THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 19, Issue of May 10, pp. 11541–11547, 1996

Printed in U.S.A.

Palmitoylation of Ha-Ras Facilitates Membrane Binding, Activation of Downstream Effectors, and Meiotic Maturation in *Xenopus* Oocytes*

(Received for publication, January 22, 1996, and in revised form, March 4, 1996)

Thomas Dudler‡ and Michael H. Gelb§

From the Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195-1700

Ras proteins serve as critical relays in signal transduction pathways that control growth and differentiation and must undergo posttranslational modifications before they become functional. While it is established that farnesylation is necessary for membrane binding and cellular functions of all Ras proteins, the significance of palmitoylation is unclear. We have studied the contribution of Ha-Ras palmitoylation for biological activity in *Xenopus* oocytes. In contrast to wild-type Ha-Ras, which binds to membranes and induces meiosis when microinjected into oocytes, a nonpalmitoylated but farnesylated and methylated mutant mislocalizes to the cytosol and fails to promote maturation. This lack of responsiveness correlates with the inability of the mutant to induce phosphorylation and activation of mitogen-activated protein kinase and maturation promoting factor, which are both strongly activated by wild-type Ha-Ras. Costimulation of oocytes with insulin increases their responsiveness to Ras and partially rescues the biological activity of the palmitoylation-resistant mutant. However, 25–50 times higher doses of mutant were required to elicit responses equivalent to wild-type Ha-Ras. These results suggest that palmitoylation and membrane association of Ha-Ras is necessary for efficient activation of the mitogen-activated protein kinase cascade *in vivo* and are consistent with a biochemical function for Ras as a membrane targeting signal for downstream effectors in this pathway.

The small guanosine nucleotide binding proteins of the *ras* gene superfamily act as molecular switches in the transduction of many extracellular signals from cell surface receptors to the nucleus and are involved in a variety of cellular processes from mitosis and differentiation to apoptosis (1, 2). Moreover, activating mutations in *ras* genes have been implicated in malignant transformation in mammals (3). Ras proteins are synthesized as cytosolic precursors and must undergo posttranslational modifications at their C termini before they become biologically functional. These modifications include farnesylation at a cysteine residue located four residues from the C terminus, followed by removal of the C-terminal tripeptide and methylation of the newly exposed C terminus (4). There is general agreement that farnesylation of Ras is indispensable for its biological functions *in vivo*. Yeast cells in which farnesylation of Ras is suppressed by pharmacological agents or by mutation are not viable (5–7). Likewise, oncogenic mammalian Ras proteins that are not farnesylated are completely nontransforming (8, 9). Since prenylated Ras proteins are predominantly localized to the cytoplasmic face of the plasma membrane, whereas the nonprenylated forms are cytosolic, prenylation of Ras has been implicated in membrane targeting where it interacts with other components of the signal transduction pathway (4, 10–15).

Some Ras proteins (Ha-Ras, N-Ras, Ras2) are further lipidated by palmitoylation at one or two cysteines near the farnesylated C terminus (7, 10). Although this modification appears to be required for high affinity binding of prenylated Ras to the plasma membrane, its biological significance remains unclear. Interestingly palmitoylation is dynamic (16), suggesting the possibility of regulation by reversible membrane-to-cytosol translocations as has been documented in the case of the α-subunit of heterotrimeric G-proteins (17). In yeast, the biological significance of Ras2 palmitoylation has been explored in detail (7). Yeast Ras2 mutants, which are farnesylated but not palmitoylated, are not localized to the plasma membrane, and a single copy of a palmitoylation-defective *RAS2* gene confers cell viability (7). Although such mutants showed no marked growth phenotype, they fail to induce a transient increase in intracellular cAMP in response to glucose addition, suggesting that nonpalmitoylated Ras2 is unable to activate adenylate cyclase, its main downstream effector in the glucose signaling pathway in yeast. In mammalian systems, earlier studies using cell lines overexpressing oncogenic Ras revealed that mutation of the palmitoylation sites of Ha-Ras caused a loss of membrane localization (8, 10). However, the mislocalized protein retains the ability to cause cell transformation as long as it is farnesylated (8). Analogous observations have been made for the polybasic domain in the hypervariable region of the K-Ras protein, which led to the interpretation that plasma membrane localization is not essential for the transforming activity of oncogenic Ras (10). This view has been challenged recently by similar transfection studies where oncogenic K-Ras was expressed at near-physiological levels in NIH-3T3 cells. The study revealed that the polybasic domain of K-Ras confers targeting to the plasma membrane and is absolutely required for cell transformation (18). The discrepancies were believed to result from different susceptibilities of particular cell clones to transformation (18).

In order to clarify the functional significance of Ha-Ras palmitoylation and membrane targeting for the activation of signal transduction cascades and biological responses *in vivo*, we have used *Xenopus laevis* oocytes as an experimental system. Oocytes are naturally arrested at the G_2-M boundary of the first meiotic cell division. When microinjected with onco-

* This work was supported by Grant CA52874 and Research Career Development Award GM562 from the National Institutes of Health (to M. H. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a postdoctoral fellowship from the Swiss National Science Foundation.

§ To whom correspondence should be addressed: Depts. of Chemistry and Biochemistry, Box 351700, University of Washington, Seattle, WA 98195-1700. Tel.: 206-543-7142; Fax: 206-685-8665.

genic Ha-Ras protein, oocytes resume meiotic maturation (19) in a process that is accompanied by activation of the mitogen-activated protein kinase (MAPK)[1] cascade (20–25) and an increase in the $p34^{cdc2}$ kinase activity of maturation promoting factor (MPF) (23). Here we describe a detailed dose-response study comparing biological properties of palmitoylated and nonpalmitoylated forms of Ha-Ras in *Xenopus* oocytes. Our results suggest an important role of Ha-Ras palmitoylation for signaling functions *in vivo*.

EXPERIMENTAL PROCEDURES

Ras Proteins—Recombinant baculovirus vectors expressing Glu-Glu-tagged wild-type (WT) human Ha-Ras and a mutant in which both palmitoylation sites (cysteine residues 181 and 184) have been mutated to serine (181/4S) have been obtained as a generous gift from E. Porfiri and J. F. Hancock (Onyx Pharmaceuticals). Non-lipidated proteins were affinity-purified from the aqueous phase of Triton X-114-partitioned Sf9 cell lysates using anti Glu-Glu mAb (kindly supplied by E. Porfiri) immobilized on protein G-Sepharose (Sigma) according to the published procedure (26). Ras protein concentrations were determined by [^{3}H]GTP binding using nitrocellulose filter binding assays as described previously (27) or by incorporation of [^{3}H]FPP catalyzed by recombinant protein farnesyltransferase (28). In this case, Ha-Ras samples (10–30 pmol) were incubated for 3 h at 30 °C with 5 pmol of recombinant protein farnesyltransferase in 30 μl of buffer containing 30 mM Tris, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 0.5 mM dithiothreitol, and 30 μM [^{3}H]FPP (DuPont NEN, 400 mCi/mmol). Reactions were terminated by boiling samples in SDS-PAGE sample buffer, and proteins were resolved on 15% polyacrylamide gels (29) and stained with Coomassie Blue. The amount of [^{3}H]farnesyl incorporated into Ras proteins was quantified by liquid scintillation counting of excised Ha-Ras bands (30).

Purified Ha-Ras proteins were loaded with GTPγS by incubation in buffer containing 20 mM Tris, pH 7.4, 10 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10–30 μM Ras, and 250 μM GTPγS (Sigma) for 20 min at 30 °C. Nucleotide exchange was stopped on ice by the addition of $MgCl_2$ to a final concentration of 25 mM, and free nucleotides were removed by repeated cycles of ultrafiltration in a Centricon-10 device (Amicon) as described previously (31). Ras proteins were finally concentrated to 1–2 mg/ml and stored at −80 °C in small aliquots in Ras buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 4 mM $MgCl_2$, 0.1 mM dithiothreitol).

Oocyte Microinjection—Oocytes were isolated from *X. laevis* females (purchased from Nasco or Xenopus I) that did not receive priming with gonadotropin. Ovaries were surgically removed from animals anesthetized with 0.25% MS-222 (Sigma), and oocytes were defoliculated by treatment with type I collagenase (Sigma). Stage VI oocytes were cultured in Barth medium (30) at 18–20 °C and allowed to recover overnight before microinjection. In experiments where oocytes were co-stimulated with Ha-Ras and suboptimal doses of insulin, the sensitivity of oocytes to insulin was determined during this overnight period, and an insulin concentration was chosen for costimulation experiments that did not induce significant maturation after overnight incubation (typically 0.25–5 μM).

Ras proteins were diluted in Ras buffer, and 50 nl was microinjected into the cytoplasm. Oocytes were kept in Barth medium and scored for germinal vesicle breakdown (GVBD) by the appearance of a white spot on the animal hemisphere. For biochemical analysis, 5–10 oocytes/group were randomly harvested and homogenized in 20 μl/oocyte of ice-cold lysis buffer (60 mM β-glycerophosphate, pH 7.4, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM NaF, 1 mM benzamidine, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). The crude lysates were either processed immediately or flash-frozen in liquid nitrogen and stored at −80 °C.

Metabolic Labeling—Farnesylation of Ha-Ras in oocytes was analyzed by coinjecting 1 pmol of Ras with 1 pmol of [^{3}H]FPP (15 Ci/mmol). After incubation for 2.5 or 24 h, groups of oocytes were homogenized in lysis buffer supplemented with 1% Triton X-100. Insoluble debris was removed by centrifugation at 10,000 × *g* for 10 min at 4 °C, and Ras was immunoprecipitated from cleared extracts using anti-Glu-Glu antibody following established procedures (30). Immunoprecipitates were resolved by SDS-PAGE, and radiolabeled proteins were visualized by fluorography on preflashed films (Kodak XAR) after treating the gels with 2,5-diphenyloxazole (Sigma) (32). Methylation was analyzed by incubating oocytes injected with 1 pmol of Ras in Barth medium supplemented with 0.4 mCi/ml of [*methyl*-^{3}H]methionine (DuPont NEN, 70 Ci/mmol). After incubation for 5 h or overnight, groups of oocytes were homogenized in lysis buffer containing 1% Triton X-100. Ras was immunoprecipitated from cleared extracts and loaded onto 15% polyacrylamide gels, and radiolabeled proteins were visualized as described above. To assess palmitoylation *in vivo*, oocytes microinjected with 1 pmol of Ha-Ras were cultured in "cold" medium for 4 h before healthy oocytes were transferred to Barth medium containing 2 mCi/ml of [^{3}H]palmitic acid (DuPont NEN, 60 Ci/mmol). 30 min after transfer, groups of oocytes were harvested and homogenized in lysis buffer containing 1% Triton X-100. Ras proteins were immunoprecipitated, and radioactivity was detected as described above.

Triton X-114 Partitioning and Oocyte Fractionation—Crude oocyte lysate (10 μl) was mixed with 100 μl of 1% Triton X-114 in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.5), and the mixture was allowed to sit on ice for 10 min with occasional mixing. After removing insoluble debris by centrifugation, the cleared lysates were phase-separated into aqueous and detergent phases as described previously (33). Both phases were adjusted to 1% Triton X-114 and 100 μl final volume, and 1/10 of each sample was subjected to immunoblotting analysis as described below. Oocytes were fractionated into cytosolic and membrane components using standard procedures (30). The membrane fraction was extracted with buffer containing 1% Triton X-100, and equal portions of cytosolic and membrane fractions were analyzed by immunoblotting.

Immunoblotting Studies—Proteins were resolved by SDS-PAGE and electroblotted onto nitrocellulose membranes (Hybond, Amersham Corp.) for 2 h at 100 V using a wet transfer system (Bio-Rad). Membranes were blocked with 5% powdered milk or 2% bovine serum albumin (phosphotyrosine blots) in TBST (Tris-buffered saline containing 0.1% Tween-20) and probed for 2 h with antibody against phosphotyrosine (PY 20) at 2 μg/ml, anti-Ras mAb at 0.25 μg/ml, anti-MAPK (anti-ERK2) mAb at 100 ng/ml (all from Transduction Laboratories, Lexington, KY) or anti-Glu-Glu mAb (gift from E. Porfiri) at 100 ng/ml diluted in blocking buffer. Immunoreactive bands were revealed with peroxidase-labeled sheep antibody against mouse IgG using the ECL detection system (Amersham Corp.).

Kinase Assays—Kinase activities were measured in cleared extracts after removing insoluble debris by centrifugation at 12,000 × *g* for 15 min at 4 °C. MAPK activity was measured using myelin basic protein (Sigma) as substrate (34). Aliquots of oocyte extracts (0.25 oocyte equivalent) were incubated for 20 min at 30 °C in a final volume of 30 μl containing 25 mM β-glycerophosphate, pH 7.4, 9 mM $MgCl_2$, 2 mM EGTA, 0.5 mM dithiothreitol, 100 μM [γ-^{32}P]ATP (DuPont NEN, 0.3–0.6 Ci/mmol), 1 mM Na_3VO_4, 30 μM Calmidazolium (Sigma), and 2 μM heat-stable inhibitor of cAMP-dependent protein kinase (Sigma) in the presence or absence of 0.34 mg/ml myelin basic protein. Assays were stopped by spotting 25 μl of the reaction mixture onto P-81 cellulose phosphate paper (Life Technologies, Inc.). After washing 5 times with 150 mM phosphoric acid and once with ethanol, the papers were dried, and radioactivity was measured by scintillation counting. The activity of MPF was measured by incubating oocyte extracts with 1 mg/ml of histones (Sigma type III-S from calf thymus) as substrate in 20 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 5 mM mercaptoethanol, 100 μM [γ-^{32}P]ATP (1 Ci/mmol), and 2 μM heat stable inhibitor of cAMP-dependent protein kinase. After incubation for 15 min at 30 °C, reactions were terminated by the addition of one-quarter volume of 5 × SDS sample buffer, and proteins were separated on 15% polyacrylamide gels. Gels were fixed, dried, and developed by autoradiography. Histone phosphorylation was quantified after excision of the H1 histone band from the gel followed by scintillation counting.

RESULTS

Like progesterone, the physiological stimulus for oocyte maturation, oncogenic Ha-Ras (Val-12), has been shown to activate the MAPK cascade (21, 22) and to induce maturation in *Xenopus* oocytes (19). To analyze the significance of Ha-Ras palmitoylation for biological activity and activation of downstream effectors *in vivo*, GTPγS-bound forms of WT Ha-Ras and the mutant 181/4S in which the palmitoylated cysteines have been substituted with serine were microinjected into oocytes.

[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; MPF, maturation promoting factor; WT Ha-Ras, nononcogenic human Ha-Ras (Gly-12) with wild-type C terminus; Ha-Ras 181/4S, nononcogenic human Ha-Ras (Gly-12) with cysteine residues 181 and 184 mutated to serine; FPP, farnesyl pyrophosphate; PAGE, polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; GVBD, germinal vesicle breakdown.

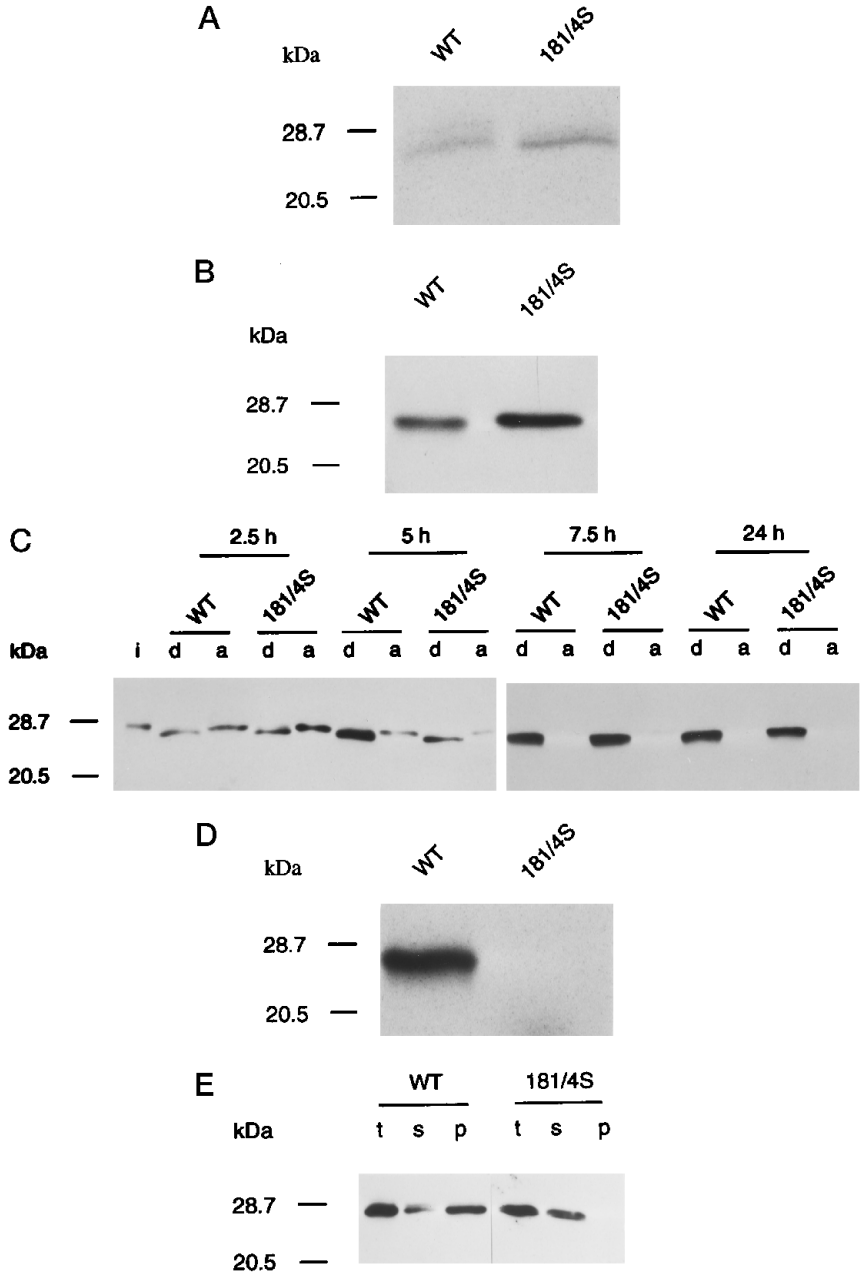

FIG. 1. **Posttranslational processing and cellular localization of WT Ha-Ras and 181/4S in *Xenopus* oocytes.** *A*, oocytes were coinjected with 1 pmol of [^{3}H]FPP and WT or mutant Ha-Ras. After a 2.5-h incubation in Barth medium, oocytes were lysed, and Ras proteins were immunoprecipitated, resolved on 15% polyacrylamide gels, and analyzed by fluorography. The exposure time was 14 days. *B*, oocytes injected with 1 pmol of WT Ha-Ras or 181/4S were labeled in Barth medium containing [*methyl*-^{3}H]methionine. After overnight culture, oocytes were lysed and Ras proteins were immunoprecipitated and analyzed by SDS-PAGE followed by fluorography. The exposure time was 4 days. 5800 (*WT*) and 8400 (*181/4S*) cpm were released as [^{3}H]methanol if the excised Ras gel slices were treated with 2 M NaOH (51). If oocytes were analyzed after a 5-h labeling period, 1900 and 2150 cpm were released from WT and mutant Ha-Ras, respectively. *C*, oocytes microinjected with 0.5 pmol of WT Ha-Ras or 181/4S were lysed 2.5, 5, 7.5, or 24 h after injection and subjected to Triton X-114 partitioning. Equal portions of detergent (*d*) or aqueous (*a*) phase were resolved by SDS-PAGE (15%), and Ras proteins were detected by immunoblotting with anti-Ras mAb as described under "Experimental Procedures." *i*, Ha-Ras before injection (purified from the aqueous phase of Sf9 cells). Similar results were obtained with anti-Glu-Glu antibody (not shown). *D*, mutant- or WT-injected oocytes were cultured in cold medium for 4 h. After a 30-min pulse in medium containing 2 mCi/ml [^{3}H]palmitic acid, oocytes were lysed, Ras was immunoprecipitated, loaded onto polyacrylamide gels (15%), and visualized by fluorography. The exposure time was 14 days. Immunoblot analysis showed that comparable amounts were immunoprecipitated (not shown) *E*, 7.5 h after microinjection with WT Ha-Ras or 181/4S, oocytes were fractionated into cytosolic (*s*) and particulate (*p*) fractions. Total cell lysate (*t*) and equivalent portions of *s* and *p* fractions were resolved by 15% SDS-PAGE and analyzed by immunoblotting with anti-Ras mAb.

Epitope-tagged, nononcogenic (Gly-12) WT Ha-Ras and its 181/4S mutant were purified in the nonprocessed form from Sf9 cells and were activated by loading with GTPγS, a nonhydrolyzable GTP analogue. *In vitro* farnesylation followed by SDS-PAGE analysis resulted in complete conversion of WT and mutant Ras proteins into the processed, faster migrating form (10, 27), and approximately 1 mol of [^{3}H]farnesyl/mol of Ras (estimated by [^{3}H]GTP binding) was incorporated into both proteins (data not shown). Thus, the C-terminal prenylation sequence, which is indispensable for farnesylation and subsequent processing steps, is functional in these Ras preparations.

Fig. 1 shows that mutation of cysteines 181 and 184 prevents palmitoylation and membrane localization but does not affect farnesylation, C-terminal proteolysis, and methylation of Ha-Ras in *Xenopus* oocytes. [^{3}H]FPP, the farnesyl donor for Ha-Ras prenylation in *Xenopus* oocytes (35), is readily incorporated into microinjected Ha-Ras proteins (Fig. 1*A*), indicating that WT and mutant Ha-Ras are comparably good substrates for the protein farnesyltransferase present in *Xenopus* oocytes. Similarly, WT Ha-Ras and 181/4S incorporate alkali-labile [*methyl*-^{3}H]methionine with comparable kinetics (Fig. 1*B*), indicating that both proteins are equally proteolyzed and carboxymethylated in oocytes. Farnesylation is also accompanied by an increase in electrophoretic mobility and hydrophobicity, which can be monitored by Triton X-114 phase-partitioning (10, 33). Immunoblot analysis of phase-partitioned oocytes microinjected with WT Ha-Ras or 181/4S shows that both proteins undergo farnesylation with comparable kinetics (Fig. 1*C*). Both proteins show a time-dependent shift from the hydrophilic, slower migrating, microinjected form to the more hydrophobic, faster migrating form with a half-life of about 2.5–5 h. After 7.5 h, farnesylation is essentially complete, and WT Ha-Ras and 181/4S are stable under these conditions for at least 24 h. Thus, as in the case of *ras*-transfected COS cells (10), mutation of the Ha-Ras palmitoylation sites does not noticeably affect the other processing steps in *Xenopus* oocytes. When oocytes microinjected with WT Ha-Ras or 181/4S were labeled with [^{3}H]palmitate, WT but not mutant Ha-Ras incorporated palmitate (Fig. 1*D*), suggesting that the same cysteine residues of Ha-Ras are palmitoylated in *Xenopus* oocytes as those identi-

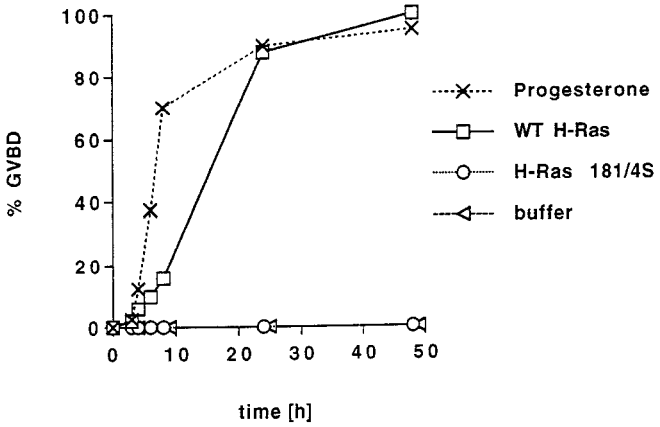

FIG. 2. **Kinetics of oocyte maturation induced by WT Ha-Ras and the 181/4S mutant.** Stage VI oocytes were either treated with progesterone (10 μM) as a positive control or microinjected with 1 pmol of WT Ha-Ras, nonpalmitoylated mutant 181/4S, or buffer alone and incubated in Barth medium at 18–20 °C. After the indicated time periods, oocytes were monitored for GVBD. Results are expressed as a percentage of the number of injected oocytes (30–40/group).

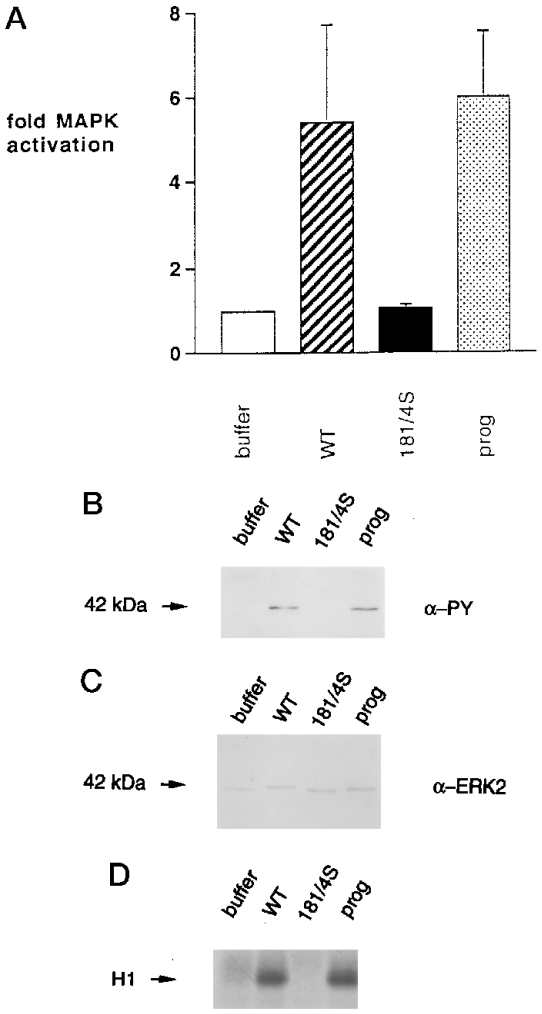

FIG. 3. **Ha-Ras 181/4S fails to activate MAPK and MPF in *Xenopus* oocytes.** Oocytes were microinjected or treated with progesterone (*prog*) as described in the legend to Fig. 2. After 4–24 h of culture in Barth medium, groups of 5–10 oocytes were randomly harvested, and cleared extracts were prepared. *A*, MAPK activity was measured by incorporation of ^{32}P into myelin basic protein (34). The -fold MAPK activation represents the cpm of Ras-injected/cpm of buffer-injected oocytes, after subtracting the minus substrate control from each data point. The mean ± S.D. from six independent experiments is shown. *B*, oocyte extracts (0.1 oocyte equivalent/lane) were separated by SDS-PAGE (10%), transferred onto nitrocellulose, and probed with antibody to phosphotyrosine (α-PY). Immunoreactive bands were visualized by ECL. *C*, the same blot was stripped and reprobed with antibody to MAPK (*α-ERK2*). *D*, MPF activity was measured by incorporation of ^{32}P into histone H1 substrate as described under "Experimental Procedures." After terminating reactions by boiling for 5 min in SDS sample buffer, reaction mixtures were separated by SDS-PAGE, and H1 phosphorylation was analyzed by autoradiography. The *arrow* indicates the position of the H1 band.

fied in COS cells (10). Thus, the processing enzymes present in *Xenopus* oocytes are functionally comparable with their mammalian counterparts. Analysis of the cellular localization of WT Ha-Ras or 181/4S by oocyte fractionation shows that 7.5 h after microinjection, about 80% of WT Ha-Ras is associated with the membrane compartment, whereas 181/4S remains entirely cytosolic (Fig. 1*E*). This pattern did not change significantly for the subsequent 24-h incubation period (not shown), suggesting that the cellular distribution has reached steady state.

A typical time-course of oocyte maturation following microinjection of 1 pmol of WT Ha-Ras or palmitoylation-site mutant 181/4S is shown in Fig. 2. While WT Ha-Ras induces near 100% maturation after a 24-h incubation period, no significant GVBD was detected in oocytes injected with 181/4S after 48 h. This lack of biologic activity of 181/4S could not be overcome by larger doses (up to 2.5 pmol) and extended culture periods (up to 72 h) in several experiments using oocytes from different frogs.

It has been shown that oncogenic Ha-Ras activates MAPK in *Xenopus* oocytes (20, 21) and that MAPK activation is necessary to mediate Ras-induced GVBD (25, 36). Thus, we investigated whether the inability of Ha-Ras 181/4S to induce oocyte maturation was associated with a lack of activation of components of the MAPK cascade. Fig. 3 shows that MAPK activity in extracts from oocytes injected with WT Ha-Ras is increased by 4–8-fold and reaches levels comparable with those measured in progesterone-treated oocytes. In marked contrast, MAPK activity in oocytes injected with 181/4S remained at control levels. MAPK activation was also evident in anti-phosphotyrosine immunoblots revealing a prominent band of ~42 kDa in WT-injected or progesterone-treated oocyte extracts, which is absent in 181/4S- or buffer-injected cells (Fig. 3*B*). This tyrosine phosphorylation was paralleled by the characteristic electrophoretic mobility shift of MAPK in anti-ERK2 immunoblots (Fig. 3*C*). Oncogenic Ha-Ras has also been shown to induce activation of MPF (23, 24), a cell cycle regulatory element that controls the G_2-M transition in eukaryotic cells and in *Xenopus* oocytes (37, 38). When the histone kinase activity associated with MPF was measured in these extracts, oocytes microinjected with WT Ha-Ras or treated with progesterone exhibited increased MPF activity, whereas 181/4S-injected cells remained at control levels (Fig. 3*D*). Taken together, these results suggest that activation of the MAPK cascade, the histone kinase activity of MPF, and the initiation of meiosis in *Xenopus* oocytes by Ha-Ras require palmitoylation.

Since the apparent lack of biological activity of 181/4S might be due to a low sensitivity of oocytes to Ras-mediated signals, Ras-injected oocytes were costimulated with insulin in a second series of experiments. Insulin has been shown to induce oocyte maturation (39) possibly by a Ras-mediated pathway (40) and was found to synergize with oncogenic Ha-Ras in the induction of oocyte maturation (41). Oocytes were microinjected with WT or mutant Ha-Ras, cultured for 8 h to allow completion of posttranslational processing, and then transferred into fresh medium supplemented with suboptimal doses of insulin. Costimulation of Ha-Ras-injected oocytes by insulin accelerated GVBD and greatly increased their sensitivity to Ras. The time

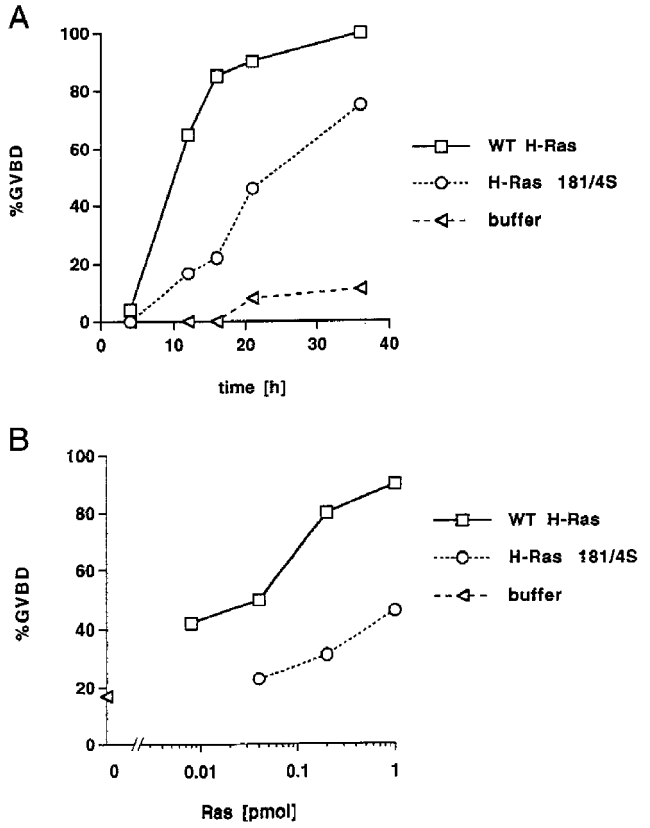

FIG. 4. **Insulin enhances the responsiveness of oocytes to Ha-Ras and partially rescues the biological activity of 181/4S.** *A*, kinetics. Oocytes microinjected with 0.5 pmol of WT Ha-Ras, 0.5 pmol of 181/4S mutant or buffer alone were cultured for 8 h in Barth medium and then transferred to medium supplemented with 5 μM insulin. Incubation was continued at 18–20 °C, and the percentage of GVBD at indicated time points was determined as in Fig. 2. *B*, dose response. Oocytes (30–40/group) were microinjected with WT Ha-Ras or 181/4S serially diluted in buffer and costimulated with suboptimal insulin concentrations as described above. After an overnight incubation, oocytes were scored for GVBD. After shorter incubation periods or when lower concentrations of insulin were used, only WT Ha-Ras at doses of 0.2–1 pmol induced maturation (not shown).

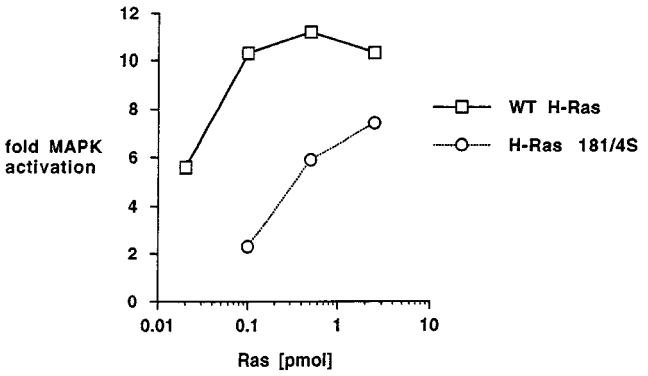

FIG. 5. **Activation of MAPK in oocytes synergistically stimulated with WT Ha-Ras or 181/4S and insulin.** Oocytes were microinjected and cultured as in Fig. 4*B*. After overnight incubation in Barth medium containing 5 μM insulin, groups of 5–10 oocytes were randomly selected and MAPK activity was measured in cleared extract. MAPK activity is expressed relative to the activity in control extracts (oocytes injected with buffer alone and treated with insulin), which was set as 1.

course of maturation (Fig. 4*A*) shows that Ha-Ras 181/4S can also promote oocyte maturation under these conditions, whereas insulin treatment on its own exhibits only marginal effects. Maturation induced by Ha-Ras 181/4S, however, proceeds with slower kinetics, and the time required to achieve 50% maturation was typically 2–3 times longer than for WT-injected cells. Insulin also substantially reduced the minimal dose of Ras needed to accelerate oocyte maturation. Fig. 4*B* shows the maturation of oocytes microinjected with various amounts of WT Ha-Ras or 181/4S and costimulated with insulin. Both WT and mutant Ha-Ras accelerated GVBD in a dose-dependent fashion, and significant maturation could be observed with as little as 10 fmol of WT Ha-Ras. However, the amount of 181/4S required to induce the same extent of maturation as WT was 25–50-fold higher. Although the sensitivity of oocytes to Ras- and insulin-induced GVBD varied considerably between different batches of oocytes, comparable differences in biological activity between WT and mutant Ha-Ras were obtained with oocytes obtained from different females.

The effect of synergistic stimulation of oocytes by various doses of Ha-Ras proteins and insulin on MAPK activity is shown in Fig. 5. Both WT Ha-Ras and 181/4S induced a dose-dependent rise in MAPK activity at a time when insulin treatment alone showed no measurable effect. However, about a 25 times higher dose of mutant Ha-Ras was needed to achieve the same degree of MAPK activation. MAPK activity in these extracts correlated with tyrosine phosphorylation of a 42-kDa protein and a reduction of the electrophoretic mobility of endogenous $p42^{ERK2}$ as revealed by immunoblot analysis (data not shown). Taken together these results show that the inability of nonpalmitoylated Ha-Ras to activate MAPK and induce meiosis in oocytes can be partially rescued by costimulation with insulin. Even under these conditions, however, about 25 times higher doses of mutant Ha-Ras are required to activate downstream effectors and induce biological responses in *Xenopus* oocytes.

DISCUSSION

We have analyzed the functional significance of Ha-Ras palmitoylation for signal transduction *in vivo* using the *Xenopus* oocyte system. Our results show that while WT Ha-Ras is predominantly membrane-bound and promotes activation of MAPK and meiotic maturation when microinjected into stage VI oocytes, a nonpalmitoylated but farnesylated and methylated mutant remains cytosolic and is inactive under these conditions (Fig. 2). This result would suggest that, like a C-terminal truncation mutant of oncogenic Ras1 that lacks all posttranslational modifications (42), the farnesylated but nonpalmitoylated mutant 181/4S is biologically inactive in *Xenopus* oocytes. These two mutants are both cytosolic, however, their biological properties are distinctly different if oocytes are costimulated with a second agonist. While the C-terminal truncation mutant of oncogenic Ras1 was shown to inhibit maturation induced by insulin-like growth factor (42), the nonpalmitoylated but farnesylated mutant 181/4S accelerates maturation if oocytes are costimulated with insulin (Fig. 4). Under these conditions, 25–50-fold higher doses of 181/4S are required to elicit responses comparable to its WT counterpart (Fig. 4*B*). These results suggest that for the induction of meiotic maturation of *Xenopus* oocytes by Ha-Ras, farnesylation alone converts an antagonist into a partial agonist, whereas farnesylation together with palmitoylation is required to achieve full biological activity. Interestingly, a C-terminally truncated form of oncogenic Ras1 in which a farnesylation site was added also showed partial biological activity in *Xenopus* oocytes (42), suggesting that this concept might also apply to other Ras proteins.

The induction of oocyte maturation correlates with phosphorylation and activation of MAPK regardless of the combination of stimuli (Figs. 2, 3, 4*B*, and 5), which is in agreement with the

pivotal role of the MAPK cascade in meiotic cell division in *Xenopus* oocytes (24, 36, 43–45). Another kinase that becomes activated during oocyte maturation is MPF, a complex of cyclin B and $p34^{cdc2}$ kinase, which controls entry of cells into mitosis (37, 38). Consistent with this functional role, MPF activity closely correlates with MAPK activity and the induction of maturation (Fig. 3*D* and not shown), further corroborating the differences in biological activity between WT Ha-Ras and 181/4S.

These results can be interpreted to mean that Ha-Ras has to be palmitoylated and consequently membrane-localized in order to efficiently activate downstream effectors. This would support a proposed biochemical function for Ras in recruiting effector molecules to the plasma membrane where they undergo activation (13–15). The Raf-1 kinase is a candidate for such an effector molecule, and translocation of Raf from the cytosol to the plasma membrane either by activated Ras or by the addition of a membrane targeting signal to Raf results in phosphorylation, activation, and stable association with the membrane compartment (13, 14, 46).

In *Xenopus* oocytes, Raf appears to be an important downstream effector in the Ras-induced activation of MAPK and meiotic maturation. Raf becomes phosphorylated during meiotic maturation induced by Ras (47), and kinase-defective Raf blocks Ras-induced maturation and activation of MAPK (22). Since Raf has to be localized to the plasma membrane for activation, our observation that nonpalmitoylated, cytosolic Ha-Ras is a poor activator of MAPK is consistent with the proposed role of Raf as an intermediate in the Ras-MAPK cascade in *Xenopus* oocytes.

A novel Ras-dependent activator of the MAPK cascade termed REKS has recently been purified from *Xenopus* oocytes (48). *In vitro*, REKS is activated by posttranslationally processed Ras in the absence of membrane components (49), and farnesylation but not palmitoylation of Ha-Ras is required for this activity (27). In light of the present study showing that farnesylated but nonpalmitoylated Ha-Ras is a poor activator of MAPK, REKS appears to play a minor role in the Ras-mediated MAPK activation *in vivo*. However, the biological activity of 181/4S detected in oocytes costimulated with insulin might be mediated by REKS. Alternatively it is also possible that small amounts of nonpalmitoylated Ha-Ras not detectable by cell fractionation are present in the membrane compartment and are responsible for the residual biological activity of 181/4S.

Our findings also reveal a striking similarity with studies in yeast. Ras is essential for viability in yeast, and farnesylation is required for viability if Ras is expressed at physiological levels (5, 7). Recent studies have shown that yeast cells containing a single copy of a *RAS2* gene defective in palmitoylation are viable but fail to activate adenylate cyclase, its presumed downstream effector in yeast (7). Thus, Ras proteins that are farnesylated but not localized to membranes appear to lack the ability to couple to some effector molecules, while they can still perform essential functions. Since farnesylation of yeast Ras has been shown to enhance interaction between Ras and adenylate cyclase *in vitro* (50), it has been suggested that distinct cytosolic and membrane-bound forms of adenylate cyclase might be involved in cell viability and glucose response (7).

In mammalian cells, previous studies by Hancock and coworkers (8) using NIH-3T3 cells transfected with oncogenic *ras* genes indicated that membrane localization might not be important for cell transformation. In the case of K-Ras, which is not palmitoylated but contains a string of polybasic residues required for membrane binding, this view has been challenged recently. Performing similar transfection studies, Jackson and co-workers (18) found that cytosol-localized K-Ras, even when farnesylated, was completely nontransforming (18); these discrepancies have been ascribed to differences in sensitivity of the particular cell clones used (18). Although the induction of meiosis in naturally G_2-arrested oocytes is not directly comparable with transformation of mammalian cell lines by Ras, our findings would suggest that palmitoylation and membrane binding might also play an important role in cell transformation by oncogenic Ha-Ras. In light of the discrepancies found with K-Ras, this issue should be addressed in future studies.

Acknowledgments—We thank Drs. E. Porfiri and J. F. Hancock (Onyx pharmaceuticals) for providing recombinant baculovirus expression constructs and anti-Glu-Glu antibody, the laboratory of Professor E. G. Krebs (University of Washington) for technical assistance to set up MAPK assays, and the laboratory of Professor W. A. Caterall (University of Washington) for technical support and helpful advice for oocyte microinjections. We also thank Dr. P. McGeady for discussions and helpful suggestions.

REFERENCES

1. Hersowitz, I. (1995) *Cell* **80,** 187–197
2. Casey, P. J. (1995) *Science* **268,** 221–225
3. Lowry, D. R., and Willumsen, B. M. (1993) *Annu. Rev.. Biochem.* **62,** 851–891
4. Schafer, W. R., and Rine, J. (1992) *Annu. Rev.. Genet.* **30,** 206–237
5. Deschenes, R. J., and Broach, J. R. (1987) *Mol. Cell. Biol.* **7,** 2344–2351
6. Schafer, W. R., Trueblood, C. E., Yang, C.-C., Mayer, M. P., Rosenberg, S., Poulter, C. D., Kim, S. -H, and Rine, J. (1990) *Science* **249,** 1133–1139
7. Bhattacharya, S., Chen, L., Broach, J. R., and Powers, S. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 2984–2988
8. Hancock, J. F., Paterson, H., and Marshall, C. J. (1990) *Cell* **63,** 133–139
9. Jackson, J. H., Cochrane, C. G., Bourne, J. R., Solski, P. A., Buss, J. E., and Der, C. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3042–3046
10. Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989) *Cell* **57,** 1167–1177
11. Clarke, S. (1992) *Annu. Rev. Biochem.* **61,** 355–386
12. Shimizu, K., Kuroda, S., Yamamori, B., Matsuda, S., Kaibuchi, K., Yamauchi, T., Isobe, T., Ire, K., Masumoto, K., and Takai, Y. (1994) *J. Biol. Chem.* **269,** 22917–22920
13. Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M., and Hancock, J. F. (1994) *Science* **264,** 1463–1467
14. Levers, S. J., Paterson, H. F., and Marshall, C. J. (1994) *Nature* **369,** 411–414
15. Hall, A. (1994) *Science* **264,** 1413–1414
16. Magee, A. I., Gutierrez, L., McKay, I. A., Marshall, C. J., and Hall, A. (1987) *EMBO J.* **6,** 3353–3357
17. Wedegaertner, P. B., and Bourne, H. R. (1994) *Cell* **77,** 1063–1070
18. Jackson, J. H., Li, J. W., Buss, J. E., Der, C. J., and Cochrane, C. G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12730–12734
19. Birchmeier, C., Broek, D., and Wigler, M. (1985) *Cell* **43,** 615–621
20. Hattori, S., Fukuda, M., Yamashita, T., Grotoh, Y., and Nishida, E. (1992) *J. Biol. Chem.* **267,** 20346–20351
21. Pomerance, M., Schweighoffer, F., Tocque, B., and Pierre, M. (1992) *J. Biol. Chem.* **267,** 16155–16160
22. Fabian, J. R., Morrison, D. K., and Daar, I. O. (1993) *J. Cell. Biochem.* **122,** 645–652
23. Barrett, C. B., Schroetke, R. M., Hoorn, F. A., Nordeen, S. K., and Maller, J. L. (1990) *Mol. Cell. Biol.* **10,** 310–315
24. Nebreda, A. R., Porras, A., and Santos, E. (1993) *Oncogene* **8,** 467–477
25. Fukoto, M., Grotoh, Y., Kosako, H., Hattori, S., and Nishida, E. (1994) *J. Biol. Chem.* **269,** 33097–33101
26. Porfiri, E., Evans, T., Bollag, G., Clark, R., and Hancock, J. F. (1995) *Methods Enzymol.* **255,** 13–20
27. McGeady, P., Kuroda, S., Shimizu, K., Takai, Y., and Gelb, M. H. (1995) *J. Biol. Chem.* **270,** 26347–26351
28. Chen, W. J., Moomaw, J. F., Overton, L., Kost, T. A., and Casey, P. J. (1993) *J. Biol. Chem.* **268,** 9675–9680
29. Laemmli, U. K. (1970) *Nature* **227,** 680–685
30. Celis, J. E. (1994) *Cell Biology: A Laboratory Manual*, Academic Press, San Diego, CA
31. Vogel, U. S., Dixon, R. A. F., Schaber, M. D., Diehl, R. E., Marshall, M. S., Scolnick, E. M., Sigal, I. S., and Gibbs, J. B. (1988) *Nature* **335,** 90–93
32. Magee, A. I., Wootton, J., and De Bony, J. (1995) *Methods Enzymol.* **250,** 330–336
33. Gutierrez, L., Magee, A. I., Marshall, C. J., and Hancock, J. F. (1989) *EMBO J.* **8,** 1093–1098
34. Ahn, N. G., Weiel, J. E., Chan, C. P., and Krebs, E. G. (1990) *J. Biol. Chem.* **265,** 11487–11494
35. Kim, R., Rine, J., and Kim, S.-H. (1990) *Mol. Cell. Biol.* **10,** 5945–5949
36. Carnero, A., Jimenez, B., and Lacal, J. C. (1994) *J. Cell Biochem.* **55,** 465–476
37. Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J. L. (1988) *Cell* **54,** 433–439
38. Gautier, J., Matsukawa, T., Nurse, P., and Maller, J. (1989) *Nature* **399,** 626–629
39. Maller, J. L., and Koontz, J. W. (1981) *Dev. Biol.* **85,** 309–318
40. Korn, L. J., Siebel, C. W., McCormick, F., and Roth, R. A. (1987) *Science* **236,** 840–842
41. Chuang, L.-M., Hausdorff, S. F., Myers, M. G., White, M. F., Birnbaum, M. J., and Kahn, R. C. (1994) *J. Biol. Chem.* **269,** 27645–27649

42. Gibbs, J. B., Schaber, M., Schofield, T. L., Scolnick, E. M., and Sigal, I. S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 6630–6634
43. Kosado, H., Grotoh, Y., and Nishida, E. (1994) *EMBO J.* **13,** 2131–2138
44. Grotoh, Y., Masuyama, N., Dell, K., Shirakabe, K., and Nishida, E. (1995) *J. Biol. Chem.* **270,** 25898–25904
45. Haccard, O., Lewellyn, A., Hartley, R. S., Erikson, E., and Maller, J. L. (1995) *Dev. Biol.* **168,** 677–682
46. Marais, R., Light, Y., Paterson, H. F., and Marshall, C. J. (1995) *EMBO J.* **14,** 3136–3145
47. Galaktionov, K., Jessus, C., and Beach, D. (1995) *Genes & Dev.* **9,** 1046–1058
48. Kuroda, S., Shimizu, K., Yamamori, B., Matsuda, S., Imazumi, S., Kaibuchi, K., and Takai, Y. (1995) *J. Biol. Chem.* **270,** 2460–2465
49. Itoh, T., Kaibuchi, K., Masuda, T., Yamamoto, T., Matsura, Y., Maeda, A., Shimizu, K., and Takai, Y. (1993) *J. Biol. Chem.* **268,** 3025–3028
50. Kuroda, Y., Luzuki, N., and Kataoka, T. (1993) *Science* **259,** 683–685
51. Stephenson, R. C., and Clarke, S. (1990) *J. Biol. Chem.* **265,** 16248–16254