°C. Open and solid arrowheads indicate positions of *Xenopus* MAP kinase and GST–ERK2, respectively. The MAP kinase activity was detected by the in-gel kinase assay. Results are representative of three independent experiments.

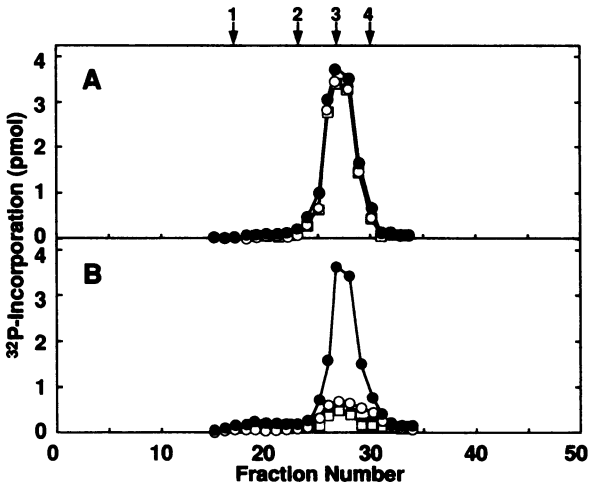

FIG. 3. Gel-filtration analysis on a Superose 12 HR10/30 column of the unadsorbed fractions from DEAE-Sephacel column chromatography. (*A*) Unadsorbed fraction from the cytosol fraction of mature oocytes. (*B*) Unadsorbed fraction from the cytosol fraction of immature oocytes. A 15-μl aliquot of each fraction from the gel filtration was incubated for 10 min at 30°C with the adsorbed fraction from the cytosol fraction (15 μg of protein) of immature oocytes and/or the GTP[γS]-bound form of Ki-*ras* p21. After addition of GST–ERK2, the reaction mixture was incubated for an additional 20 min at 30°C and then applied to the in-gel kinase assay mixture. ^{32}P incorporated into MBP by GST–ERK2 was measured. ●, Adsorbed fraction and Ki-*ras* p21; ○, adsorbed fraction alone; ■, Ki-*ras* p21 alone; □, without adsorbed fraction and Ki-*ras* p21. Elution positions of gel-filtration standards are indicated by numbers: 1, thyroglobulin (M_r, 670,000); 2, IgG (M_r, 158,000); 3, ovalbumin (M_r, 44,000); 4, myoglobin (M_r, 17,000). Results are representative of three independent experiments.

likely to be a protein molecule(s). We tentatively referred to this protein as REKS (*ras* p21-dependent ERK-kinase stimulator). The inactive form of endogenous MAP kinase, which was detected by immunoblot analysis, appeared as a single peak with a M_r of ≈40,000 and was separated from REKS on the same gel filtration (data not shown).

REKS-Dependent Activation of MAP Kinase Kinase Through Its Phosphorylation. The active form of MAP kinase kinase has been shown to be a phosphorylated protein and inactivated by treatment with phosphatase 2A (17–20). This

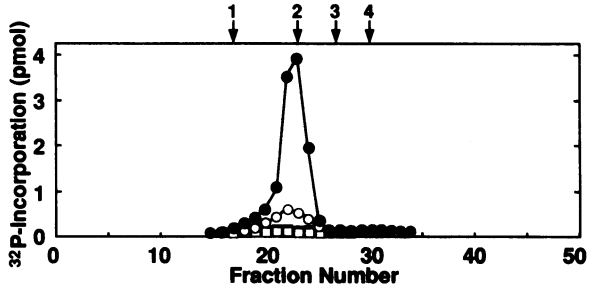

FIG. 4. Gel-filtration analysis on a Superose 12 HR10/30 column of the adsorbed fraction from the cytosol fraction of immature oocytes. A 15-μl aliquot of each fraction from the gel filtration was incubated with the inactive form of MAP kinase kinase (15 μl of fraction 27 from Fig. 3*B*) and/or the GTP[γS]-bound form of Ki-*ras* p21. After addition of GST–ERK2, the reaction mixture was incubated for an additional 20 min at 30°C and then applied to the in-gel kinase assay mixture. ●, Inactive form of MAP kinase kinase and Ki-*ras* p21; ○, inactive form of MAP kinase kinase alone; ■, Ki-*ras* p21 alone; □, without the inactive form of MAP kinase kinase and Ki-*ras* p21. Elution positions of gel-filtration standards are the same as in Fig. 3. Results are representative of three independent experiments.

indicates that MAP kinase kinase is regulated by its phosphorylation on serine/threonine residues. Therefore, we examined whether REKS activated MAP kinase kinase in a phosphorylation-dependent manner in the presence of Ki-*ras* p21. The active form of MAP kinase kinase phosphorylated and activated the inactive form of MAP kinase and GST–ERK2 (data not shown) as described (18–20). When the active form of MAP kinase kinase was treated with phosphatase 2A, it was inactivated to <50% of its original activity (Fig. 5). Okadaic acid prevented this inactivation. When the phosphatase-treated sample was incubated with REKS and the GTP[γS]-bound form of Ki-*ras* p21, the treated MAP kinase kinase was reactivated. On the other hand, REKS and the GTP[γS]-bound form of Ki-*ras* p21 did not further activate the untreated active form of MAP kinase kinase.

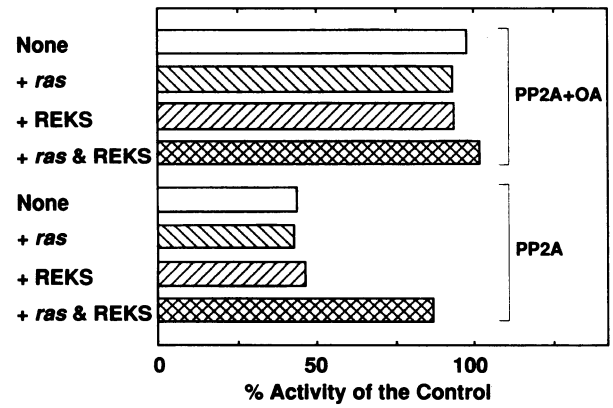

FIG. 5. REKS-dependent activation of MAP kinase kinase through its phosphorylation. The active form of MAP kinase kinase was treated with phosphatase 2A (PP2A) in the presence or absence of okadaic acid (OA) for 10 min at 30°C. Then, a 15-μl aliquot of the sample was incubated with REKS (15 μl of fraction 23 from Fig. 4) and the GTP[γS]-bound form of Ki-*ras* p21. MAP kinase activity is expressed as percentage of control activity derived from the untreated active form of MAP kinase kinase. Results are representative of three independent experiments.

DISCUSSION

We have recently found that Ki-*ras* p21 activates endogenous MAP kinase and recombinant ERK2 in the cell-free system by using the cytosol fraction of *Xenopus* oocytes (35). In this paper, to identify the factors necessary for Ki-*ras* p21 to activate MAP kinase, we first attempted to fractionate the cytosol fraction of immature oocytes by DEAE-Sephacel column chromatography into two fractions—the unadsorbed and adsorbed fractions. Both fractions are necessary for Ki-*ras* p21 to activate MAP kinase and recombinant ERK2. A factor required for MAP kinase activation in the unadsorbed fraction is identified as MAP kinase kinase. The adsorbed fraction contains another factor, which activates MAP kinase in the presence of the inactive form of MAP kinase kinase and Ki-*ras* p21. This molecule is distinct from endogenous MAP kinase and MAP kinase kinase, and its M_r value is 150,000–200,000 as estimated by gel filtration (see Fig. 4). Since it is trypsin sensitive and heat labile, it is most likely a protein molecule(s). We have tentatively referred to this protein as REKS (*ras* p21-dependent ERK-kinase stimulator).

MAP kinase is regulated by its phosphorylation on threonine and tyrosine residues by the active form of MAP kinase kinase (16–20, 32, 33). It has also been shown that MAP kinase kinase is a phosphorylated protein and that dephosphorylation of serine and/or threonine residues inactivates MAP kinase kinase (17–20). We have confirmed that treatment of MAP kinase kinase with phosphatase 2A inactivates its activity and shown that REKS and Ki-*ras* p21 reactivate the phosphatase-treated MAP kinase kinase. Taken together, it is tempting to speculate that REKS is either a *ras* p21-dependent serine/threonine kinase that phosphorylates and activates the inactive form of MAP kinase kinase or the regulator of a still unidentified protein kinase that phosphorylates and activates MAP kinase kinase. It has recently been reported that Raf-1 kinase activates MAP kinase kinase by phosphorylating it (34). Therefore, it is possible that Raf-1 kinase serves as MAP kinase kinase kinase. We cannot exclude the possibility that REKS is a complex of two or more proteins or that it is/contains Raf-1 kinase. It is obvious that *ras* p21 does not directly activate MAP kinase kinase. Thus, it is speculated that REKS contains the targeting site or the target molecule of *ras* p21. Purification of REKS remains to be done.

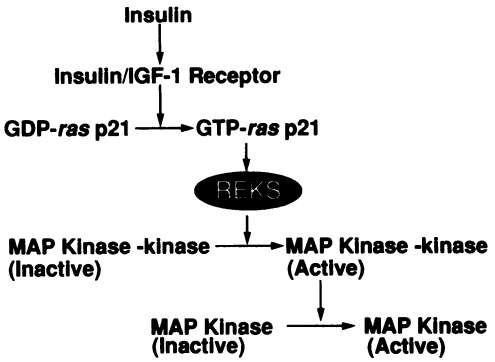

FIG. 6. Model for *ras* p21-dependent activation of MAP kinase in *Xenopus* oocytes. IGF-1, insulin-like growth factor 1.

The model depicted in Fig. 6 is one of the simplest but generally acceptable candidates to explain the signal transduction cascade from extracellular signals such as insulin to MAP kinase in *Xenopus* oocytes. According to this model, insulin induces activation of *ras* p21 through its receptor by stimulating the conversion of the GDP-bound inactive form of *ras* p21 to the GTP-bound active form. The GTP-bound form of *ras* p21 then activates REKS, which subsequently activates MAP kinase kinase. Finally, the active form of MAP kinase kinase activates MAP kinase. Since several growth factors such as PDGF, EGF, insulin, and nerve growth factor have been shown to induce the activation of *ras* p21 and to stimulate MAP kinase in mammalian cells (4–7, 12–15), a similar signal transduction cascade may also exist in mammalian cells.

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1. Barbacid, M. (1987) *Annu. Rev. Biochem.* **56**, 779–827.
2. McCormick, F. (1989) *Cell* **56**, 5–8.
3. Takai, Y., Kaibuchi, K., Kikuchi, A. & Kawata, M. (1992) *Int. Rev. Cytol.* **133**, 187–230.
4. Satoh, T., Endo, M., Nakafuku, M., Nakamura, S. & Kaziro, Y. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5993–5997.
5. Downward, J., Graves, J. D., Warne, P. H., Rayter, S. & Cantrell, D. A. (1990) *Nature (London)* **346**, 719–723.
6. Gibbs, J. B., Marshall, M. S., Scolnick, E. M., Dixon, R. A. F. & Vogel, U. S. (1990) *J. Biol. Chem.* **265**, 20437–20442.
7. Burgering, B. M. T., Medema, R. H., Maassen, J. A., van de

Wetering, M. L., van der Eb, A. J., McCormick, F. & Bos, J. L. (1991) *EMBO J.* **10**, 1103–1109.
8. Molloy, C. J., Bottaro, D. P., Fleming, T. P., Marshall, M. S., Gibbs, J. B. & Aaronson, S. A. (1989) *Nature (London)* **342**, 711–714.
9. Ellis, C., Moran, M., McCormick, F. & Pawson, T. (1990) *Nature (London)* **343**, 377–381.
10. Feig, L. A. & Cooper, G. M. (1988) *Mol. Cell. Biol.* **8**, 3235–3243.
11. Szeberényi, J., Cai, H. & Cooper, G. M. (1990) *Mol. Cell. Biol.* **10**, 5324–5332.
12. Ray, L. B. & Sturgill, T. W. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1502–1506.
13. Hoshi, M., Nishida, E. & Sakai, H. (1988) *J. Biol. Chem.* **263**, 5396–5401.
14. Gotoh, Y., Nishida, E., Yamashita, T., Hoshi, M., Kawakami, M. & Sakai, H. (1990) *Eur. J. Biochem.* **193**, 661–669.
15. Gotoh, Y., Nishida, E., Matsuda, S., Shiina, N., Kosako, H., Shiokawa, K., Akiyama, T., Ohta, K. & Sakai, H. (1991) *Nature (London)* **349**, 251–254.
16. Ahn, N. G., Seger, R., Bratlien, R. L., Diltz, C. D., Tonks, N. K. & Krebs, E. G. (1991) *J. Biol. Chem.* **266**, 4220–4227.
17. Gómez, N. & Cohen, P. (1991) *Nature (London)* **353**, 170–173.
18. Matsuda, S., Kosako, H., Takenaka, K., Moriyama, K., Sakai, H., Akiyama, T., Gotoh, Y. & Nishida, E. (1992) *EMBO J.* **11**, 973–982.
19. Nakielny, S., Cohen, P., Wu, J. & Sturgill, T. (1992) *EMBO J.* **11**, 2123–2129.
20. Seger, R., Ahn, N. G., Posada, J., Munar, E. S., Jensen, A. M., Cooper, J. A., Cobb, M. H. & Krebs, E. G. (1992) *J. Biol. Chem.* **267**, 14373–14381.
21. Leevers, S. J. & Marshall, C. J. (1992) *EMBO J.* **11**, 569–574.
22. Thomas, S. M., DeMarco, M., D'Arcangelo, G., Halegoua, S. & Brugge, J. S. (1992) *Cell* **68**, 1031–1040.
23. Wood, K. W., Sarnecki, C., Roberts, T. M. & Blenis, J. (1992) *Cell* **68**, 1041–1050.
24. de Vries-Smits, A. M. M., Burgering, B. M. T., Leevers, S. J., Marshall, C. J. & Bos, J. L. (1992) *Nature (London)* **357**, 602–604.
25. Nori, M., L'Allemain, G. & Weber, M. J. (1992) *Mol. Cell. Biol.* **12**, 936–945.
26. Pomerance, M., Schweighoffer, F., Tocque, B. & Pierre, M. (1992) *J. Biol. Chem.* **267**, 16155–16160.
27. Mizuno, T., Kaibuchi, K., Yamamoto, T., Kawamura, M., Sakoda, T., Fujioka, H., Matsuura, Y. & Takai, Y. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6442–6446.
28. Usui, H., Kinohara, N., Yoshikawa, K., Imazu, M., Imaoka, T. & Takeda, M. (1983) *J. Biol. Chem.* **258**, 10455–10463.
29. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
30. Gotoh, Y., Moriyama, K., Matsuda, S., Okumura, E., Kishimoto, T., Kawasaki, H., Suzuki, K., Yahara, I., Sakai, H. & Nishida, E. (1991) *EMBO J.* **10**, 2661–2668.
31. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
32. Anderson, N. G., Maller, J. L., Tonks, N. K. & Sturgill, T. W. (1990) *Nature (London)* **343**, 651–653.
33. Seger, R., Ahn, N. G., Boulton, T. G., Yancopoulos, G. D., Panayotatos, N., Radziejewska, E., Ericsson, L., Bratlien, R. L., Cobb, M. H. & Krebs, E. G. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6142–6146.
34. Kyriakis, J. M., App, H., Zhang, X., Banerjee, P., Brautigan, D. L., Rapp, U. R. & Avruch, J. (1992) *Nature (London)* **358**, 417–421.
35. Itoh, T., Kaibuchi, K., Masuda, T., Yamamoto, T., Matsuura, Y., Maeda, A., Shimizu, K. & Takai, Y. *J. Biol. Chem.*, in press.