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Activation of Intracellular Kinases in *Xenopus* Oocytes by p21^{ras} and Phospholipases: a Comparative Study

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Signal transduction induced by generations of second messengers from membrane phospholipids is a major regulatory mechanism in the control of cell proliferation. Indeed, oncogenic p21^{ras} alters the intracellular levels of phospholipid metabolites in both mammalian cells and *Xenopus* oocytes. However, it is still controversial whether this alteration it is biologically significant. We have analyzed the *ras*-induced signal transduction pathway in *Xenopus* oocytes and have correlated its mechanism of activation with that of the three most relevant phospholipases (PLs). After microinjection, *ras*-p21 induces a rapid PLD activation followed by a late PLA_2 activation. By contrast, phosphatidylcholine-specific PLC was not activated under similar conditions. When each of these PLs was studied for its ability to activate intracellular signalling kinases, all of them were found to activate maturation-promoting factor efficiently. However, only PLD was able to activate MAP kinase and S6 kinase II, a similar pattern to that induced by p21^{ras} proteins. Thus, the comparison of activated enzymes after microinjection of p21^{ras} or PLs indicated that only PLD microinjection mimetized p21^{ras} signalling. Finally, inhibition of the endogenous PLD activity by neomycin substantially reduced the biological activity of p21^{ras}. All these results suggest that PLD activation may constitute a relevant step in *ras*-induced germinal vesicle breakdown in *Xenopus* oocytes.

The *ras* family is highly conserved in evolution, being present in organisms ranging from yeasts to humans (5). Ras proteins activated by point mutations are found in a significant fraction of human and carcinogen-induced animal tumors (2, 27). A number of previous studies have demonstrated alterations in the phospholipid metabolism induced by p21^{ras} proteins (reviewed in reference 22). Ras-transformed cells show a significant increase in the basal levels of diacylglycerol (DAG) and phosphorylcholine (PCho) (21). We have recently shown that these metabolites are generated by a complex pathway involving phospholipase D (PLD) activation followed by choline kinase and phosphatidic acid (PA) hydrolase (6). Furthermore, no significant activation of a PC-specific PLC (PC-PLC) was observed. While a constitutively activated PC-PLD enzyme and a twofold increase in the basal levels of PA were observed in *ras*-transformed cells, very small alterations of these parameters were detected at late times after serum stimulation of quiescent cells (6). Therefore, cell proliferation induced by *ras* oncogenes in fibroblasts may be functionally linked to activation of a PC-PLD enzyme.

Cell proliferation in eukaryotic cells is triggered by events initiated at the plasma membrane that control reentry into the cell cycle. The subsequent biochemical pathways activated actually direct the process of cell division itself. Both of these aspects of cell growth regulation can be studied in *Xenopus* oocytes undergoing meiotic maturation in response to mitogenic stimulation by hormones and mitogens (18, 31). *Xenopus* oocytes are arrested at the G_2/M border of the first meiotic prophase. Several signals can induce reentry into the cell cycle, which can be followed by the activation of different kinases, used as biochemical markers of meiotic maturation. Several inducers of oocyte maturation can activate a preexisting factor, the pre-MPF (maturation-promoting factor) which is an heterodimer of cyclin B and the p34^{cdc2} protein kinase. Activation of pre-MPF promotes reentry into the cell cycle (reviewed in references 18, 31, and 37). As a consequence of MPF activation, there is a burst of phosphorylation of intracellular substrates 30 to 60 min before the germinal vesicle breakdown (GVBD). After the stimulation with hormones, MAP kinase (MAP K) is activated during the M-phase transition under the control of MPF, and it may constitute part of the kinase cascade downstream of MPF (13, 15, 31). However, while p21^{ras} induces MAP K activation without MPF activation (8, 34), MAP K activation still seems to be necessary for GVBD induced by progesterone and p21^{ras} (8, 20). Moreover, p42^{MAPK} phosphorylates and activates S6 kinase II (KII) (48), the *Xenopus* homolog of mammalian pp90^{rsk} (11). Thus, MPF, MAP K, and S6 KII can be used as biochemical markers for Ras and PL pathways in *Xenopus* oocytes.

Phospholipids play an important role in the control of cellular responses such as secretion, platelet aggregation, macrophage function, and fertilization (4, 36, 40). They also play an important role in signal transduction, since generation of second messengers from membrane phospholipids is a major regulatory mechanism in control of cell proliferation. We have reported that in the *Xenopus laevis* oocytes model, microinjection of the three most relevant types of PLs acting on membrane phospholipids (PLA_2, PLC, and PLD) are capable of inducing oocyte maturation (9). This effect is mediated by the generation of known second messengers such as lysophospholipids, arachidonic acid, DAG, and PA. Furthermore, using specific inhibitors of protein kinase C (PKC), we have identified alternative independent signalling pathways for the induction of oocyte maturation. Our results indicate that while PLC seems to be dependent on PKC, PLA_2 and PLD are completely independent of PKC function (9). In this study, we further investigated the functional relationship between p21^{ras} and PLs in the *X. laevis* oocyte system. Our results suggest that PLD is an important component of the *ras* signalling pathway in this system, in keeping with our previous findings that *ras*

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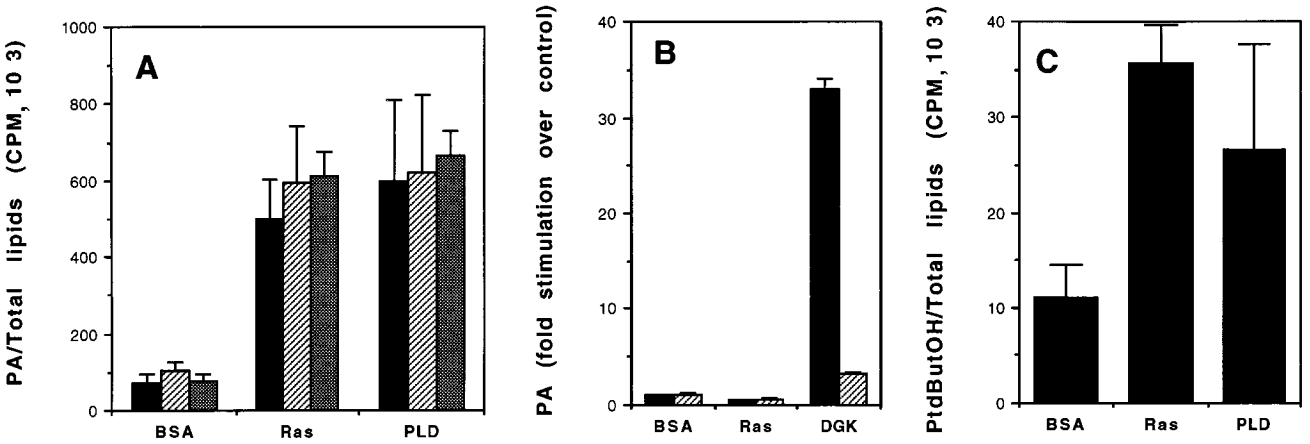

FIG. 1. Activation of PLD by p21^{ras}. (A) Oocytes were prepared as described in Materials and Methods. After 18 h of incubation with 0.5 mCi of $^{32}P_i$ per ml at room temperature, oocytes were washed and incubated for 2 h in the presence of 10 μM GF109203X (▨), 100 μM (final concentration) R59022 (▨), or buffer alone (■). Oocytes were further given microinjections of 50 ng of purified p21^{ras} or BSA or 25 μU of PLD. After 1 h of incubation, PA was analyzed as described in Materials and Methods. (B) Oocytes were given microinjections of 100 μM R59022 (▨) or buffer alone (■). After 2 h of incubation, the oocytes were given microinjections of 25 nCi of [α-^{32}P]ATP per oocyte and 50 ng of BSA or p21^{ras} per oocyte or 25 nl of purified DGK. After 30 min of incubation at room temperature, the oocytes were processed for PA production as described in Materials and Methods. (C) Oocytes were incubated for 18 h with 0.5 mCi of $^{32}P_i$ per ml at room temperature, washed, given microinjections of 50 ng of purified p21^{ras} or BSA, and incubated for 1 h in Ringer's buffer containing 0.5% *n*-butanol. The phosphatidylbutanol produced was analyzed as described in Materials and Methods. Data represent the average of triplicate samples (10 oocytes per sample) ± standard deviation SD.

and PLD lie within the same signalling pathway in the NIH 3T3 system.

MATERIALS AND METHODS

Oocyte maturation and microinjection. Stage VI oocytes were selected by manual dissection. Series of 30 to 50 oocytes were treated for hormonal induction of maturation with 1 μg of progesterone per ml in Ringer's buffer (100 mM NaCl, 1.8 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 4 mM $NaHCO_3$ [pH 7.8]). Microinjection of 25 μU of each PL or 25 ng of the p21^{ras} protein was performed as previously described (21). After overnight incubation at 18 to 20°C in Ringer's buffer, oocytes were lysed for biochemical characterization or fixed in 16% trichloroacetic acid (TCA). Visual verification of nuclear vesicle breakdown was performed by splitting the oocytes open after fixation.

Protein purification. The v-H-*ras* p21 protein was purified as described previously (25). After protein induction, 7 M urea extracts were subjected to further purification by chromatography through a Sephadex G-100 column (90 by 2.5 cm) in 7 M urea–20 mM morpholineethanesulfonic acid (MES; pH 7.0). Fractions of 3 ml were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to estimate purity and by a GTP-binding assay to determine the activity of p21^{ras}. Fractions containing up to 95% purified p21^{ras} were pooled and dialyzed against 20 mM MES (pH 7.0), and the concentrations were estimated by the Bradford assay system (Bio-Rad).

PLs were obtained from Sigma. Their respective origins are as follows: bee venom for PLA_2, *Bacillus cereus* type III for PLC, and peanut type II for PLD.

Analysis of PA and phosphatidylbutanol production. Oocytes were incubated for 8 h in Ringer's buffer containing 0.5 mCi of ^{32}P per ml and rinsed twice to remove unincorporated isotope. Following microinjection of 50 nl of a solution containing either 1 mg bovine serum albumin (BSA) per ml, 1 mg of p21^{ras} per ml, or 0.5 U of PC-PLD per ml, the reactions were stopped at the indicated times in an ethanol bath kept at −70°C. PA and phosphatidylbutanol were extracted with 160 μl of water and 0.6 ml of chloroform-methanol (1:1, vol/vol). The organic phase was lyophilized under a nitrogen stream, resuspended in 100 μl of chloroform and resolved on thin-layer chromatography plates with the upper phase (100 ml) consisting of a mixture of ethyl acetate, trimethyl pentane, acetic acid, and H_2O (90:50:20:100, by volume), to which 1 ml of acetic acid was added. The thin-layer chromatography plates were exposed to an X ray film, and the radioactive spots were quantified by scratching and scintillation counting.

MPF assays. MPF assays were carried out with total extracts from series of 10 oocytes treated with progesterone or given microinjections of p21^{ras} protein or PLs. After incubation for 18 to 20 h, oocytes were homogenized in BLO buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.0), 10 mM β-glycerophosphate, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid EGTA, 5 mM $MgCl_2$, 50 mM NaF, 2 mM dithiothreitol, 10 μg of leupeptin per ml, 25 μg of aprotinin per ml, and 100 μM phenylmethylsulfonyl fluoride. Following centrifugation at 13,000 × *g* for 15 min, extracts were assayed for 15 min at 30°C in a final reaction volume of 50 ml containing 20 mM HEPES (pH 7.0), 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, 100 μM [α-^{32}P]ATP (2 to 5 dpm/fmol), 0.2 μg of PKA inhibitor, and 1 mg of type III S calf thymus histone (Sigma) per ml. Reactions were stopped by addition of PAGE sample buffer and boiling for 5 min. The samples were subjected to PAGE (15% polyacrylamide), dried, and exposed at −70°C. The band corresponding to H1 was excised from the gel and quantitated by a scintillation counter.

S6 KII assay. To determine S6 KII activity in total extracts, series of 20 oocytes were homogenized in BLO buffer in a final volume of 200 μl. Extracts were centrifuged in a Biofuge A (Heraeus Sepatech) at 13,000 rpm for 15 min, and 30 μl of supernatant was assayed with pp90^{rsk} substrate peptide. The reaction mixture, in a final volume of 50 μl, contained 250 μM rsk substrate peptide (UBI), 50 mM glycerophosphate (pH 7.3), 7 mM NaF, 0.3 mM EDTA, 150 mM $MgCl_2$, 2 mM dithiothreitol, 50 μM [α-^{32}P]ATP (3,000 Ci/mmol; Amersham), and 7 μM PKA inhibitor peptide (Sigma). The assay mixtures were incubated at 30°C for 20 min, and the reactions were stopped with ice-cold TCA to a final concentration of 16% TCA. Samples were maintained for 15 min at 4°C and centrifuged in a Biofuge A (Heraeus Sepatech) at 13,000 rpm for 15 min. The supernatants were spotted onto Whatman p81 phosphocellulose paper filters and washed extensively with 1% orthophosphoric acid and once with 95% ethanol. The radioactivity retained on the filters was quantified in a scintillation counter.

RESULTS

Activation of PLD by microinjection of p21^{ras}. We have recently shown that in *ras*-transformed fibroblasts there is a sustained activation of PLD activity, resulting in constitutive elevation of PA levels (6). This could be generated as a consequence of direct or indirect activation of PLD mediated by Ras protein. To investigate whether p21^{ras} induces the early activation of PLD, we analyzed PA production after microinjection of p21^{ras} into *X. laevis* oocytes. Oocytes were labeled with 0.5 mCi of $^{32}P_i$ per ml at room temperature for 18 h, washed extensively, and given microinjections of 25 μU of PLD, 50 ng of the purified p21^{ras} protein, or equivalent amounts of BSA. PA levels were analyzed 30 min after injection. As shown in Fig. 1A, microinjection of either p21^{ras} or PLD induced a rapid increase in the level of PA.

There are alternative mechanisms for the generation of PA, such as direct activation of a PKC-independent PLD, activation of a PLD by a PKC-dependent pathway, or activation of a DAG kinase (DGK). Therefore, the levels of PA were analyzed in oocytes pretreated with the PKC inhibitor bisindolylmaleimide or the DGK inhibitor R59022. Ras- and PLD-induced production of PA was not affected by the PKC inhibitor (Fig. 1A), suggesting that p21^{ras} can induce PA production in

a PKC-independent form. Moreover, the PA production induced by p21^{ras} is not affected by R59022, suggesting that PA was generated by a mechanism involving PLD activation rather than by activation of DGK.

To further confirm this conclusion, we have analyzed PA production under conditions where it can proceed only from DGK activity. Oocytes were given microinjections of 100 μM R59022 or buffer alone. After 2 h of incubation, oocytes were given microinjections of 25 nCi of [γ-^{32}P]ATP per oocyte and 50 ng of BSA or p21^{ras} per oocyte, or 25 nl of purified DGK. After 30 min of incubation at room temperature, the oocytes were processed for PA production as described in Materials and Methods. Under these conditions, the observed labeled PA would originate from DGK activity since insufficient radioactivity is incorporated into phospholipids (data not shown). As shown in Fig. 1B, microinjection of DGK induced a 33-fold increase in the PA levels, and this increase was inhibited by R59022, an indication of its generation through the DGK enzyme. However, under the same conditions, p21^{ras} was unable to induce PA generation, indicating that *ras*-induced PA originated directly from phospholipid hydrolysis by PLD activation. These results were confirmed when we analyzed the *ras*-induced PLD activity directly by its specific transphosphatidylating activity. Oocytes were labeled with 0.5 mCi of $^{32}P_i$ per ml at room temperature for 18 h, washed extensively, and then given microinjections of 50 ng of purified p21^{ras} protein or BSA. Oocytes were further incubated for 1 h in the presence or absence of 0.5% *n*-butanol, and the [^{32}P]phosphatidylbutanol levels were analyzed. Microinjection of p21^{ras} induced a rapid increase in the transphosphatidylating activity (Fig. 1C), confirming that the *ras*-induced PA production is due to activation of a PLD enzyme.

Inhibition of p21^{ras}-induced GVBD. Microinjection of p21^{ras} protein into *X. laevis* oocytes induces the production of DAG (21, 23). It has been suggested that this DAG production is a consequence of PC-PLC activation (10, 14, 30) and that this activation is necessary for *ras*-induced GVBD. We have previously demonstrated that microinjection of a *B. cereus* PLC is able to induce GVBD and this effect is dependent on PKC, since PKC inhibitors efficiently block PLC-, DAG-, and tetradecanoyl phorbol acetate-induced GVBD (9). Moreover, GVBD induced by PLD and PLA2 seems to be independent of the PKC pathway. To study whether p21^{ras} requires a functional PC-PLC enzyme, we have microinjected p21^{ras} in the presence of different concentrations of bisindolylmaleimide (GF109203X). As shown in Fig. 2A, this PKC inhibitor efficiently blocks tetradecanoyl phorbol acetate-induced GVBD but does not interfere with the GVBD induced by progesterone or p21^{ras}. This result indicate that *ras*-induced GVBD is independent of the PLC-PKC pathway and that the *ras*-induced DAG production is most probably not necessary for oocyte maturation.

In NIH 3T3 cells, transformation by activated p21^{ras} induces PLD activation, with a significant increase in the PA levels (6). Thus, we have analyzed whether PLD activation is necessary for the GVBD induced by Ras proteins. Neomycin has been reported to inhibit the PC-PLD activity with a 50% inhibitory concentration of 65 μM in vitro, and it is able to block the PC-PLD activity in vivo at concentrations near to 1 mM (29). Therefore, we investigated whether PLD activity was necessary for *ras* function in this system. Neomycin was able to block the p21^{ras}-induced GVBD in a dose-dependent manner (Fig. 2B) and with comparable results to those shown by others (38). This effect was parallel with a drastic reduction in *ras*-induced PLD activation (data not shown). Thus, the *ras*-induced GVBD seems to be independent of PC-PLC activity but dependent on PC-PLD activity. However, the lack of specificity of this drug made it necessary to explore other approaches to the problem. Therefore, we analyzed the PLD-induced biochemical pathway in the *X. laevis* oocytes and compared it with that of p21^{ras} and the other PLs (PLC and PLA_2).

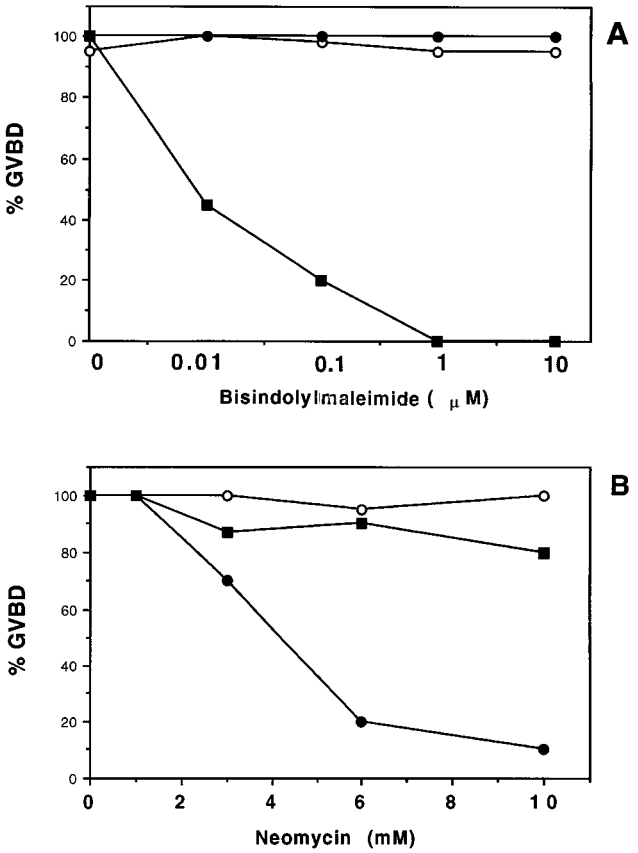

FIG. 2. Effects of the PKC inhibitor GF109203X and neomycin in Ras-induced GVBD. Oocytes were prepared as described in Materials and Methods. p21^{ras} (25 ng) (●) was microinjected per oocyte in a volume of 25 nl in 20 mM MES buffer. Treatments were carried out with 10 μg of progesterone per ml (○) or 100 nM TPA (■). Oocytes were incubated with different concentrations of the indicated inhibitor. GVBD was analyzed 18 h after microinjection or treatment by fixing in 10% TCA.

MPF activity associated with PL-induced GVBD. A number of phospholipid metabolites have been proven capable of triggering mitogenic signals in eukaryotic cells. Microinjection of PLA_2, PLC, or PLD into *X. laevis* oocytes induces the G_2-to-M phase transition through the generation of second messengers generated from phospholipid breakdown, since DAGs, PA, arachidonic acid, lyso-PC, and lysophosphatidylinositol each induce the same effects (9). This effect is comparable to the biological activity of progesterone, phorbol esters, or p21^{ras}.

Mitogenic stimulation of *X. laevis* oocytes can be monitored by measuring posttranslational modification of the key enzymes involved in the process. Quiescent stage VI oocytes contain inactive MPF. Upon stimulation with progesterone or the oncogenic p21^{ras} protein, the MPF complex is activated. When we measured the p34^{cdc2} kinase activity in extracts of PL-injected oocytes, we observed a similar H1 kinase activity in all of them when compared with that observed in extracts from oocytes treated with progesterone or given microinjections of p21^{ras} proteins (results not shown).

A more detailed analysis of the time course of MPF activa-

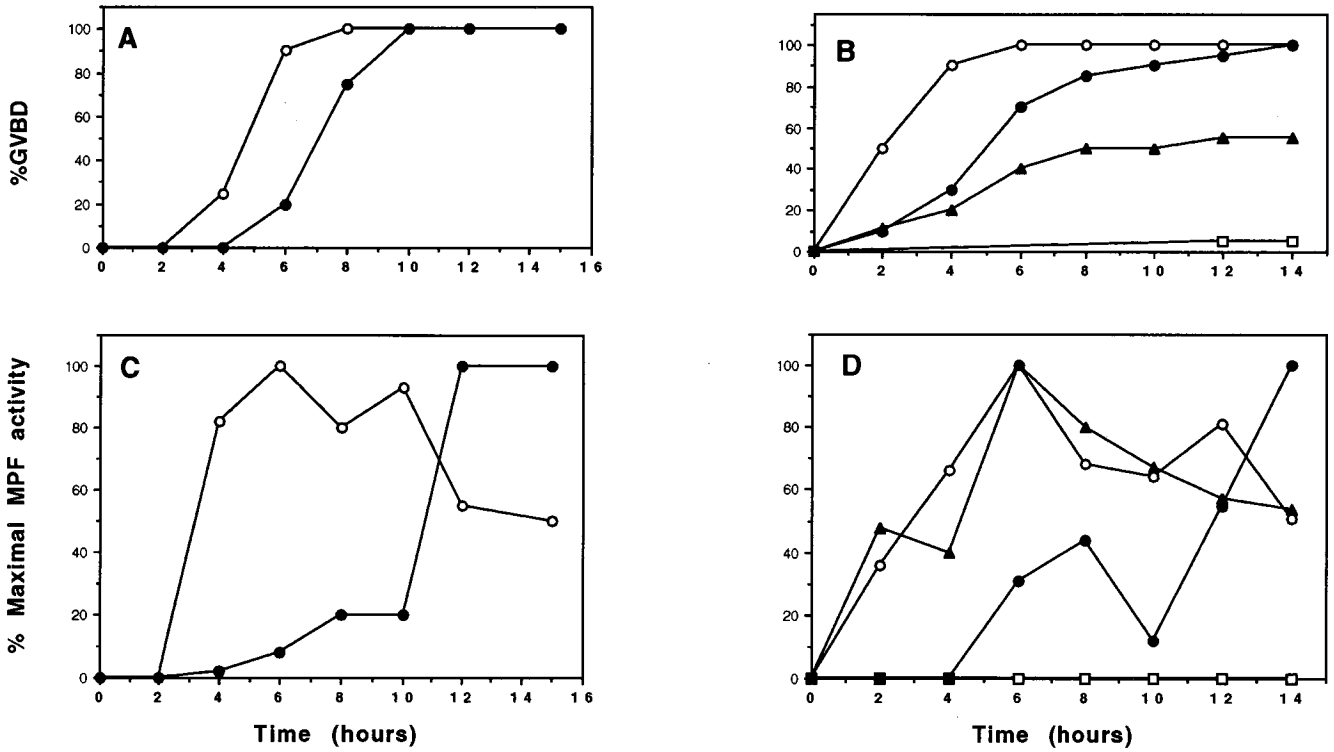

FIG. 3. Time course of p21^{ras}- and PL-induced activation of MPF. (A) Time course of GVBD induced by progesterone (○) or p21^{ras} (●). (B) Time course of GVBD induced by PLA_2 (○), PLD (●), PLC (▲), or BSA (□). (C) Time course of MPF activation induced by progesterone (○) or p21^{ras} (●). (D) Time course of MPF activation induced by PLA_2 (○), PLD (●), PLC (▲), or BSA (□). Oocytes were treated with 10 μg of progesterone per ml or given microinjections of 25 μU of PLA_2, PLC, or PLD per oocyte or 25 ng of v-H-*ras* of p21 protein (Ras) or BSA per oocyte (C). At the indicated times, oocytes were put in ice and processed for the MPF assay (C and D) or fixed in 16% TCA for GVBD analysis (A and B). The MPF assay was performed as described in Materials and Methods with extracts from treated or microinjected oocytes as indicated. The phosphorylated band corresponding to the H1 protein was cut and counted.

tion shows that progesterone induced the H1 phosphorylation at least 2 h before the GVBD took place, correlating with the results reported by other groups (reviewed in reference 31). However, p21^{ras} induced MPF activity only after a significant level of GVBD was reached, indicating that *ras*-induced MPF activation does not precede GVBD. When MPF activation was analyzed after microinjection of PLs (Fig. 3) and compared with the GVBD, we observed that PLA_2 induced a rapid GVBD (almost 100% at 4 h). However, PLD and PLC induced GVBD more slowly. Activation of MPF by PLA_2 was rapid but did not precede the GVBD, since it was maximal at 6 h, 2 h after complete GVBD induction was observed. PLD also showed a late MPF activation but only after GVBD was observed. Finally, PLC showed a partial activation of MPF that preceded GVBD. Thus, a comparison between p21^{ras}- and PL-induced activation of MPF showed that *ras* and PLD had similar kinetics.

MAP K is activated by PLD but not by PLC or PLA_2. Mammalian MAP K is a serine/threonine kinase whose activation and phosphorylation on Tyr and Thr residues are rapidly induced by a variety of mitogens (16, 17, 26, 43–45). This kinase is considered to have a critical role in a network of protein kinases in mitogenic signal transduction (see references 32, 35, and 39 for reviews). *Xenopus* p42^{MAPK} is also a Ser/Thr kinase closely related to the mammalian MAP K. It is phosphorylated on Tyr and Thr residues, a mechanism for its activation which takes place during entry into the M phase in the cell cycle of the *Xenopus* oocyte.

We have investigated the activation of MAP K by microinjection of PLs. As shown in Fig. 4A, PLD was able to induce MAP K activation, as indicated by a net shift in electrophoretic mobility observed on SDS-PAGE. By contrast, neither PLA_2 nor PLC was capable of activating this enzyme. A similar effect on MAP K activation was also observed after microinjection of 1-stearoyl-2-arachidonoyl-PA, strengthening our conclusion that PLD induces activation of MAP K.

To further demonstrate the activation of MAP K after PLD microinjection, an alternative assay was performed as shown in Fig. 4B. After microinjection of either Ras, PLD, or BSA into oocytes or after treatment of oocytes with progesterone, the oocytes were incubated for 9 h in Ringer's buffer. Then the oocytes were lyzed in BLO buffer and samples were immunoprecipitated with an anti-phosphotyrosine antibody. The resulting immunoprecipitates were then resolved by PAGE (10% polyacrylamide), transferred to nitrocellulose, and Western immunoblotted with an anti-MAP K antibody. Either progesterone, Ras, or PLD treatment was sufficient to induce tyrosine phosphorylation of MAP K. These results suggest that the three PLs follow different pathways for induction of GVBD and that only PLD activates p42^{MAPK} like progesterone or p21^{ras} does.

S6 KII activation is induced by PLD but not by PLC or PLA_2. Phosphorylation of the 40S ribosomal protein S6 is common to all known oocyte maturation inducers. This process is one of the last known events in the kinase pathway that leads to reentry into mitosis in oocytes (31). In *X. laevis* oocytes, the S6 protein phosphorylation is mostly mediated by S6 KII, a member of the pp90^{rsk} family (11). We have analyzed the time course of S6 KII activation induced by microinjection of PLs with a specific peptide substrate for this family. As expected

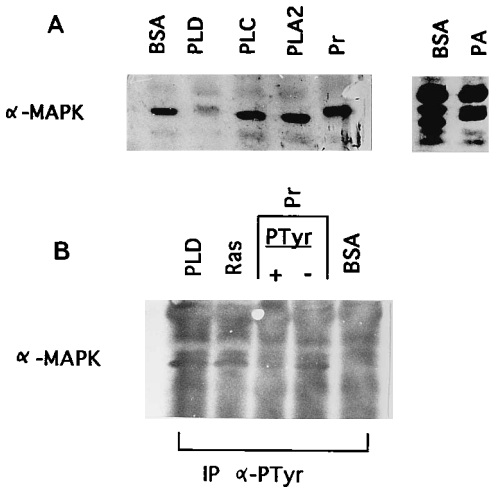

FIG. 4. Activation of $p42^{MAPK}$ by PLs and PA. (A) Activation of $p42^{MAPK}$ was measured by Western blot as the mobility shift of the kinase after electrophoresis in polyacrylamide gels. Total extracts of control (BSA), PLA_2-, PLC-, PA-, or PLD-microinjected oocytes or oocytes treated with progesterone were run on PAGE (10% polyacrylamide), transferred to nitrocellulose, and incubated in the presence of an α-MAP K polyclonal antibody followed by a biotinylated anti-rabbit antibody and streptavidin-peroxidase conjugated protein. (B) Oocytes were given microinjections of $p21^{ras}$, PLD, or BSA or treated with progesterone (Pr) and incubated for 9 h in Ringer's buffer. After this time, oocytes were lyzed in BLO buffer and immunoprecipitated with an anti-phosphotyrosine (α-PTyr) antibody as follows. Oocyte extracts were incubated for 8 h at 4°C with the anti-phosphotyrosine antibody and then for 30 min with protein A-Sepharose beads, linked to a goat anti-mouse antibody. Immunoprecipitates were washed three times in BLO buffer, and the resulting pellets were resolved by PAGE (10% polyacrylamide). Proteins were transferred to nitrocellulose and immunoblotted with the anti-MAP K (α-MAPK) polyclonal antibody. Where indicated, extracts were inhibited with phosphotyrosine at the time of incubation with the anti-phosphotyrosine antibody. Blots were developed by the ECL system as recommended by the manufacturer (Amersham).

from the results of MAP K activation, PLD was able to induce activation of this kinase with a similar kinetics to that of Ras or progesterone (Fig. 5). By contrast, neither PLC nor PLA_2 was able to significantly activate S6 KII. Finally, PA alone was also able to induce S6 KII activation at 16 h after injection, with a

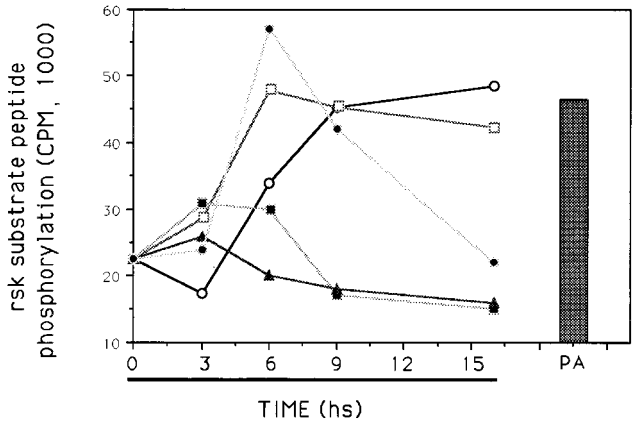

FIG. 5. Activation of S6 KII by PLs. Activation of the S6 KII was determined as described in Materials and Methods by phosphorylation of the rsk substrate peptide. Total-oocyte extracts were analyzed from unstimulated oocytes (zero time point), oocytes treated with 10 μg of progesterone per ml (□) or given microinjections of 25 μU of PLA_2 (■), PLC (▲), or PLD (●) per oocyte or 25 ng of v-H-*ras*-p21 protein per oocyte (○), or 300 ng of PA per oocyte. Data shown for PA microinjection were determined at 16 h of microinjection.

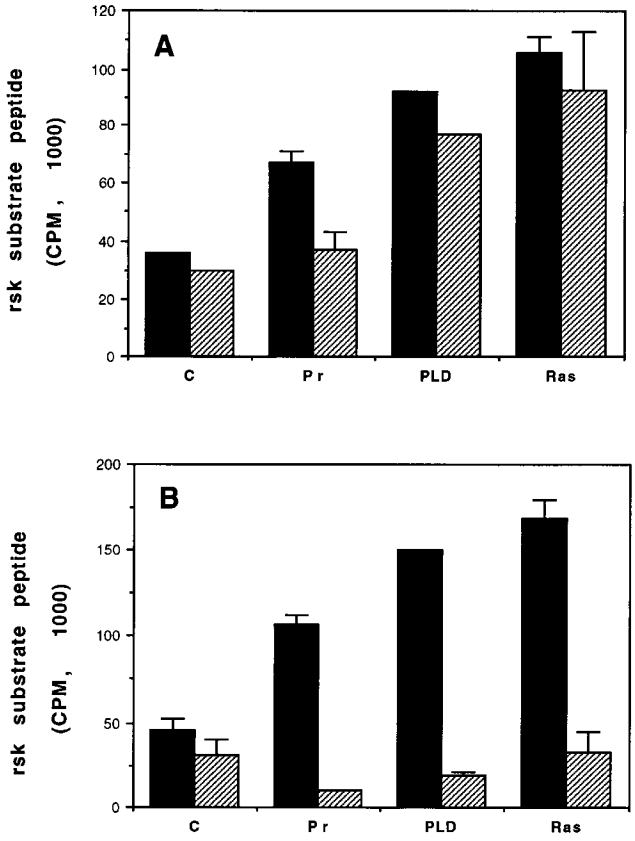

FIG. 6. Effect of CHX or 2-AP treatment on the activation of S6 KII. Phosphorylation of the rsk substrate peptide in total-oocyte extracts was performed as described in Materials and Methods. Extracts were obtained from unstimulated oocytes (C), oocytes treated with 10 μg of progesterone (Pr) per ml, or oocytes given microinjections of 25 μU of PLD per oocyte or 25 ng of v-H-*ras*-p21 protein (Ras) per oocyte. Solid bars indicate untreated oocytes; striped bars indicate CHX-treated (A) or 2-AP-treated (B) oocytes. Error bars indicate the standard error of duplicate samples.

similar efficiency to that of PLD, Ras, or progesterone, suggesting that PLD-mediated activation of S6 KII is mediated by the generation of PA.

Since cycloheximide (CHX) was able to block MPF-dependent signals (8), we used this inhibitor to study whether S6 KII activation in progesterone-, PLD- and *ras*-induced oocytes follows similar pathways. As shown in Fig. 6A, when oocytes were treated with CHX and then stimulated with progesterone, no activation of the S6 KII enzyme could be detected, indicating that S6 KII activation requires protein synthesis. In contrast, when the oocytes were given microinjections of the $p21^{ras}$ protein or PLD, the S6 KII enzymatic activity could be detected at a similar rate to that in control CHX-untreated oocytes. Moreover, microinjection of 200 ng of PA per oocyte resulted in a similar pattern of phosphorylation to that observed by microinjection of $p21^{ras}$ or PLD (data not shown). These results indicate that S6 KII activation induced by *ras*, PLD, or PA follows routes that do not require protein synthesis.

2-Aminopurine (2-AP) is a purine analog that has been described as a rather selective inhibitor of protein kinases activated by certain growth factors (49, 50). In particular, 2-AP blocks *ras*-induced activation of $p44^{ERK1}$ in PC12 cells (42) and in *X. laevis* oocytes (8). Therefore, we studied the effects of 2-AP treatment on the intracellular signals generated by progesterone and PLs and compared them with those induced by

TABLE 1. Effect of CHX or 2-AP treatment on *Xenopus* oocyte maturation[a]

Oocyte stimulation	GVBD[b] after treatment with:	
	CHX	2-AP
Progesterone	15 ± 3	8 ± 5
PLA_2	111 ± 12	100 ± 10
PLC	91 ± 10	70 ± 20
PLD	100 ± 2	10 ± 5
p21^{ras}	100 ± 5	12 ± 4

[a] Oocytes were prepared as described in Materials and Methods. A 25-μU portion of each PL was microinjected per oocyte in a volume of 25 nl in MES buffer. BSA (25 ng) as negative control or p21^{ras} (50 ng) as a positive control were microinjected per oocyte in a volume of 25 nl in 20 mM MES buffer. Treatments were carried out with 10 μg of progesterone per ml. Induction of GVBD was analyzed 18 h after microinjection or treatment by fixing in 10% TCA.

[b] Data represent the mean and standard deviation of two independent experiments and are shown as a percentage of the value for untreated oocytes.

TABLE 2. Effect of CHX or 2-AP treatment on the induction of *Xenopus* oocyte maturation by PLs[a]

Oocyte stimulation[b]	GVBD[c] after treatment with:	
	CHX	2-AP
PLA_2 metabolites		
Lyso-PI	93 ± 7	87 ± 14
Lyso-PC	103 ± 2	65 ± 5
AA	89 ± 8	111 ± 8
PLC metabolite		
S-A-Gly	100 ± 10	117 ± 12
PLD metabolite		
PA	99 ± 1	36 ± 4

[a] Oocytes were prepared as described in Materials and Methods. Each metabolite (200 ng) was microinjected per oocyte in a volume of 25 nl in MES buffer.

[b] Abbreviations: Lyso-PC, lysophosphatidylcholine; Lyso-PI, lysophosphatidylinositol; S-A-Gly, stearoyl-arachidonoyl-glycerol; AA, arachidonic acid.

[c] GVBD was analyzed 18 h after microinjection or treatment by fixing in 10% TCA. Data represent the mean and standard deviation of two independent experiments and are shown as a percentage of the value for untreated oocytes.

p21^{ras} proteins. When oocytes were treated with 2-AP and then stimulated with progesterone, a complete inhibition of S6 KII activity was observed (Fig. 6B), indicating that S6 KII activation by progesterone depends on MAP K activity. Similar results were also observed in PLD- and *ras*-microinjected oocytes, indicating that PLD- and *ras*-induced activation of S6 KII may also be mediated by activation of the MAP K. Finally, when we analyzed the effect of 2-AP treatment on PA-induced S6 KII activation, we found that it was also blocked (data not shown), supporting the functional connection of p21^{ras}, PLD, and PA in this system.

Protein synthesis and MAP K requirement for PL-induced GVBD. GVBD induced by the three PLs analyzed is independent of CHX (Table 1), as has been previously shown for *ras* (1). By contrast, GVBD induced by progesterone is dependent on protein synthesis. Inhibition of GVBD by 2-AP seems to be related to the requirement of MAP K, since only inducers that activate MAP K are blocked by this drug: progesterone, PLD, and p21^{ras} (Table 1). In contrast, PLA_2 and PLC can bypass the 2-AP effect, indicating that they follow a different pathway from that of MAP K activation. The fact that PLA_2 and PLC can bypass the drug effect indicates that inhibition of the biological activity of PLD, *ras*, and progesterone by 2-AP treatment is not a result of a nonspecific effect or toxicity.

PL microinjection can induce GVBD through generation of their respective known metabolites (9). Therefore, we analyzed whether active lipid metabolites were sensitive to CHX and 2-AP inhibition. As shown in Table 2, none of the metabolites generated by PLA_2, PLC, or PLD were significantly sensitive to CHX. By contrast, PA, the only biologically active metabolite in *X. laevis* oocytes generated by PLD, was significantly sensitive to 2-AP treatment, as shown for PLD microinjection.

DISCUSSION

PLs are key enzymes responsible for the generation of important intracellular lipid metabolites and second messengers involved in the regulation of the most relevant signaling processes in a large variety of cells (3, 12, 36). While the PL-induced processes in somatic cells have been extensively studied, much less information is available about the regulation of germinal cell cycles by PLs. We provide evidence that PLA_2, PLC, and PLD are also powerful biological effectors in the *Xenopus* oocyte system and activate intracellular signalling pathways.

PLA_2, PLC, and PLD were able to activate p34^{cdc2} kinase as did p21^{ras}. However, the time course of p34^{cdc2} kinase activation showed clear differences among PLs. While PLC and PLA_2 showed a rapid activation, PLD, like p21^{ras}, showed a similar delayed H1 kinase activation. Moreover, none of the PLs studied were inhibited by CHX in their biological activity, suggesting that, like p21^{ras} proteins, PLs do not need protein synthesis for oocyte maturation. p42^{MAPK} is activated by progesterone and p21^{ras}, and this activation is necessary for oocyte maturation (8, 20). These results are in agreement with previous observations indicating that oocyte maturation requires MPF in progesterone- but not *ras*-induced GVBD (34). PLD, like progesterone and *ras*, activates p42^{MAPK}. In contrast, PLC and PLA_2 do not, indicating that *ras* and PLD follow a different pathway from PLC and PLA_2. Thus, both MPF and MAP K seems to be sufficient elements to distinguish among signalling routes in oocytes, clearly indicating that *ras* is not related to PLC.

Cytosolic PLA_2 is phosphorylated and activated by MAP K (28). This suggests that PLA_2 is located downstream of MAP K in the pathway and can explain the lack of detected activation of MAP K by PLA_2 injection. Indeed, when oocytes were treated with 2-AP, a MAP K inhibitor, the activation of PLA_2 by p21^{ras} or PLD microinjection was abolished, indicating that PLA_2 is located downstream of MAP K activation (7). Finally, since PLC was unable to activate p42^{MAPK} and PLA_2 in oocytes, it must be located within an alternative pathway to MAP K activation for the induction of GVBD in *Xenopus* oocytes.

MAP K phosphorylates and activates S6 KII (48). Therefore, inducers that activate MAP K should also activate S6 KII. Progesterone and oncogenic p21^{ras} activate S6 KII through activation of MAP K (8). We show that PLD, which activates p42^{MAPK}, also activates S6 KII. By contrast, in *X. laevis* oocytes, PLA_2 and PLC failed to activate pp90^{rsk}, another indication that p21^{ras} and PLD follow different pathways from PLC in this system.

We have also analyzed the dependency of S6 KII activation on protein synthesis and MAP K activity. Oocytes stimulated with progesterone showed S6 KII activation which was sensitive to CHX treatment, indicating that its activation is dependent on protein synthesis. In contrast, PLD- or *ras*-induced oocytes showed S6 KII activation and GVBD independent of protein synthesis. These results are consistent with the previous studies suggesting that although progesterone needs protein synthesis for p42^{MAPK} activation, *ras*-induced p42^{MAPK}

activation is independent of protein synthesis (8, 46). We report that PLD is also insensitive to CHX for S6 KII activation, again a good correlation to the function of Ras proteins.

2-AP, a reported protein kinase inhibitor, was able to block the S6 KII activation by *ras* and progesterone. We have observed that 2-AP blocks *ras*- and progesterone-induced activation of MAP K in oocytes (8), suggesting that MAP K is necessary for the induction of S6 KII activation. Our results indicate that PLD-induced activation of S6 KII is also sensitive to 2-AP.

It has been suggested that $p21^{ras}$ is functionally dependent on a PC-PLC activity (10, 14, 30). However, analysis of the biochemical pathways of cell cycle-dependent kinases followed by the three more important PLs compared with the *ras* pathway indicates that this hypothesis does not appear to be correct. $p21^{ras}$ activates $p42^{MAPK}$ and S6 KII. In contrast, a nonspecific PLC that is able to hydrolyze both PC and phosphatidylinositol (9) does not activate $p42^{MAPK}$ and S6 KII. PLD, as well as $p21^{ras}$, activates $p42^{MAPK}$ and S6 KII, and its ability to induce GVBD is independent of protein synthesis. We also show in this study that PLD- but not PLA_2- or PLC-induced GVBD was sensitive to 2-AP treatment. Moreover, the PKC inhibitor bisindolylmaleimide inhibits the PLC- and DAG-induced GVBD (7) but not the *ras*-induced GVBD (Fig. 2A). Therefore, our results suggest that $p21^{ras}$ does not activate PLC but may be functionally related to PLD, since both proteins show a similar pattern of signalling during GVBD induction.

When we analyzed the functional relationship between these two proteins, we found that oncogenic $p21^{ras}$ was able to induce the PLD activity with a net PA production, which would be sufficient to induce GVBD (9). Moreover, treatment with propanolol, a well-known inhibitor of the PA-phosphohydrolase, inhibits DAG production but does not alter *ras*-induced GVBD (results not shown), indicating that this biological effect is not mediated by DAG. Also, we have recently shown that in *ras*-transformed NIH 3T3 fibroblasts there is a sustained activation of PLD (6), further supporting the hypothesis of a functional link between $p21^{ras}$ and PLD.

It has been shown that *src*-induced transformation may be connected to activation of PLD in murine fibroblasts through a GTP-binding protein (19, 47). PDGF may be also functioning through activation of PLD (41) rather than activation of a PC-PLC. Since microinjection of Y13-259, a neutralizing antibody against $p21^{ras}$ proteins, efficiently blocks both PDGF and *src* function (33), it is reasonable to postulate that all these mitogenic signals may have a common mechanism that implies the generation of PA. However, the exact implication of this metabolite in the *ras*-induced pathways in different systems is still unknown; further research is required to elucidate this.

A clear connection between insulin and *ras* has been reported for both mammalian cells and *Xenopus* oocytes (reviewed in reference 22). We have also observed that insulin-induced GVBD in oocytes is associated with a rapid production of PA, similar to that observed after $p21^{ras}$ microinjection (data not shown). These results suggest the putative involvement of $p21^{ras}$ as the coupling system of insulin receptor and PLD; this deserves further investigation.

In summary, in *X. laevis* oocytes, microinjection of purified oncogenic $p21^{ras}$ protein induces activation of PLD, and inhibition of this enzyme by neomycin blocks the *ras*-induced GVBD. Moreover, the pathway activated by $p21^{ras}$ that leads to GVBD correlates with the PLD-induced pathway. Thus, activation of PLD by $p21^{ras}$ proteins may be an essential step in the pathway that leads to DNA synthesis in *X. laevis* oocytes, as we have previously demonstrated in the NIH 3T3 cell system.

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