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Role of Phosphatidylinositide Metabolism in *ras*-Induced *Xenopus* Oocyte Maturation

BIN-TAO PAN AND GEOFFREY M. COOPER*

Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

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Microinjection of *Xenopus* oocytes with *ras* protein (p21) was used to investigate the role of phospholipid metabolism in *ras*-induced meiotic maturation. Induction of meiosis by *ras* was compared with induction by progesterone, insulin, and the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Neomycin, which specifically binds to phosphatidylinositides and inhibits their metabolism, blocked meiotic maturation induced by *ras* or insulin but not by progesterone or TPA. In addition, p21 and TPA, but not insulin or progesterone, stimulated the incorporation of $^{32}P_i$ into oocyte lipids. *ras* protein specifically stimulated ^{32}P incorporation into phosphatidylinositides, whereas both *ras* and TPA stimulated ^{32}P incorporation into phosphatidylcholine and phosphatidylethanolamine. The stimulatory effect of p21 on phosphatidylinositide metabolism correlated with the dose response and kinetics of *ras*-induced meiotic maturation. In addition, the *ras* oncogene protein was more potent than the proto-oncogene protein both in inducing meiotic maturation and in stimulating phosphatidylinositide metabolism. These results indicate that phosphatidylinositide turnover is required for *ras*-induced meiosis and suggest that phosphatidylinositide-derived second messengers mediate the biological activity of *ras* in *Xenopus* oocytes.

The *ras* proto-oncogene family is highly conserved in evolution, being present in organisms ranging from yeasts to humans (see reference 2 for a review). In mammals, this gene family consists of three members that encode closely related proteins of 21,000 daltons, designated p21s. *ras* oncogenes activated by point mutations are found in a significant fraction of human and carcinogen-induced animal neoplasms, indicating their frequent contribution to tumor development (2). In addition, *ras* proto-oncogenes appear to be involved in the normal proliferation of both yeast (22, 40) and mammalian (15, 31) cells and may also function in cell differentiation (3, 21, 33).

The *ras* genes encode plasma membrane proteins that bind GTP and GDP with high affinity and possess GTP hydrolysis activity (2). Most mutations that activate *ras* transforming potential result in single-amino-acid substitutions that increase the fraction of p21 in the GTP-bound state, which appears to be the physiologically active form (2). The regulation of p21 by guanine nucleotide binding is similar to the regulation of G proteins (20), suggesting that *ras* proteins function as signal-transducing molecules that regulate the metabolism of intracellular second messengers.

In *Saccharomyces cerevisiae*, *ras* proteins stimulate the activity of adenylate cyclase (8, 42). In vertebrate cells, however, adenylate cyclase does not appear to be affected by p21 (5), and the physiologically relevant target(s) for *ras* function has not been established. However, alterations in phosphatidylinositol (PI) metabolism and increased levels of diacylglycerol (DAG) have been observed in *ras*-transformed cells, suggesting the possibility that p21 regulates the turnover of phospholipid-derived second messengers (17, 25, 35, 44, 47). In the well-characterized PI pathway, phosphatidylinositol 4,5-bisphosphate (PIP_2) is hydrolyzed by phospholipase C to yield two second messengers, DAG and inositol 1,4,5-triphosphate (IP_3). DAG activates protein kinase C, and IP_3 mediates the release of calcium from intracellular stores (6, 32). The activity of this second-messenger system is closely correlated with cell proliferation, since PIP_2 hydrolysis is stimulated by a variety of growth factors and direct activation of protein kinase C by phorbol ester analogs of DAG is mitogenic (29, 32). It is not clear, however, whether the reported alterations of PI and DAG metabolism in *ras*-transformed cells are a primary or secondary result of p21 action.

We have used microinjection of *Xenopus* oocytes as a model to investigate *ras* function in a vertebrate cell. These oocytes, which are arrested in the first meiotic prophase, can be induced to resume meiosis by exposure to progesterone or insulin (4, 30). Treatment of *Xenopus* oocytes with progesterone, the natural inducer of meiosis, leads to inhibition of adenylate cyclase, and the resultant decrease in intracellular cyclic AMP levels triggers meiotic maturation (36). However, it appears that stimulation of the PI pathway can also result in resumption of meiosis, since meiotic maturation can be induced by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (40). Since microinjection of activated *ras* proteins also induces meiotic maturation of *Xenopus* oocytes (7), this system seemed to provide a useful model in which the function of *ras* could be compared with that of other inducers of meiotic maturation with respect to alterations in second-messenger metabolism. We report here that p21 specifically stimulates PI metabolism in microinjected oocytes and that PI turnover is a specific requirement for meiotic maturation induced by p21. These results implicate the PI second-messenger system as a mediator of *ras* action in *Xenopus* oocytes.

MATERIALS AND METHODS

Preparation of *rasH* proteins. Proteins encoded by the normal human *rasH* proto-oncogene and a human *rasH* oncogene activated by the substitution of leucine for glutamine at codon 61 were expressed in bacteria as previously described (16). The insoluble pellet containing p21 was dissolved in 7 M urea in buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2), 1 mM dithiothreitol, 0.1 mM $MgCl_2$, 50 mM NaCl,

* Corresponding author.

and 1% trasylol. The urea was then removed by dialysis against the same buffer followed by gel filtration through a Sephadex G-150 column. All p21 preparations used for microinjection were >95% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Oocyte microinjection and maturation. *Xenopus laevis* frogs were purchased from Nasco and primed with 25 to 50 IU of pregnant mare serum gonadotropin (Sigma Chemical Co.) 1 to 2 weeks before isolation of oocytes. The frogs were anesthetized in ice-cold water, and ovary fragments were surgically removed and placed in modified Barth medium (110 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM HEPES [pH 7.8]). Oocytes were recovered by manual dissection, and stage VI oocytes (14) were selected for assay. For analysis of *ras*-induced maturation, oocytes were microinjected in the cytoplasm with 50 to 100 nl of p21 in the buffer described above. For analysis of hormonally induced maturation, oocytes were incubated in the presence of progesterone (10 μM), insulin (50 μg/ml), or TPA (0.1 μg/ml). Oocytes were cultured at room temperature in modified Barth medium, and germinal vesicle (nuclear) breakdown (GVBD) was assessed by the appearance of a white spot in the animal pole (46). In some cases, nuclear breakdown was confirmed by dissection of oocytes that had been fixed in 10% trichloracetic acid.

Analysis of $^{32}P_i$ incorporation into lipids. Oocytes were incubated with $^{32}P_i$ (9,000 Ci/mmol; 10 to 20 μCi/ml) and washed in fresh medium, and groups of four to six oocytes were homogenized in 0.8 ml of 10 mM HEPES (pH 7.5)–1 mM dithiothreitol–1 mM ethylene glycol-bis(β-amino-ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)–0.1 mM vanadate–1% leupeptin. HCl (1 N; 100 μl) was added to the homogenate, followed by 3 ml of chloroform-methanol (2:1, vol/vol) and 0.1 ml of 2 M KCl (37). The chloroform phase was saved, and 0.2 ml was used to quantitate ^{32}P incorporation into total lipids by scintillation counting. For thin-layer chromatography, 0.2-ml samples of the organic phase were dried under nitrogen and applied to silica gel 60 thin-layer chromatography plates (Sigma) that had been impregnated with 1% potassium oxalate and activated at 110°C for 15 min. Solvent A (chloroform-methanol-acetic acid-water, 75:45:12:3) was used to resolve phosphatidylcholine (PC), phosphatidylethanolamine (PE), and PI, and solvent B (chloroform-acetone-methanol-acetic acid-water, 160:60:52:48:32) was used to resolve phosphatidylinositol 4-phosphate (PIP) and PIP_2 (11, 38, 41). [^{14}C]PI, [^{14}C]PC, and [^{14}C]PE (Du Pont, NEN Research Products) and unlabeled PIP and PIP_2 (Sigma) were used as markers. After chromatography, the plates were subjected to autoradiography.

RESULTS

Induction of oocyte maturation by p21, hormones, and TPA. We initially characterized the kinetics and dose dependence of *Xenopus* oocyte maturation induced by *ras* proteins to provide a framework for biochemical analyses. The time course of meiotic maturation after microinjection of p21 is compared with that observed after exposure of oocytes to progesterone, insulin, or TPA in Fig. 1A. As reported by Stith and Maller (39), TPA as well as insulin and progesterone induced GVBD in all treated oocytes. Oncogene p21 (c-*rasH* 61-leu), but not proto-oncogene p21, also induced GVBD in all oocytes by 15 h after microinjection. As reported by Birchmeier et al. (7), induction of meiotic maturation by microinjected p21 was 3 to 4 h slower than induction by insulin or progesterone. This time lag appeared

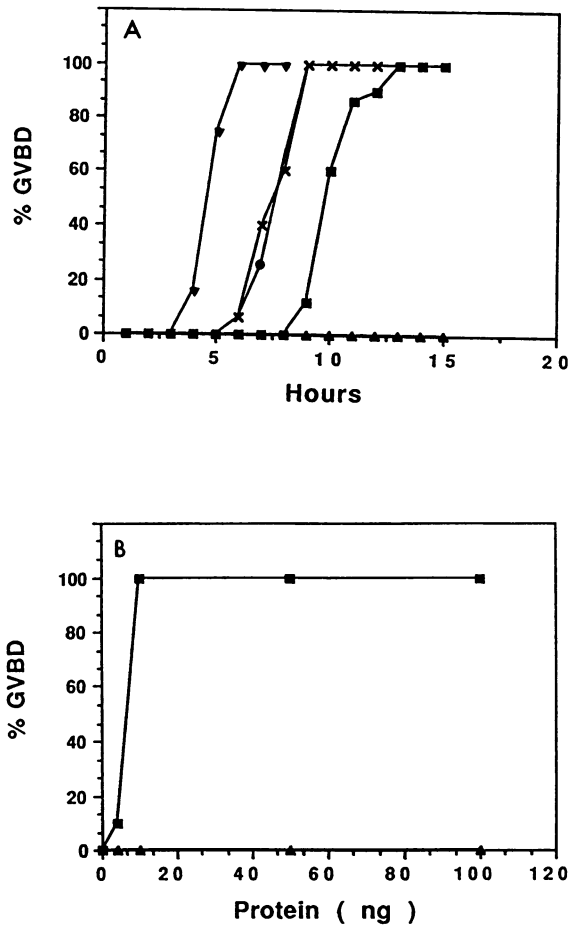

FIG. 1. Induction of oocyte maturation by p21, hormones, and TPA. (A) Groups of 10 to 15 oocytes were incubated with 10 μM progesterone (X), 50 μg of insulin per ml (●), or 0.1 μg of TPA per ml (▼) or microinjected with 100 ng of oncogene (61-leu) (■) or proto-oncogene (▲) p21. Oocytes were incubated at room temperature, and GVBD was assessed at the indicated times by the appearance of a white spot in the animal pole. (B) Oocytes were microinjected with the indicated amounts of either oncogene (■) or proto-oncogene (▲) p21. GVBD was scored after 18 h of incubation.

to correspond to the posttranslational processing of microinjected bacterially expressed p21 required for membrane localization and biological activity of *ras* proteins (7). To confirm these kinetics of processing, we microinjected radiolabeled p21 and determined the time required for the protein to become membrane associated. Consistent with the results of Birchmeier et al. (7) and with the lag time between progesterone- and p21-induced GVBD (Fig. 1A), membrane-bound p21 was first detected approximately 3 h after microinjection (data not shown).

Microinjection of ca. 10 ng of oncogene 61-leu p21 was sufficient to induce GVBD in all microinjected oocytes, and GVBD in ca. 10% of oocytes was induced by 2 ng of oncogene p21 (Fig. 1B). In contrast, no activity was observed following microinjection of up to 100 ng of proto-oncogene p21 (Fig. 1). The biological activities of the oncogene- and proto-oncogene-encoded proteins thus differed by >50 fold.

Inhibition of p21-induced GVBD by neomycin. Induction of *Xenopus* oocyte maturation by the natural inducer progesterone apparently results from the inhibition of adenylate

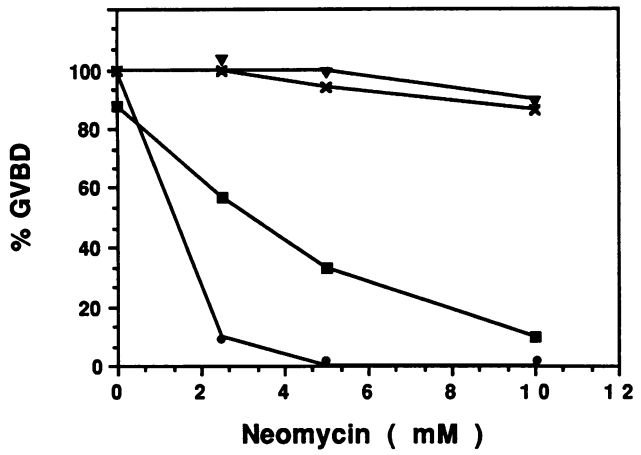

FIG. 2. Inhibition of oocyte maturation by neomycin. Oocytes were incubated in the indicated concentrations of neomycin starting 15 min before exposure to progesterone (X), insulin (●), or TPA (▼) or microinjection of 100 ng of oncogene p21 (■). Neomycin was present throughout maturation, and GVBD was assessed after 18 h.

cyclase (36). However, the ability of TPA to induce GVBD indicates that activation of protein kinase C can also trigger meiotic maturation. Since microinjection of *ras* proteins does not appear to affect cyclic AMP metabolism (7; our unpublished observations) but has been reported to result in increased PI turnover (24), we investigated the possible role of PI-derived second messengers in the induction of maturation by p21. Since neomycin specifically binds to PI derivatives and inhibits the hydrolysis of PIP_2 to DAG and IP_3 (26, 38), we analyzed the effect of neomycin to determine whether PI turnover was required for p21 induced oocyte maturation.

Neomycin effectively inhibited the induction of GVBD by p21 (Fig. 2). The concentration range of neomycin that was active in these experiments (1 to 10 mM) is similar to that found to specifically inhibit PI turnover in other systems (9, 10, 13). As reported previously (39), neomycin also inhibited the induction of GVBD by insulin but not by progesterone (Fig. 2). In addition, neomycin did not inhibit the induction of GVBD by TPA (Fig. 2). Neomycin therefore specifically inhibited the induction of GVBD by *ras* and insulin, rather than being a nonspecific inhibitor of the maturation process. The lack of effect of neomycin on GVBD induced by progesterone and TPA is consistent with their acting to inhibit adenylate cyclase and directly activate protein kinase C, respectively, since PI turnover would then not be an expected requirement for intracellular transmission of either progesterone- or TPA-initiated signals. In contrast, the specific inhibitory effect of neomycin indicates that the PI second-messenger pathway is required for transmission of intracellular signals initiated by *ras* and insulin.

Stimulation of phospholipid metabolism by p21. To directly analyze the effect of p21 on phospholipid metabolism, we assayed the incorporation of $^{32}P_i$ into lipids during oocyte maturation (Fig. 3). ^{32}P incorporation into the lipid fraction was significantly increased (two- to fivefold) in oocytes that were microinjected with the *ras* oncogene protein compared

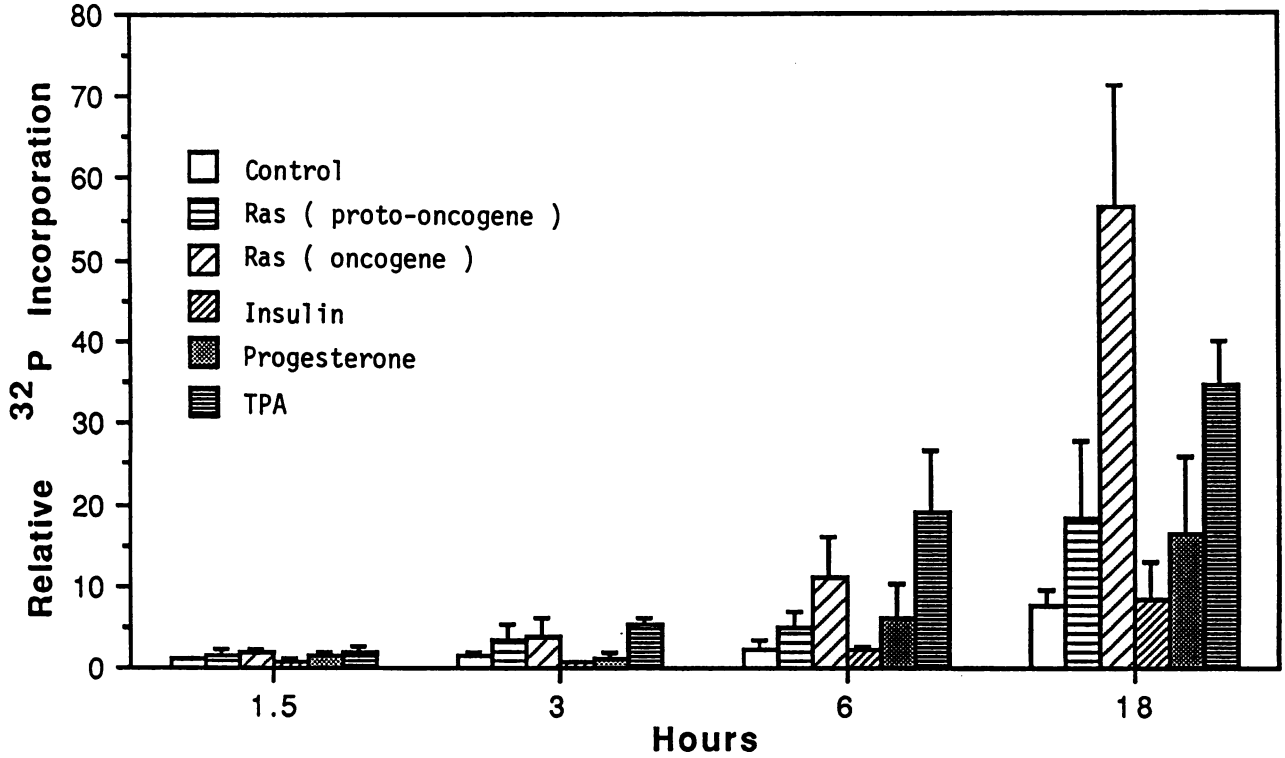

FIG. 3. Incorporation of $^{32}P_i$ into lipid. Untreated control oocytes, oocytes incubated with progesterone, insulin, or TPA, and oocytes microinjected with 100 ng of oncogene or proto-oncogene p21 were incubated with $^{32}P_i$ (20 μCi/ml) for the indicated times. Groups of four oocytes were then washed, and lipids were extracted and counted as described in Materials and Methods. The data are presented relative to ^{32}P incorporation into control oocyte lipids at 1.5 h and represent the average of three or four independent experiments. The actual incorporations at 18 h in a representative experiment were as follows: control, 1,830 cpm; progesterone, 1,940 cpm; insulin, 1,610 cpm; TPA, 9,040 cpm; proto-oncogene p21, 3,480 cpm; oncogene p21, 8,220 cpm.

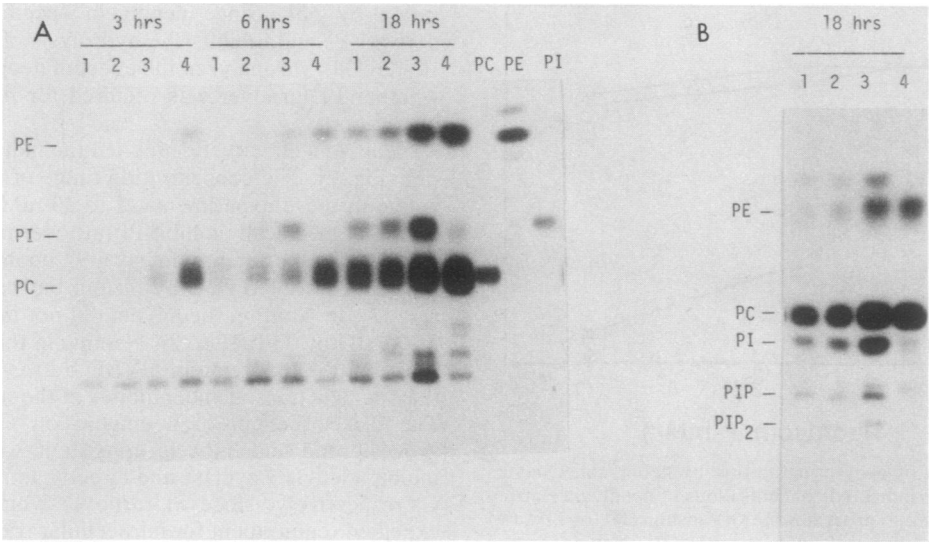

FIG. 4. Thin-layer chromatography of ^{32}P-labeled lipids. Oocytes were labeled with $^{32}P_i$, and lipids were extracted as described in the legend to Fig. 3. (A) Lipids from control oocytes (lane 1), proto-oncogene p21-injected oocytes (lane 2), oncogene p21-injected oocytes (lane 3), and TPA-treated oocytes (lane 4) extracted at 3, 6, and 18 h after initiation of maturation were analyzed by thin-layer chromatography in solvent A as described in Materials and Methods to resolve PI, PC, and PE. (B) An 18-h sample was also analyzed by using solvent B to resolve PIP and PIP_2.

with oocytes microinjected with buffer or with the *ras* proto-oncogene protein. This increased ^{32}P incorporation was first detected 6 h after microinjection and persisted up to 18 h, when maturation of the oncogene p21-injected oocytes was complete. Oocytes that were induced to mature by treatment with progesterone or insulin did not display a similar increase in ^{32}P incorporation, so the stimulation induced by p21 was not simply a reflection of alterations in phospholipid metabolism during oocyte maturation. Treatment of oocytes with TPA, however, resulted in an increase in ^{32}P incorporation into lipid similar to that observed following microinjection of p21. Thus, both p21 and TPA appeared to specifically stimulate phospholipid metabolism.

To determine whether the stimulation of ^{32}P incorporation into lipid by p21 and TPA represented an effect on ^{32}P uptake or incorporation into the ATP pool, we quantitated [^{32}P]ATP in control oocytes, in oocytes that were treated with progesterone, insulin, or TPA, and in oocytes that were microinjected with buffer, proto-oncogene p21, or oncogene p21. Neither the total pool of ATP (assayed by luciferase bioluminescence) nor the amount of [^{32}P]ATP (assayed by autoradiography after thin-layer chromatography on polyethyleneimine plates [43]) differed significantly between these groups of oocytes at 3, 6, and 18 h after microinjection and ^{32}P labeling. Increased incorporation of ^{32}P into lipids thus appeared to represent a specific effect on phospholipid metabolism.

Thin-layer chromatographic analysis of the ^{32}P-labeled phospholipids from representative samples is shown in Fig. 4. Three major phospholipids comigrating with PI, PC, and PE were resolved by using the solvent system shown in Fig. 4A. Compared with control oocytes and oocytes microinjected with proto-oncogene p21, TPA stimulated ^{32}P incorporation into PC and PE, but not into PI. In contrast, oncogene p21 stimulated ^{32}P incorporation into PI as well as into PC and PE. Increased PI labeling was first detectable 3 h after p21 microinjection and increased during the next 3 h, consistent with the lag time resulting from posttranslational modification and membrane localization of microinjected p21 discussed above. Increased labeling of PI continued for at least 18 h, perhaps suggesting a persistent interaction of microinjected p21 with its substrate. Consistent with the results presented in Fig. 3, no significant change in phospholipid labeling was observed in progesterone- or insulin-treated oocytes compared with controls (data not shown). Thus, PI metabolism was specifically stimulated by oncogene p21, whereas both oncogene p21 and TPA stimulated the metabolism of PC and PE.

Further analysis of ^{32}P-labeled lipids by using a different solvent system to resolve PI, PIP, and PIP_2 was performed; the results are presented in Fig. 4B. The incorporation of ^{32}P into PIP and PIP_2, in addition to PI, is increased by oncogene p21, indicating that the *ras* oncogene protein specifically stimulates metabolism of all three of these PI derivatives.

To further establish the specificity of p21-stimulated ^{32}P incorporation into phospholipids, we coinjected anti-*ras* monoclonal antibody Y13-259 (18) to neutralize the activity of p21. Coinjection of Y13-259, but not control rat immunoglobulin G, completely blocked the induction of GVBD by p21, whereas Y13-259 had no effect on the induction of GVBD by progesterone or insulin (data not shown). In addition, Y13-259 specifically blocked p21 stimulation of phospholipid metabolism (Fig. 5).

To correlate the stimulation of phospholipid biosynthesis with the biological activity of p21, we compared the effects of various doses of *ras* oncogene and proto-oncogene proteins. Near-maximal stimulation of ^{32}P incorporation was induced by 20 ng of oncogene p21 (Fig. 6), which induced GVBD in 100% of injected oocytes (Fig. 1B). Partial stimulation of ^{32}P incorporation was induced by 4 ng of oncogene p21 (Fig. 6), which induced GVBD in only a fraction of injected oocytes (Fig. 1B and data not shown). The dose responses for induction of GVBD and stimulation of phospholipid metabolism by oncogene p21 were therefore well correlated. Partial stimulation of ^{32}P incorporation into PC, PE, and PI was observed only after microinjection of 100 ng of proto-oncogene p21 (Fig. 6), a dose that was still insufficient to induce meiosis (Fig. 1). Interestingly, no increase in

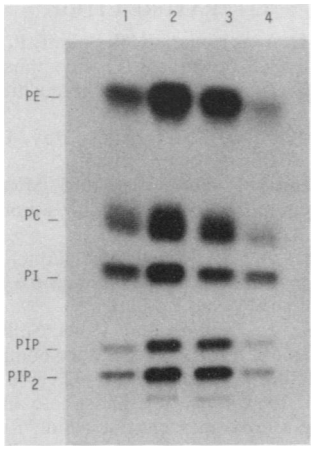

FIG. 5. Neutralization of *ras*-stimulated phospholipid turnover by anti-p21 antibody. Oocytes were microinjected with 100 ng of proto-oncogene p21 (lane 1), 100 ng of oncogene p21 (lane 2), 100 ng of oncogene p21 plus 1.2 μg of control rat IgG (lane 3), or 100 ng of oncogene p21 plus 1.2 μg of anti-*ras* monoclonal antibody Y13-259 (lane 4). ^{32}P-labeled lipids were extracted 18 h after microinjection and analyzed by thin-layer chromatography in solvent B. GVBD was induced in all oocytes microinjected with oncogene p21 with or without control rat immunoglobulin G but in none of the oocytes microinjected with proto-oncogene p21 or oncogene p21 plus Y13-259.

labeled PIP or PIP_2 was detected after injection of the proto-oncogene protein, whereas ^{32}P labeling of these PI derivatives was significantly increased by doses of the oncogene protein that induced GVBD (Fig. 6B). Stimulation of PIP and PIP_2 metabolism thus appeared to correlate closely with the biological activity of p21 in inducing oocyte maturation.

DISCUSSION

A number of previous studies have demonstrated alterations of PI and DAG metabolism in *ras*-transformed somatic cells, but these effects have not been directly related to the primary site of action of p21 in inducing a biological response (17, 25, 35, 44, 47). Moreover, it has been reported that normal *ras* function is required for induction of mitosis by phorbol esters and phospholipid-derived second messengers, suggesting that *ras* proteins act downstream of phospholipid turnover in a mitogenic signal transduction pathway (48). Our present studies of the activity of p21 in inducing *Xenopus* oocyte maturation, however, indicate that PI metabolism is necessary for *ras*-induced meiosis and suggest a direct correlation between biological response and stimulation of PI turnover. This conclusion is based on the specific inhibition of *ras*-induced GVBD by neomycin in addition to the specific stimulatory effect of microinjected *ras* protein on PI metabolism.

Neomycin binds to and inhibits the metabolism of PI derivatives (26, 38) and has therefore been used to study the role of PI turnover in a variety of systems (9, 10, 13, 36). Neomycin inhibition of *ras* action in *Xenopus* oocytes was neither a consequence of nonspecific toxicity nor a reflection of a general requirement for PI turnover in meiotic maturation, since neomycin did not inhibit GVBD induced by progesterone or TPA. These results therefore imply a direct and specific role of PI-derived second messengers in transmission of the p21-initiated signal leading to resumption of meiosis.

Stimulation of PI metabolism by microinjected p21 was demonstrated directly by assaying the incorporation of $^{32}P_i$ into phospholipids. Biosynthesis of phosphatidylinositides was stimulated by p21, but not by progesterone, insulin, or TPA, indicating that this is a specific effect of *ras* action rather than a general consequence of meiotic maturation. Oncogene p21 was much more potent than proto-oncogene p21 in inducing both GVBD and ^{32}P incorporation into phospholipid, and the dose of p21 required to induce GVBD correlated well with that required to stimulate ^{32}P incorporation into PIP and PIP_2. This increase in phospholipid metabolism was first detected ca. 3 h after p21 microinjection, corresponding to the time required for posttranslational modification and membrane localization of p21. These re-

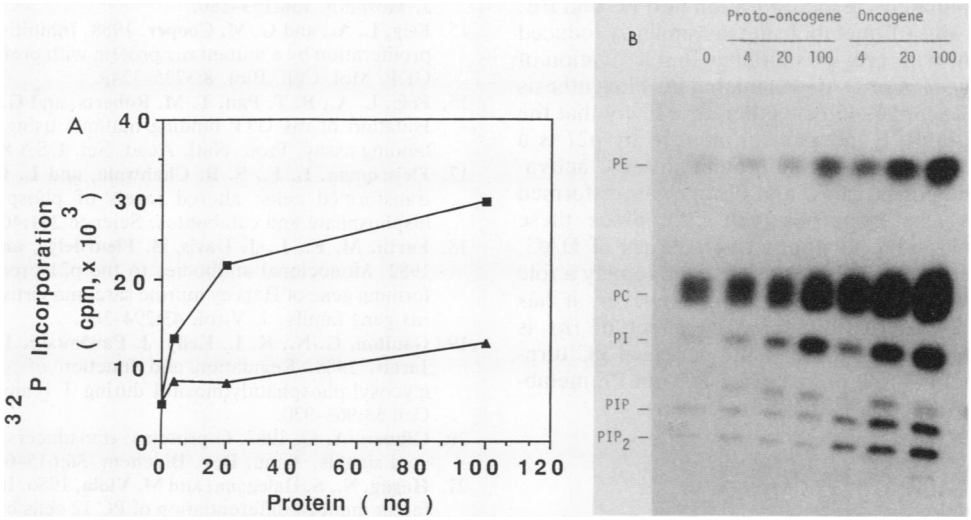

FIG. 6. Dose response of *ras*-stimulated phospholipid turnover. Oocytes were microinjected with the indicated amounts (4, 20, and 100 ng) of either proto-oncogene (▲) or oncogene (■) p21 and labeled for 18 h with $^{32}P_i$. Lipids were extracted, counted to determine total ^{32}P incorporation (A), and analyzed by thin-layer chromatography in solvent B (B). In this experiment, GVBD was induced in all oocytes microinjected with 20 or 100 ng of oncogene p21 but in none of the oocytes microinjected with proto-oncogene p21 or with 4 ng of oncogene p21.

sults therefore suggest that stimulation of PI turnover is an early and direct event in the signal transduction cascade initiated by membrane bound p21.

A previous study reported increased levels of PIP_2, DAG, and inositol phosphates in *Xenopus* oocytes microinjected with p21 (24). In contrast to our experiments, however, these alterations were detected by Lacal et al. (24) within 20 min of microinjection. Since this is much shorter than the several hours required for posttranslational modification and membrane association of microinjected p21 (7) (see above), such rapid effects may reflect activity of unmodified p21. Nonetheless, the stimulation of PI metabolism observed in our studies is consistent with these results.

The inhibition of insulin induced GVBD by neomycin (36) (see above) suggests the involvement of PI metabolism in transduction of insulin- as well as *ras*-initiated signals. This is consistent with the finding that insulin stimulates hydrolysis of PI-containing glycolipids in a variety of systems; this hydrolysis may play a role in signal transduction (11, 19, 27). The finding that insulin did not stimulate ^{32}P incorporation into oocyte phospholipids may reflect a transient response to insulin, compared with a sustained response to microinjected p21. In addition, the PI-glycan that is hydrolyzed in response to insulin represents only a portion of the total PI pool.

The step in PI metabolism that is affected by p21 was not identified in the present experiments. We have been unable to detect alterations in the levels of either DAG or inositol phosphates following p21 microinjection, perhaps due to the rapid metabolism of these compounds. It is possible that increased PI turnover reflects p21 stimulation of phospholipase C, resulting in PIP_2 hydrolysis and increased activity of the PI cycle. Alternatively, p21 might stimulate one or more enzymes involved in biosynthesis of the phosphatidylinositides, thereby regulating the availability of PI substrates for hydrolysis. For example, both *src* and the platelet-derived growth factor receptor tyrosine kinases are associated with novel PI kinases that phosphorylate the 3-position of the inositol moiety, generating distinct phosphatidylinositides with potentially novel second-messenger activities (1).

In addition to stimulating PI biosynthesis, microinjection of *ras* proteins stimulated ^{32}P incorporation into PC and PE. Stimulation of PC and PE metabolism was similarly induced by TPA, consistent with previous findings that activation of protein kinase C by TPA or DAG stimulated PC biosynthesis in somatic cells (12, 23, 34, 45). It is therefore likely that the stimulation of PC and PE metabolism induced by p21 is a secondary result of PI turnover and protein kinase C activation. Increased metabolism of PC and PE in *ras*-transformed somatic cells has also been observed (25). Since these phospholipids could serve as alternative sources of DAG, their increased metabolism has been suggested to play a role in *ras*-mediated signal transduction (25). However, it has recently been reported that the primary effect of *ras* is activation of choline kinase rather than increased PC turnover (28). The potential role of increased PC and PE metabolism in signal transduction is therefore unclear.

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