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Diacylglycerol Production in *Xenopus laevis* Oocytes after Microinjection of p21^{ras} Proteins Is a Consequence of Activation of Phosphatidylcholine Metabolism

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Microinjection of p21$^{Ha\text{-}ras}$ proteins into *Xenopus laevis* oocytes induces a rapid increase of 1,2-diacylglycerol (DAG) levels. The observed alterations in DAG levels were consistent with the ability of the protein to induce maturation, measured by germinal vesicle breakdown (GVBD). Both the increase in DAG levels and GVBD activity were dependent on the ability of the proteins to undergo membrane translocation. Alterations of DAG levels or GVBD activity did not correlate with changes in the levels of inositol phosphates. However, at minimal doses sufficient to achieve maximal biological response, a biphasic increase in the amounts of phosphocholine and CDP-choline was observed. The first burst of phosphocholine and CDP-choline preceded the increase in DAG levels. The second peak paralleled the appearance of DAG. Choline kinase activity was also increased in oocyte extracts after p21^{ras} microinjection. These results suggest that both the synthesis and degradation of phosphatidylcholine are activated after microinjection of *ras* proteins into *Xenopus* oocytes, resulting in a net production of DAG.

ras genes code for 21-kilodalton proteins that are thought to play a critical role in the regulation of signal transduction mechanisms in a variety of cell systems (for reviews, see references 1 and 14). The p21 proteins bind GTP and GDP with high affinity and are able to hydrolyze GTP with a very low intrinsic efficiency. Recently, a cytoplasmic protein designated GTPase-activating protein has been shown to increase both in vivo and in vitro the intrinsic GTPase of the normal protein by a factor of at least 100-fold, with no effect on the GTPase activity of mutant p21 proteins (23). Single-point mutations at defined positions alter biochemical and biological properties of the p21^{ras} products. Such alterations are thought to play an important role in malignant transformation. Despite intensive investigations to determine the pathway in which *ras* proteins function, their role as regulating factors remains elusive.

ras genes are highly conserved in evolution from yeast to mammals, with a great degree of homology of their products (1, 14). Several systems have been used to investigate the biochemical properties of the *ras* proteins, including yeast, *Dictyostelium discoideum*, *Drosophila melanogaster*, and *Xenopus laevis*. Microinjection of recombinant p21^{ras} proteins into *X. laevis* oocytes has been shown to induce maturation. This effect seems to be different from the mechanism induced by progesterone, since it is independent of the regulation of the levels of cyclic AMP (3). I have used *X. laevis* oocytes for analysis of alterations in the phospholipid metabolism induced by *ras* proteins. In a previous study, it was reported that microinjection of *ras* proteins into *Xenopus* oocytes induced a rapid formation of 1,2-diacylglycerol (DAG) and inositol phosphates (IPs) (11). However, there are some controversial studies on the involvement of p21^{ras} proteins in the regulation of a phospholipase C (PLC) (7, 9, 13, 21, 25, 29). In this study, the relationship between oocyte maturation, production of DAG, and phospholipid metabolism is investigated.

MATERIALS AND METHODS

Estimation of germinal vesicle breakdown (GVBD). Stage VI oocytes were manually dissected and selected by standard procedures as previously described (11). After microinjection with different amounts of the indicated proteins, oocytes were incubated at 23°C for 24 h and fixed for 20 min in 10% trichloroacetic acid (TCA). Oocytes were then split open with a scalpel and analyzed for the presence of the germinal vesicle. In most cases, the appearance of a white spot in the animal pole of the oocyte correlated with the absence of the germinal vesicle.

Analysis of IP production. Oocytes were prepared as indicated above and microinjected with 50 nl of [^{3}H]myoinositol (500 nCi per oocyte), followed by incubation for 5 h as previously described (11). This amount resulted in an estimated concentration of approximately 50 μM radioactive isotope. After microinjection of either normal or mutated proteins, 10 to 20 oocytes were processed at the indicated times for IP content. Oocytes were homogenized in 440 μl of methanol–1 N HCl (10:1, vol/vol), and phases were split by addition of 0.6 ml of chloroform and 160 μl of deionized water. Organic phases were collected, and aqueous phases were extracted with 400 μl of chloroform. Both organic phases were washed with 220 μl of methanol–1 N HCl (10:1, vol/vol) and dried under nitrogen. Radioactivity was estimated by scintillation counting in 10 ml of Aquasol (Dupont, NEN Research Products). Aqueous phases were passed through Dowex 1X8 columns (formate form) and eluted with 8 ml of 1.2 M ammonium formate–0.1 M formic acid. Results were normalized to values for the radioactivity in the organic phases and expressed as percentage of the value for control, uninjected oocytes. Typical values were from 10,000 to 25,000 cpm in IPs and from 30,000 to 80,000 cpm in total lipids. All values shown are mean values from one experiment performed in triplicate, with less than 10% variation. Experiments were repeated

two to three times with oocytes from different animals and yielded similar results.

Analysis of DAG levels. Oocytes prepared as described above were microinjected with 50 nl of [^{3}H]glycerol (50 nCi per oocyte), resulting in a final concentration of 26 μM radioactive isotope. After incubation at 23°C for 5 h, oocytes were microinjected with the indicated amounts of p21^{ras} proteins and processed for DAG determination. Oocytes were homogenized in 160 μl of deionized water, and then 0.6 ml of chloroform-ethanol (1:2, vol/vol) was added. Phases were separated by addition of 0.2 ml of chloroform and 0.2 ml of deionized water. Samples were centrifuged, and organic phases were saved. Aqueous phases were washed with 0.5 ml of chloroform; organic phases were dried under nitrogen and suspended in 50 μl of chloroform containing purified DAG as a standard, and 25 μl was resolved by thin-layer chromatography (TLC) in hexane-diethyl ether-acetic acid (60:40:1, vol/vol/vol). Lipids were visualized by exposure to iodine vapor, and DAG levels were determined by scintillation counting. DAG production was normalized to the level of radioactivity remaining in the origin (total phospholipids) and expressed as percentage over the value for control, uninjected oocytes. Values in DAG were 250 to 1,500 cpm; those in total phospholipids were 5,000 to 10,000 cpm. Data are from one experiment performed in triplicate, with less than 5% variation. Experiments were repeated two to three times, with similar results. Each experiment was performed in triplicate, using oocytes extracted from the same animal, but different animals were used for each independent experiment.

Analysis of PC metabolites. Oocytes were prepared as described above and microinjected with 50 nl of [^{3}H]choline (500 nCi per oocyte) under conditions resulting in a 12.5 μM concentration of the radioactive compound. After 5 h of incubation, oocytes were microinjected with different amounts of p21^{ras} proteins and homogenized in 400 μl of deionized water, and then 92 μl of 80% TCA was added to a final concentration of 15%. Samples were kept on ice for 15 min and centrifuged for 10 min at 12,000 rpm. Supernatants were then washed four times with 4 volumes of water-saturated ethyl ether, lyophilized, and suspended into 50 μl of deionized water. Choline metabolites were resolved by TLC in 0.9% NaCl-methanol-ammonium hydroxide (50:70:5, vol/vol/vol). Appropriate radioactive standards ([*methyl*-^{14}C]choline chloride [56 mCi/mmol]; phosphoryl-[*methyl*-^{14}C]choline [50 mCi/mmol]; and CDP-[*methyl*-^{14}C]choline [50 mCi/mmol]; Amersham Corp.) were also run in the same TLC plates in parallel, and corresponding spots were scraped off and counted in 10 ml of Aquasol. Incorporation into phosphatidylcholine (PC), phosphocholine, and CDP-choline was approximately 10% of total radioactivity microinjected, with only 2% incorporated into PC. On basis of these results, PC, phosphocholine, and CDP-choline levels were normalized to the level of the total intracellular unincorporated [^{3}H]choline pool. Total counts in phosphocholine and CDP-choline were 30,000 to 90,000.

When indicated, the levels of [^{3}H]choline incorporated in phosphocholine or CDP-choline were expressed relative to the levels incorporated into PC. In this case, oocytes were treated and processed as described for phosphocholine and CDP-choline analysis. After centrifugation, supernatants were processed as described above, and choline metabolites were resolved by TLC. Pellets from 15% TCA precipitates were washed with 1 ml of ice-cold 15% TCA and suspended into 200 μl of 0.5 N NaOH–1% sodium dodecyl sulfate for several hours. Samples were then neutralized with 200 μl of 0.5 N HCl, and radioactivity was estimated by scintillation counting. Typical values for incorporation into PC were 10,000 to 30,000 cpm. Analysis of phospholipids by TLC revealed that more than 95% of total radioactive lipid corresponded to PC. Data presented are from a single experiment performed in triplicate. The experiment was repeated once more, using oocytes from a different animal, with similar results.

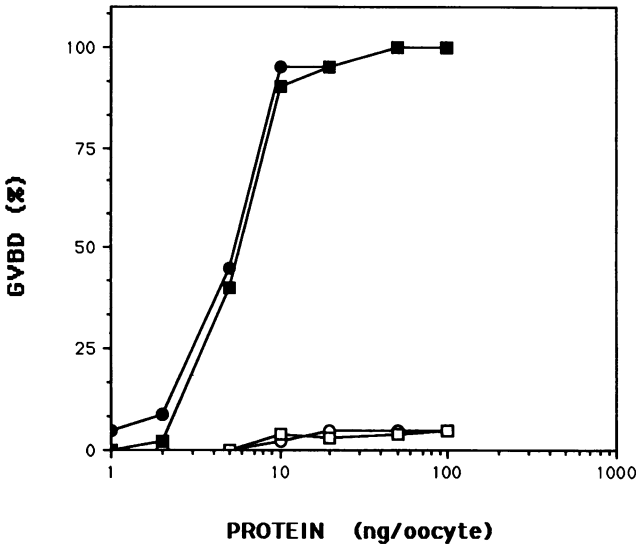

FIG. 1. Induction of GVBD after microinjection of p21^{ras} proteins. *ras* proteins were expressed in *E. coli* and purified as previously described (12). Stage VI oocytes were manually dissected and selected from ovarian fragments by standard procedures (11). After microinjection of indicated amounts of each protein, oocytes were incubated at 23°C for 24 h, fixed for 20 min in 10% TCA, and analyzed for the presence of the germinal vesicle. For each point, 20 to 30 oocytes were microinjected. Shown are results for normal (○) and transforming (●) H-*ras* protein, mutated p21$^{Ser-186}$ (□), and chimeric p21-SR (■). Data are representative of three independent experiments that yielded similar results.

Choline kinase activity. Oocytes were prepared as described above and microinjected with 50 nl containing 100 ng of either normal or transforming v-H-*ras* protein. At indicated times, oocytes were homogenized mechanically and centrifuged at 12,000 rpm in an Eppendorf microfuge for 15 min. The resulting supernatants were analyzed for choline kinase activity at 30°C for 15 min in 400 μl of reaction buffer (100 mM Tris hydrochloride [pH 8.0], 10 mM $MgCl_2$, 10 mM ATP, 0.25 mM [*methyl*-^{3}H]choline). Reactions were stopped by the addition of 80% TCA to a final concentration of 15%. Samples were kept on ice for 15 min, centrifuged for 15 min at 12,000 rpm, washed four times with 4 volumes of water-saturated ethyl ether, lyophilized, and suspended in 10 μl of deionized water. Phosphocholine was resolved and estimated by TLC as described above.

RESULTS

Previous studies have demonstrated the ability of transforming *ras* proteins to induce maturation of *X. laevis* oocytes (3, 11). Figure 1 shows the induction of oocyte maturation, measured by GVBD, 24 h after microinjection of different amounts of either normal or transforming p21^{ras} protein. Microinjection of as little as 10 ng per oocyte was sufficient to fully induce the biological activity of the pro-

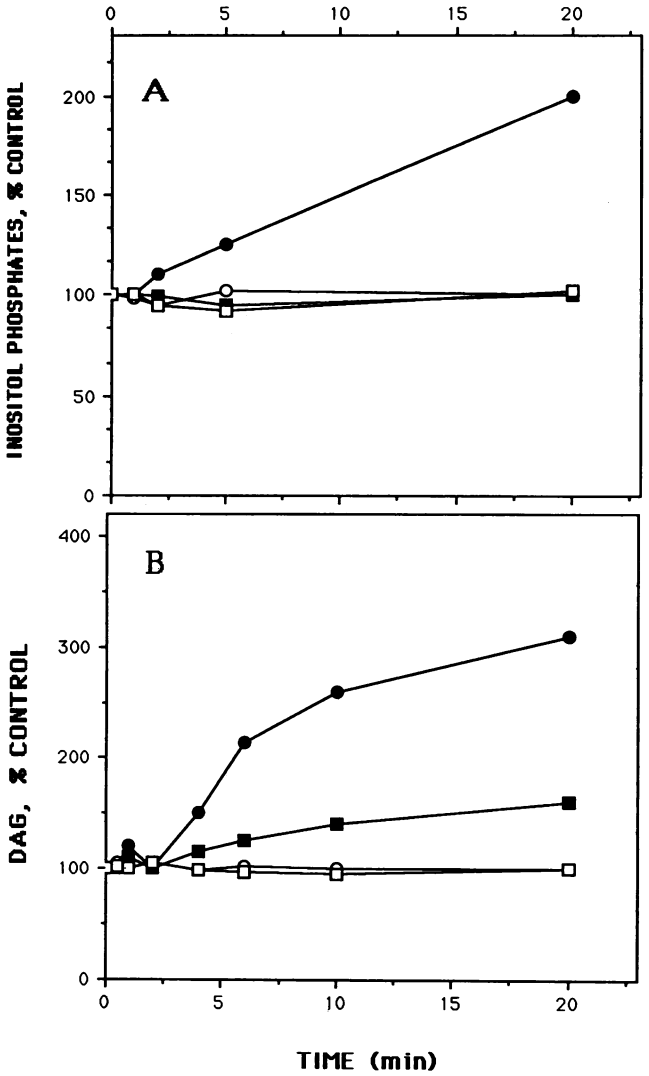

FIG. 2. Effects of microinjection of p21^{ras} proteins in *Xenopus* oocytes on IP and DAG production. Oocytes were labeled by microinjection of 50 nl of water containing either [^{3}H]myoinositol (500 nCi per oocyte) or [^{3}H]glycerol (50 nCi per oocyte) for 5 h as previously described (11). Assuming a free solvent volume of 500 nl per oocyte, isotope concentrations after microinjection were 50 μM [^{3}H]myoinositol and 26 μM [^{3}H]glycerol. After microinjection of normal or transforming p21 protein, 10 to 20 oocytes were processed at the indicated times for IP or DAG determination. Shown are results for normal protein at 100 ng per oocyte (○) and for transforming protein at 100 (●), 10 (■), and 2 (□) ng per oocyte.

teins. Amounts of less than 10 ng per oocyte were only partially active, and less than 2 ng per oocyte was completely inactive. By contrast, as much as 100 ng of normal p21 per oocyte was inactive when microinjected under identical conditions.

ras proteins are synthesized as soluble precursors that undergo posttranslational modifications leading to translocation to the plasma membrane (5, 22). Translocation is associated with the covalent attachment of a molecule of palmitic acid to residue Cys-181 or Cys-184 (8). For this process, the presence of residue Cys-186 is absolutely required (8). It has been demonstrated that both palmitoylation and membrane translocation are required for the biological function of the proteins, since p21^{ras} proteins with a truncated carboxyl terminus or a single amino acid substitution of residue Cys-186 were unable to induce transformation in the NIH 3T3 transfection assay (27, 28). Palmitoylation can be replaced by myristoylation, since the biological activity of a nonpalmitoylable p21^{ras} protein carrying a Cys–to–Ser-186 substitution can be recovered by a peptide sequence harboring the myristoylation signal of the p60$^{v\text{-}src}$ protein in its amino-terminal region (15). Therefore, the requirement of acylation of *ras* proteins for the induction of GVBD was investigated.

For this study, we first expressed in *Escherichi coli* a viral p21$^{H\text{-}ras}$ protein in which Cys-186 was mutated to Ser-186 (p21$^{Ser\text{-}186}$) as well as a chimeric viral p21$^{H\text{-}ras}$ protein in which the 15-amino-acid amino-terminal sequence from p60$^{v\text{-}src}$ was fused in frame to the complete sequence of the Ser-186 mutant (p21-SR). The proteins were then purified to homogeneity and microinjected into *Xenopus* oocytes, and GVBD analysis was performed at 24 h after microinjection. Figure 1 shows the results obtained after microinjection of different amounts of p21$^{Ser\text{-}186}$ and p21-SR proteins. Whereas the nonpalmitoylable p21^{ras} protein was inactive for induction of GVBD, activity was fully recovered by fusion to the myristoylation sequence of the *src* protein. These results are in complete agreement with those observed in the mammalian system by analysis of transforming activity upon transfection of NIH 3T3 cells (13, 15). Thus, processing and membrane localization are essential for the biological activity of *ras* proteins in *X. laevis* oocytes.

In an effort to correlate the ability of *ras* proteins to induce GVBD with alterations in phospholipid metabolism, the effects of microinjection of different amounts of p21^{ras} proteins on IP and DAG production were investigated. Whereas high doses of the transforming *ras* protein induced generation of IPs, no alterations were detected at lower concentrations of proteins that were shown sufficient to fully induce maturation of microinjected oocytes (Fig. 2A). Anal-

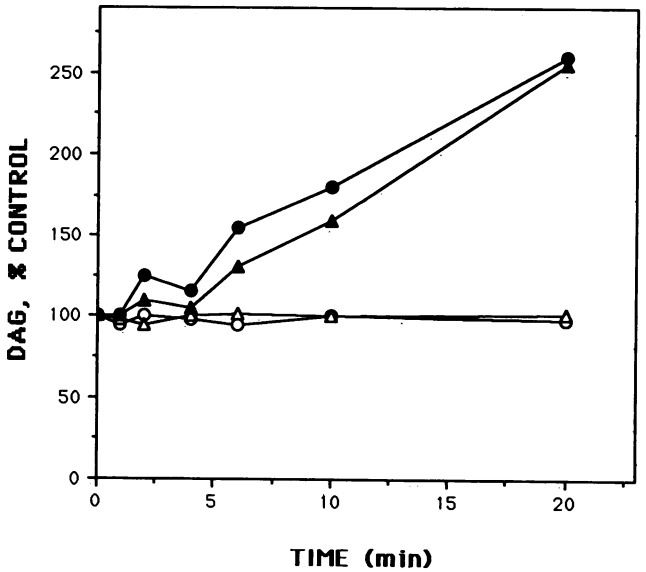

FIG. 3. Effects of microinjection of p21^{ras} mutants on DAG levels. Purified p21^{ras} proteins were microinjected at 100 ng per oocyte into [^{3}H]glycerol-labeled oocytes and analyzed for DAG production at the indicated times. Shown are results for normal (○), and transforming (●) viral H-*ras* proteins, mutated p21$^{Ser\text{-}186}$ (△), and chimeric p21-SR (▲).

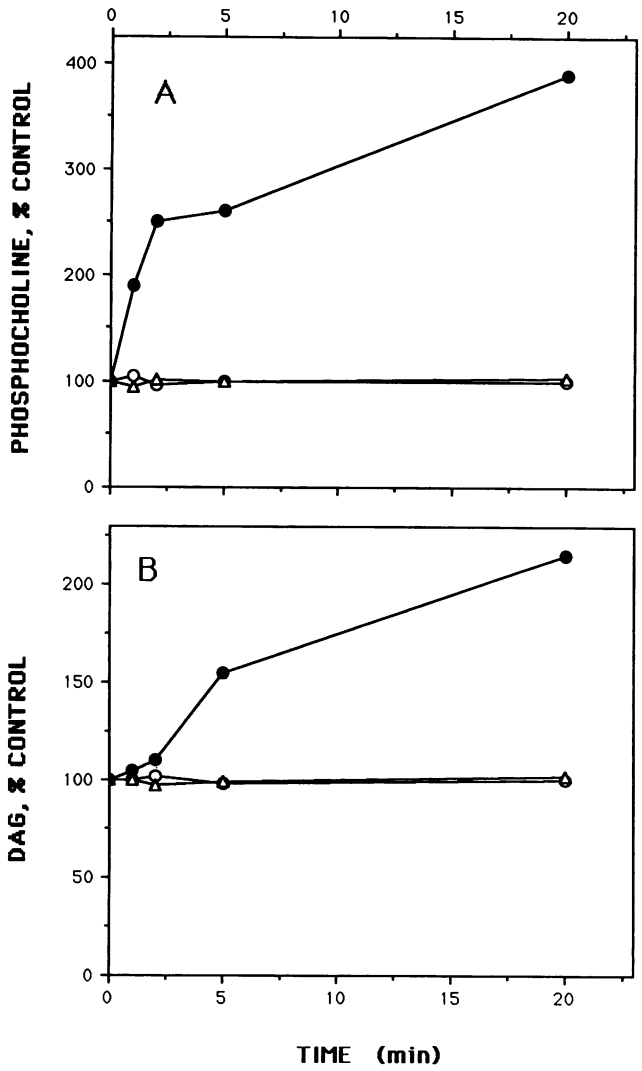

FIG. 4. Time course of phosphocholine and DAG production after microinjection of *ras* proteins into *Xenopus* oocytes. Oocytes were labeled with [^{3}H]glycerol as described in the legend to Fig. 1 or with 500 nCi of [^{14}C]choline (final concentration, 12.5 μM) and then microinjected with 100 ng of normal (○), transforming (●), or nonpalmitoylable p21$^{Ser-186}$ (△) protein per oocyte. At the indicated times, 10 (choline samples) or 20 (glycerol samples) oocytes were processed for determination of phosphocholine or DAG levels. Data for the phosphocholine samples were normalized to the level of the unincorporated intracellular pool of [^{3}H]choline.

ysis of IP levels even at 2 h after microinjection showed similar results (data not shown). Thus, alterations in IP levels did not correlate with the ability of *ras* proteins to induce oocyte maturation.

Figure 2B shows the levels of DAG after microinjection of different amounts of *ras* proteins. When 10 ng of the transforming protein was microinjected per oocyte, an increase of around 50% over the basal levels was observed at 20 min after microinjection. Higher levels of microinjected protein induced increases of up to 300 to 500% over the control level. By contrast, no increase was observed when as much as 100 ng of normal protein was microinjected.

The ability of p21$^{Ser-186}$ and chimeric p21-SR proteins to induce production of DAG was also investigated. DAG production was detectable only when p21-SR or wild-type viral p21$^{H\text{-}ras}$ was microinjected (Fig. 3). No effects were observed when normal or p21$^{Ser-186}$ protein were analyzed. These results strongly suggest a correlation between DAG production and oocyte maturation induced by *ras* proteins.

A number of reports have suggested that p21^{ras} proteins are involved in the regulation of the hydrolysis of phosphatidylinositols (PIs) as a result of the activation of PLC activity (7, 9, 25), an important regulatory component on the signal transduction mechanism (2). However, we and others have observed that *ras* activation is not always associated with an increase in the basal levels of IPs (13, 21, 29), suggesting that it could be a secondary effect rather than a primary effect of the p21^{ras} product. These results also suggest that DAG production induced by microinjection of p21^{ras} proteins into *X. laevis* oocytes originates from a source other than hydrolysis of PIs. We have previously found that in *ras*-transformed NIH 3T3 cells, the levels of phosphocholine and

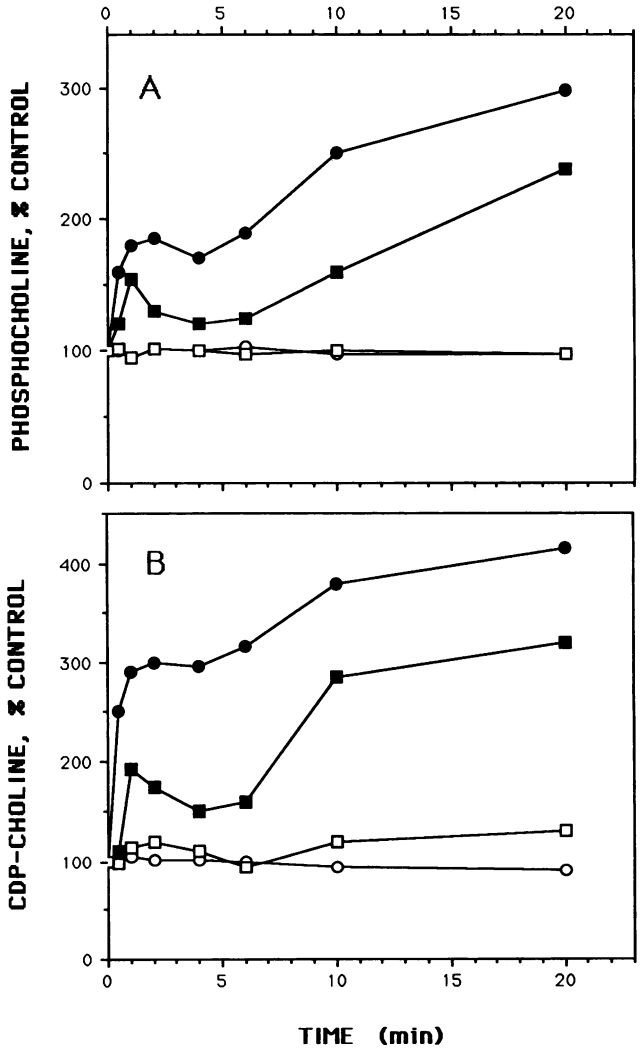

FIG. 5. Phosphocholine and CDP-choline levels in *Xenopus* oocytes after p21^{ras} microinjection. Oocytes were labeled with [^{3}H]choline as described in the legend to Fig. 4 and microinjected with normal protein at 100 ng per oocyte (○), or with transforming p21$^{v\text{-}Ha\text{-}ras}$ at 100 (●), 10 (■), or 2 (□) ng per oocyte. Data were normalized to values for the unincorporated intracellular pool of [^{3}H]choline.

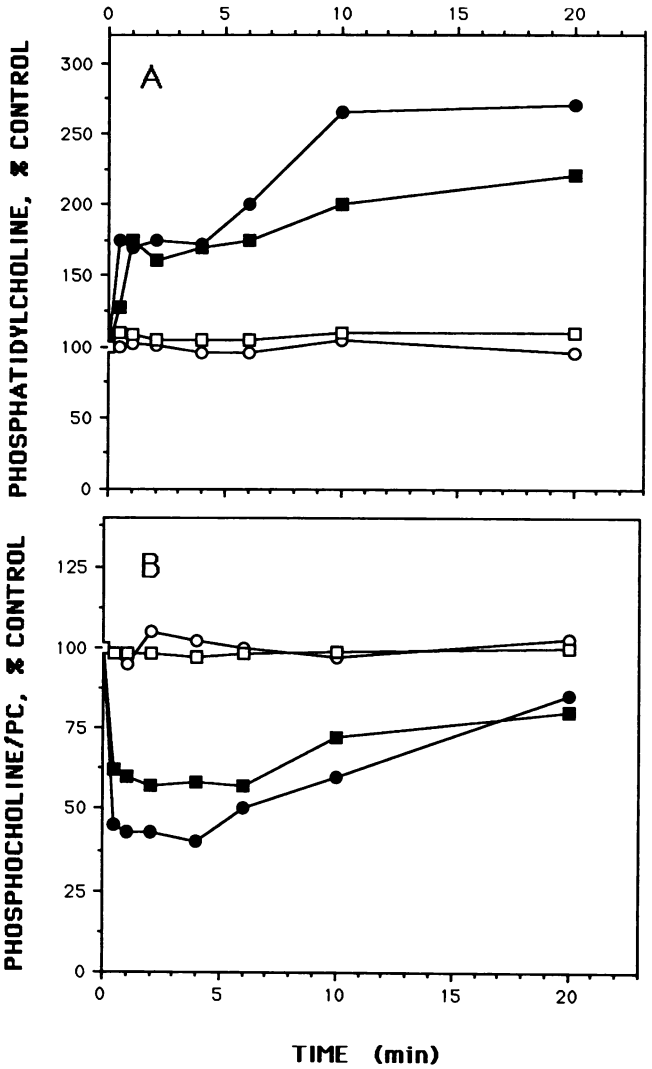

FIG. 6. Incorporation of [^{3}H]choline into PC and phosphocholine after microinjection of p21^{ras} proteins into *Xenopus* oocytes. Oocytes were labeled as described in the legend to Fig. 4 with [^{3}H]choline and microinjected with the indicated amounts of p21^{ras} proteins. At the indicated times, 10 oocytes were processed for determination of PC or phosphocholine levels. Total PC content was normalized to values for the intracellular pool of unincorporated [^{3}H]choline; phosphocholine levels were normalized to levels of radioactivity incorporated into PC. Shown are results for normal protein at 100 ng per oocyte (○) and for transforming protein at 100 (●), 10 (■), and 2 (□) ng per oocyte.

phosphoethanolamine are substantially higher than those of their normal counterparts (13).

The levels of phosphocholine after microinjection of either normal or transforming p21^{ras} proteins into oocytes were analyzed. Figure 4 shows the correlation between phosphocholine production and generation of DAG after microinjection of 100 ng of transforming, normal, or mutated p21$^{Ser-186}$ protein per oocyte. A rapid increase in this metabolite was observed when the transforming protein was injected, but no alterations were observed when the normal or the nonpalmitoylable protein was injected. The alteration induced by the transforming p21 protein was observed at earlier time points than was the increase in DAG at equivalent amounts of protein microinjected per oocyte.

Phosphocholine can be directly generated as a result of the activation of two different enzymes. Activation of a PC-specific PLC would yield both DAG and phosphocholine in a process similar to that for the well-documented activation of a PI-specific PLC (2). Activation of choline kinase will also produce phosphocholine without generating DAG (10, 17, 24). To investigate which of these enzymes was responsible for the generation of phosphocholine after microinjection of *ras* proteins into oocytes, a time course experiment was performed after microinjection of different amounts of *ras* proteins. The transforming *ras* protein at 10 ng per oocyte was sufficient to induce a rapid increase in the production of phosphocholine at time points when very little or no detectable DAG was observed (compare Fig. 5A and 2B). This amount of *ras* protein was also sufficient to fully induce GVBD (Fig. 1). However, when the transforming protein at 2 ng per oocyte and the normal protein at 100 ng per oocyte were analyzed, no detectable phosphocholine increase was observed.

Phosphocholine is converted to CDP-choline by a specific CTP-phosphocholine cytidylyltransferase and constitutes a second metabolite in the anabolic pathway of PC synthesis. The levels of CDP-choline were also investigated as a time course after microinjection of different amounts of *ras* proteins. Figure 5B shows a good correlation between the levels of CDP-choline and phosphocholine at 10 and 100 ng of the transforming protein per oocyte. Again, no changes were observed with 2 ng of the transforming protein or 100 ng of the normal protein per oocyte. Since the levels of both phosphocholine and CDP-choline metabolites were increased at earlier times than DAG was produced, these results suggested that synthesis of PC was activated by p21^{ras} proteins. Indeed, the levels of [^{3}H]choline incorporated in PC were stimulated after microinjection of the transforming *ras* protein at a high dose but not at a low dose or when a high dose of the normal protein was microinjected (Fig. 6).

In a parallel series of experiments, the levels of phosphocholine after microinjection of oocytes by normal or transforming *ras* proteins were related to the radioactivity incorporated into PC. An apparent rapid reduction in the levels of phosphocholine was observed (Fig. 6B) at time points as early as 30 s after microinjection (the first time considered), suggesting that the production of phosphocholine was independent of the hydrolysis of PC. At later times (10 to 20 min), an apparent relative increase of the levels of phosphocholine coinciding with the generation of DAG was observed. These results suggested that at least partially at late

TABLE 1. Choline kinase activity in oocyte extracts after p21^{ras} microinjection[a]

Protein	Time (min)	Choline kinase activity (nmol/min per mg of protein)	
		Expt 1	Expt 2
BSA	20	2.50 ± 0.22 (100)	1.78 ± 0.11 (100)
Normal p21	2	2.53 ± 0.15 (101)	1.88 ± 0.14 (106)
	20	2.65 ± 0.24 (106)	1.82 ± 0.09 (102)
v-H-*ras* p21	2	4.67 ± 0.52 (187)	3.98 ± 0.27 (224)
	20	6.51 ± 0.58 (260)	5.32 ± 0.49 (299)

[a] Oocytes were prepared as indicated, and each was microinjected with 100 ng of carrier protein (BSA) or of normal or transforming p21^{ras}. At indicated times after microinjection, oocytes were homogenized and soluble extracts analyzed for choline kinase activity as described in Materials and Methods. Numbers in parentheses indicate percentages of control, BSA-injected oocytes.

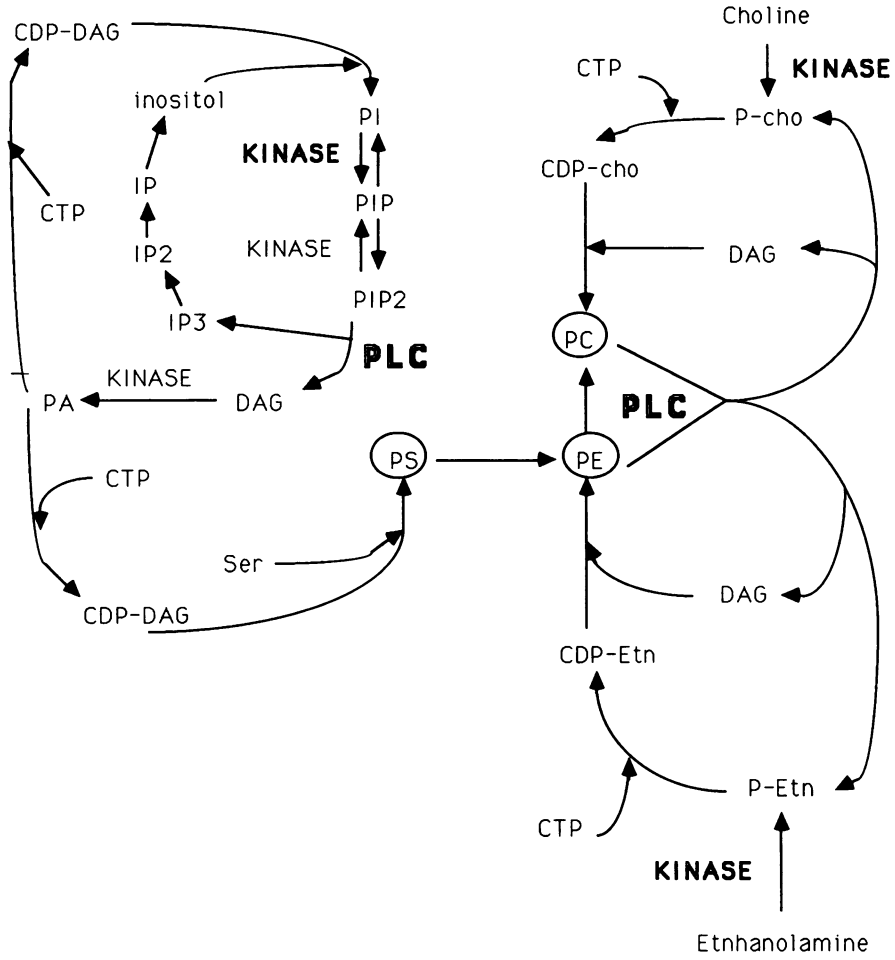

FIG. 7. Schematic representation of enzymes involved in phospholipid metabolism potentially activated by p21^{ras} proteins. Metabolism of the four major phospholipids of eucaryotic membranes is schematically represented to indicate the relationship among them. The enzymes potentially activated directly or indirectly by p21^{ras} proteins are in bold characters as follows: PLC, phospholipase C; KINASE, PI kinase, choline kinase, or ethanolamine kinase (see text for details). Abbreviations not given in text: PIP, phosphatidylinositol phosphate; PS, phosphatidylserine; PE, phosphatidylethanolamine; P-cho, phosphocholine; P-Etn, phosphoethanolamine; IP3, inositol 1,4,5-trisphosphate; IP2, inositol bisphosphate; CDP-cho, CDP-choline; CDP-Etn, CDP-ethanolamine.

time points, phosphocholine could also be generated by hydrolysis of PC.

The enzymatic activity of choline kinase was investigated in oocytes extracts in an effort to establish whether this activity was altered after microinjection of p21^{ras} proteins. At either 2 or 20 min after microinjection of transforming *ras* protein, a two- to threefold increase in choline kinase activity was observed (Table 1). By contrast, no alteration in choline kinase activity was detected when normal p21 protein was injected. These results demonstrate that choline kinase activation is an early event of microinjection of p21^{ras} proteins into *Xenopus* oocytes.

DISCUSSION

The results presented in this report are consistent with the activation by p21^{ras} proteins of the production of DAG from a source other than hydrolysis of PIs in *X. laevis* oocytes. Generation of DAG correlated with the ability of the proteins to induce GVBD as well as with their ability to undergo acylation and to localize to the plasma membrane. These results are in full agreement with previous observations in NIH 3T3 cells transformed by *ras* oncogenes, where transforming activity correlated with elevated levels of DAG but not PI hydrolysis (13, 29). Moreover, membrane localization is required for biological activity in both systems (8, 22, 27, 28).

The nature of the elevated levels of DAG found in NIH 3T3 cells transformed by *ras* oncogenes, as well as the increased DAG levels in *Xenopus* oocytes after microinjection with recombinant *ras* proteins, remains to be elucidated. However, the results shown here suggest that DAG could be originated, at least in part, as a consequence of activation of PC metabolism, since the first alterations observed were generation of phosphocholine and CDP-choline and stimulation of the incorporation of [^{3}H]choline into PC. These alterations were observed before any increase in the levels of DAG could be detected. Moreover, microinjection of transforming p21^{ras} into oocytes induced a rapid increase in the choline kinase activity of oocyte extracts. Therefore, activation of choline kinase by *ras* p21 proteins, directly or indirectly, seems to be a reasonable explanation for this effect. In agreement with this hypothesis are the recent

findings that *ras*-transformed NIH 3T3 cells show increased choline kinase activity (16).

Although a role for phosphocholine as second messenger cannot be completely ruled out, the relatively high intracellular concentration of this metabolite makes it unlikely. However, the most significant effect of $p21^{ras}$ microinjection could be the generation of DAG, since this metabolite seems to be associated with the GVBD induced by *ras*. PC is one of the major components of plasma membranes in mammalian cells (6). Small changes in the total amount of this lipid, which would not be detected, may account for the relatively modest DAG increases (50%) observed in *ras*-transformed cells (20). In fact, the amount of PC in mammalian cells is around 35 nmol/100 nmol of total phospholipids (6), whereas DAG levels are around 0.5 to 1 nmol/100 nmol (6, 20). Hydrolysis of 5% of total PC would induce an increase of DAG to around 2 to 3 nmol/100 nmol of total phospholipids, a two- to sixfold increase over basal levels. Thus, to achieve a 50% increase in DAG, the hydrolysis of only 1% of total PC would be required. Moreover, activation of both choline kinase activity and a PC-specific PLC could generate increased levels of DAG without detectable alterations in total PC content.

Oocytes microinjected with $p21^{ras}$ showed a much more drastic increase in DAG (300 to 500%) than did *ras*-transformed mammalian fibroblasts (50% increase). This alteration correlates with a readily detectable, dose-dependent increase in [^{3}H]choline incorporation into PC and the observed release of phosphocholine. Besides the obvious differences that might derive from a diverse enzymatic pool involved in the regulation of lipid metabolism in frog oocytes and mammalian fibroblasts and the differences between chronic and acute conditions, a specific pool of PC might be regulated in a manner similar to that found in PI metabolism (2).

The regeneration of PIs from released IPs can be achieved only through conversion of IPs to inositol. To my knowledge, no kinase has been found that phosphorylates inositol to directly produce IPs. However, a kinase has been purified that is able to phosphorylate both choline and ethanolamine to phosphocholine and phosphoethanolamine, respectively (10). Some authors have been able to separate both activities as different entities (reviewed in reference 18). Since phosphocholine can enter directly the anabolic pathway of PC, this finding implies a substantial difference between PI and PC metabolism (revised in 19).

My results suggest the possibility of $p21^{ras}$-mediated activation of a series of kinases involved in the regulation of phospholipid metabolism, resulting in the generation of DAG and other metabolites. This possibility implies that $p21^{ras}$ does not activate a PLC per se, but it could affect the levels of IPs and phosphocholine by different mechanisms (Fig. 7). This hypothesis is based on two observations. First, microinjection of *ras* proteins leads to the activation of a choline kinase before the generation of detectable levels of DAG. Second, the first detectable change in PI metabolism after microinjection of high levels of $p21^{ras}$ into *Xenopus* oocytes before increased levels of IPs are detected is a decrease of PI levels paralleled by an increase in phosphoinositol 4,5-bisphosphate (PIP_2) (11). This could be a consequence of activation of a PI kinase.

There is a higher efficiency of choline kinase activation by microinjected transforming $p21^{ras}$ protein than of conversion of PI to PIP_2. However, generation of PIP_2 at high levels of microinjection of $p21^{ras}$ implies the ability of the protein to intervene in this route. An alternative explanation is that DAG, generated by activation of PC metabolism, could activate PI metabolism through protein kinase C (PKC). Evidence for a functional relationship between $p21^{ras}$ and PKC has been previously reported (12). Activation of PKC could be the link between PC and PI metabolism, as judged from previous observations implying a role of PKC in regulation of PI kinase activity (26). The results presented in this study are consistent with a complex regulatory mechanism for phospholipid metabolism in which $p21^{ras}$ proteins have an important regulatory function.

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