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Role of GTPase activating protein in mitogenic signalling through phosphatidylcholine-hydrolysing phospholipase C

Isabel Dominguez[1], Mark S.Marshall[2], Jackson B.Gibbs[2], Antonio García de Herreros[1], Maria E.Cornet[1], Grazia Graziani[1], Maria T.Diaz-Meco[1], Terje Johansen[3], Frank McCormick[4] and Jorge Moscat[1,5,6]

[1]Medicina y Cirugía Experimental, Hospital General Gregorio Marañón, Dr Esquerdo 46, 28007 Madrid, Spain, [2]Department of Cancer Research, Merck Sharp and Dohme Research Laboratories, West Point, PA 19486, USA, [3]Institute of Medical Biology, University of Tromso, 9001 Tromso, Norway, [4]Department of Molecular Biology, Cetus Corporation, Emeryville, CA 94608, USA and [5]Centro de Biología Molecular UAM-CSIC, Canto Blanco, 28049 Madrid, Spain.
[6]Corresponding author

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Recent evidence has accumulated showing that activation of PLC-catalysed hydrolysis of phosphatidylcholine (PC-PLC) is a critical step in mitogenic signal transduction both in fibroblasts and in oocytes from *Xenopus laevis*. The products of *ras* genes activate PC-PLC, bind guanine nucleotides, have intrinsic GTPase activity, and are regulated by a GTPase-activating protein (GAP). It has been suggested that, in addition to its regulatory properties, GAP may also be necessary for *ras* function as a downstream effector molecule. In this study, evidence is presented that strongly suggests that the functional interaction between *ras* p21 and GAP is sufficient and necessary for activation of maturation promoting factor (MPF) H1-kinase activity in oocytes, and that PC hydrolysis is critically involved in this mechanism. Therefore, we identify GAP as a further step required for signalling through PC-PLC, and necessary for the control of oocyte maturation in response to *ras* p21/insulin but not to progesterone.
Key words: GAP/maturation/oocytes/PC-PLC/*ras*

Introduction

Considerable effort has been invested to identify critical steps in mitogenic signal transduction pathways. Particularly, phospholipid degradation, which is potently activated following stimulation with growth factors is the core of intense research (Besterman *et al.*, 1986; Berridge, 1987; Exton, 1990; Pessin *et al.*, 1990). Recently, evidence has accumulated showing that activation of PLC-catalysed hydrolysis of phosphatidylcholine (PC-PLC) is sufficient to mimic a significant portion of the PDGF mitogenic signal (Larrodera *et al.*, 1990). PLC-mediated PC hydrolysis has also been shown to be stimulated by the product of *ras* oncogene, *ras* p21 (Lacal *et al.*, 1987a; Price *et al.*, 1989; Diaz-Laviada *et al.*, 1990; Lopez-Barahona *et al.*, 1990), whose role in mitogenic cascades has been demonstrated (Mulcahy *et al.*, 1985; Smith *et al.*, 1986).

Oocytes from *Xenopus laevis* are a suitable system for investigating the involvement of different enzymatic activities in relevant signal transduction mechanisms (Korn *et al.*, 1987; Lacal *et al.*, 1987b). *Xenopus* oocytes undergo a maturation program following stimulation with either insulin or progesterone. Several lines of evidence indicate the specific involvement of *ras* p21 in the maturation signalling cascades activated by insulin/IGF-1: (i) microinjection of *ras* p21 activates maturation in oocytes (Birchmeier *et al.*, 1985); (ii) microinjection of a neutralizing anti-*ras* p21 antibody (Y13-259) blocks the maturation program induced by insulin but not by progesterone (Korn *et al.*, 1987). A more definitive proof of the involvement and importance of PC-PLC in cell growth has recently been obtained in *Xenopus* oocytes (Garcia de Herreros *et al.*, 1991), where we have shown that PLC-mediated hydrolysis of PC is both necessary and sufficient for activation of maturation by insulin/*ras* p21 (Garcia de Herreros *et al.*, 1991). Thus, microinjection of a permanently activated PC-PLC triggers a potent maturating response, whereas microinjection of a neutralizing anti-PC-PLC antibody specifically blocks the maturation program induced by insulin/*ras* p21 (Garcia de Herreros *et al.*, 1991). Stimulation of maturation by progesterone was not affected by microinjection of anti-PC-PLC. Of note is that whereas insulin/*ras* p21 promote a potent induction of PLC-mediated PC hydrolysis in *Xenopus* oocytes, progesterone is unable to do so (Garcia de Herreros *et al.*, 1991). The mechanisms utilized by *ras* p21 to activate PC-PLC are as yet unknown.

The products of *ras* genes bind guanine nucleotides (Scolnick *et al.*, 1984), have intrinsic GTPase activity (McGrath *et al.*, 1984), and are regulated by GAP (Trahey and McCormick, 1987). This molecule appears to bind to the effector domain of both normal and transforming *ras* p21 and is capable of modulating the GTPase activity of only the normal *ras* p21 (Adari *et al.*, 1988; Cales *et al.*, 1988; Vogel *et al.*, 1988; McCormick, 1989). It has been suggested that, in addition to its regulatory properties, GAP may also be necessary for *ras* function as a downstream effector molecule (Cales *et al.*, 1988; McCormick, 1989). In the study presented here we demonstrate that the functional interaction between *ras* p21 and GAP is sufficient and necessary for activation of maturation promoting factor (MPF) H1-kinase activity in oocytes, and that PC hydrolysis is critically involved in this mechanism. Therefore, we identify GAP as a further step required for signalling through PC-PLC.

Results and discussion

In addition to its down-modulatory properties on *ras*, GAP has been suggested to play a role as an effector molecule in the transmission of the mitogenic signals generated by this oncogene. If this model is correct and GAP is not just a mere down-modulator of *ras*, and taking into account that PC-PLC is both activated by *ras* p21 (Lacal *et al.*, 1987a; Price

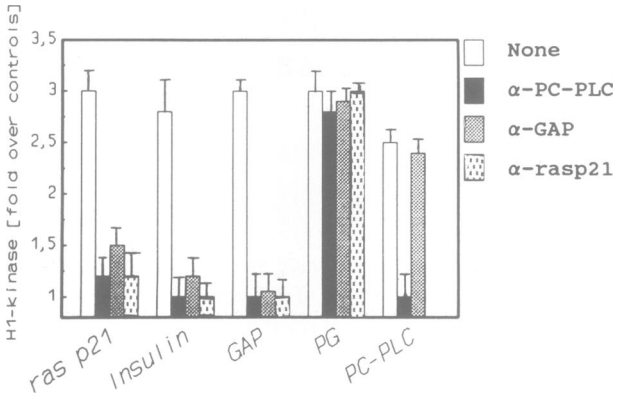

Fig. 1. Induction of H1-kinase activity in *Xenopus laevis* oocytes. Stage VI oocytes prepared following standard procedures were microinjected either with buffer control or with anti-*B.cereus* PC-PLC (60 ng), anti-*ras* p21 antibody (Y13-259; 50 ng), or neutralizing anti-GAP antibody (60 ng). Afterwards, they were microinjected with either transforming v-H-*ras* p21 (10 ng), *B.cereus* PC-PLC (25 μU), or bovine recombinant GAP purified from *E.coli*. The amount of GAP microinjected was 100 pg which gives a maximum response (not shown). Oocytes were also incubated with either 1 μM insulin or 1 μM progesterone. Reactions were stopped by 4–6 h after microinjection of different stimuli. The preparation of extracts and H1-kinase assays were carried out as described under Materials and methods. Control level of H1-kinase was 76 fmol/min/oocyte, and was not affected by microinjection with different antibodies. Likewise microinjection of non-relevant IgG antibodies produced no effects on the stimulation of H1-kinase by different stimuli. Results are means ± standard deviation of three independent experiments with incubations in duplicate.

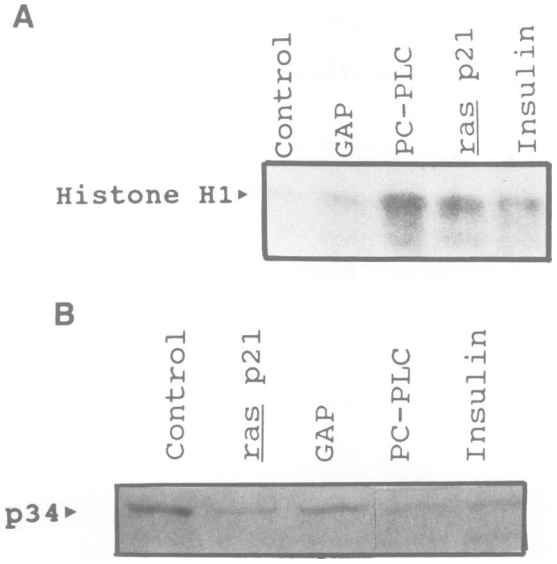

Fig. 2. Induction of p34 dephosphorylation and H1 kinase in *Xenopus laevis* oocytes. (**A**) extracts from oocytes microinjected either with bovine recombinant GAP (100 pg), *B.cereus* PC-PLC (25 μU), or with transforming v-H-*ras* p21 (10 ng), or stimulated with insulin (1 μM), were precipitated with $p13^{suc1}$–agarose, and H1 kinase activity was determined as described in Materials and methods, followed by separation of histone H1 by SDS–PAGE. (**B**) extracts from oocytes treated as described above were separated by SDS–PAGE and immunoblotted with an anti-phosphotyrosine antibody as described previously. Essentially identical results were obtained in three other experiments.

et al., 1989; Diaz-Laviada *et al.*, 1990; Lopez-Barahona *et al.*, 1990; Garcia de Herreros *et al.*, 1991) and necessary for induction of the oocyte's maturation pathway controlled by this oncogene (Garcia de Herreros *et al.*, 1991), it is conceivable that GAP might be responsible for the generation of maturation signals through stimulation of PC breakdown. To examine this possibility, we initially microinjected *Xenopus laevis* oocytes with either transforming v-H-*ras* p21 or GAP, or with 25 μU of a highly purified, permanently activated PC-PLC from *Bacillus cereus* (Larrodera *et al.*, 1990; Little, 1988). Oocytes were also incubated either with 1 μM insulin or 1 μM progesterone. Following these treatments, extracts were obtained and H1-kinase was determined. This kinase activity has been thoroughly characterized as a component of MPF which includes $p34^{CDC28/cdc2+}$ (Murray and Kirschner, 1989), and is an excellent marker of the biochemical mechanisms controlling oocyte maturation. Results from Figure 1 clearly show that transforming v-H-*ras* p21, PC-PLC and insulin, potently stimulate H1-kinase activity, in good agreement with previously published results (Garcia de Herreros *et al.*, 1991). Importantly, microinjection of bovine recombinant GAP (expressed and purified from *E.coli*; Figure 1) or human recombinant GAP (from baculovirus-infected Sf9 insect cells, not shown) also stimulates H1-kinase activity. After the solution containing GAP was boiled for 2 min, this effect was lost. Interestingly, the effect of GAP on H1-kinase is completely abolished when GAP is microinjected in the presence of a neutralizing anti-GAP antibody (Yatani *et al.*, 1990) (Figure 1). Taken together all these results suggest that, in this system, GAP may be contributing to the generation of mitogenic signals by *ras*.

It is known that the product of the *suc1* gene, $p13^{suc1}$, specifically interacts with p34, and this interaction does not affect its H1-kinase activity '*in vitro*' (Draetta *et al.*, 1987). Therefore, in order to demonstrate that the H1-kinase activity stimulated by GAP is actually due to p34, extracts from oocytes either control stimulated with insulin or microinjected with GAP, transforming v-H-*ras* p21, or PC-PLC, were incubated with p13 linked to agarose beads. H1-kinase activity was determined in the corresponding precipitates. Results from Figure 2A show that precipitates from GAP-microinjected oocytes gave increased H1-kinase activity indicating that GAP actually activates p34. Again, PC-PLC, transforming v-H-*ras* p21, and insulin potently activated this parameter. Numerous studies demonstrate that p34 kinase activity is subject to control by complex changes in its pattern of phosphorylation. In this regard, it has been demonstrated extensively that activation of p34 kinase is associated with dephosphorylation on tyrosine 15 (Morla *et al.*, 1989; Krek and Nigg, 1991). This can be easily followed in immunoblots of oocyte extracts with a specific antiphosphotyrosine antibody (Ferrel *et al.*, 1991). Results from Figure 2B show that insulin, transforming v-H-*ras* p21 and PC-PLC, all trigger tyrosine dephosphorylation of p34. Of note is that GAP also promotes a dramatic tyrosine dephosphorylation of this protein.

Yatani *et al.* (1990) have recently demonstrated that GAP actions on K^+[ACh] channels required the interaction with its known target, *ras* p21. To investigate whether a similar mechanism is operating in our system, GAP was microinjected into oocytes in the presence of the neutralizing anti-*ras* antibody, Y13-259, and H1-kinase activity was determined thereafter. Results shown in Figure 1 indicate that the presence of Y13-259 dramatically inhibited GAP-induced H1-kinase, suggesting that the interaction of GAP

with endogenous *ras* p21 is required for the induction of this kinase. Interestingly, microinjection of neutralizing anti-GAP antibody also inhibited transforming v-H-*ras* p21-induced H1-kinase (Figure 1). These results would be consistent with the notion that GAP transduces mitogenic signals channelled by *ras* p21, and that the direct or indirect interaction of both molecules is required for H1-kinase activity to be triggered. Additional results shown in Figure 1 are in keeping with this model. Thus, not only anti-*ras* p21 but also anti-GAP blocks insulin but not progesterone-induced H1-kinase.

If, as has been demonstrated, PC-PLC transduces the mitogenic signals generated by insulin/*ras* p21 (Garcia de Herreros *et al.*, 1991), conceivably this phospholipase would also control H1-kinase activity in response to GAP. Therefore, the specific blockade of PC-PLC should lead to the inhibition of GAP-induced H1-kinase. Conversely, the blockade of GAP should not affect the activation of this parameter by *B.cereus* PC-PLC. Results from Figure 1 show that this is actually the case. Thus, microinjection of a neutralizing affinity-purified anti-*B.cereus* PC-PLC antibody, which has previously been described (Larrodera *et al.*, 1990; Garcia de Herreros *et al.*, 1991), significantly inhibits not only insulin/*ras* p21-induced H1-kinase (Garcia de Herreros *et al.*, 1991) but also the activation of this kinase by GAP. However, microinjection of neutralizing anti-GAP antibody does not affect the activation of H1-kinase by *B.cereus* PC-PLC (Figure 1). As a control, activation of this kinase by progesterone is not affected by any of these antibodies. Taken together, all these results are consistent with a model whereby PC-PLC is directly or indirectly involved in the downstream transduction of mitogenic signals generated by *ras* p21/GAP. In accordance with this conclusion are the results from Figure 3 where it is shown that microinjection of GAP promotes PC-PLC activation although to a lesser extent than by transforming v-H-*ras* p21 (Garcia de Herreros *et al.*, 1991). This is consistent with the notion that GAP is less efficient than transforming v-H-*ras* p21 in activating H1-kinase, and that PC-PLC is critical in the stimulation of this enzymatic activity. Therefore, the results presented here suggest that GAP plays an important role in the transmission of signals generated by *ras*. The fact that endogenous *ras* p21 is required for GAP-mediated activation of H1-kinase indicates that, presumably, part of that *ras* is in the GTP-bound state at resting conditions. This could also explain the fact that microinjection of GAP mimics only partially the response of transforming v-H-*ras*, probably because for GAP to act *ras* has to generate transducing signals. If this model is correct it can be predicted that microinjection of GAP will synergistically interact with transforming v-H-*ras* in activating mitogenic signals in oocytes. To test this hypothesis the following experiment was carried out. Oocytes were microinjected either with GAP, transforming v-H-*ras* p21, or with GAP plus transforming v-H-*ras* p21, and H1-kinase and the breakdown of the germinal vesicle (GVBD, a good parameter of oocyte maturation) were determined at different times thereafter. Results from Figure 4A indicate that H1-kinase activity was significantly elevated only 3 h after microinjection of transforming v-H-*ras* p21. Microinjection of GAP stimulated H1-kinase by 4–6 h. However, microinjection of transforming v-H-*ras* p21 and GAP together reproducibly activates H1-kinase by 1 h, suggesting a synergistic interaction between both molecules. Consistent with this notion are the results from Figure 4B which demonstrate that although microinjection

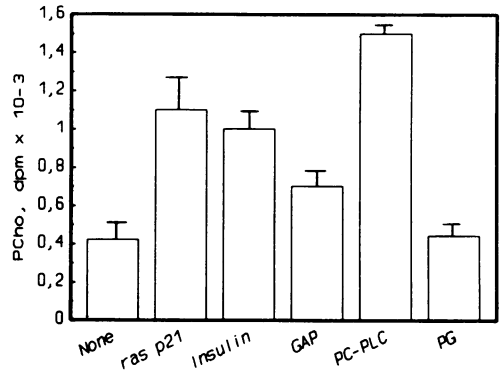

Fig. 3. PCho release in response to different stimuli. Stage VI *Xenopus* oocytes were labelled with [methyl-^{14}C]choline, after which they were either incubated with 1 μM insulin, or with 1 μM progesterone, or were microinjected with 10 ng of transforming v-H-*ras* p21, recombinant bovine GAP (as described in the legend to Figure 1), or with 25 μU of *B.cereus* PC-PLC. Reactions were stopped, either 20 min following microinjection of *ras* p21, GAP, and PC-PLC, or 4 h after addition of insulin or progesterone. PCho levels were determined by thin layer chromatography, followed by autoradiography of plates in which standards corresponding to the different water-soluble choline metabolites were included as described under Materials and methods. GAP and *ras* preparations were free of any contaminant phospholipase activity (not shown). Results, expressed in d.p.m./five oocytes, are means ± standard deviation of three independent experiments with incubations in duplicate.

of GAP alone is unable to promote oocyte maturation, it dramatically accelerates the induction of GVBD by transforming v-H-*ras* p21.

All these results suggest that although GAP by itself is unable to promote a full maturation response in oocytes, in keeping with previously published results (Gibbs *et al.*, 1989), it synergistically enhances the action of transforming v-H-*ras*. This may be interpreted as, that the partial activation by GAP of p34-associated H1-kinase is not enough to drive a full maturation response in the oocyte. This is in good agreement with preliminary data (not shown) indicating that GAP alone is also unable to stimulate other activities that, like MAP-2 kinase, are critically involved in mitogenic signal transduction (Anderson *et al.*, 1990). Of note is that results from Figure 4C demonstrate that GAP is necessary for the specific activation by insulin/*ras* p21 of maturation in oocytes, since microinjection of neutralizing anti-GAP antibody inhibits the induction of GVBD by transforming v-H-*ras* and insulin but not by progesterone.

It has recently been shown that overexpression of GAP in fibroblast cell lines inhibits transformation induced by c-*ras* but does not affect transformation by either transforming v-*ras* or v-*mos* (Zhang *et al.*, 1991). Those findings may be interpreted as that GAP is functioning solely as a negative modulator of *ras*. The results so far shown here would not be consistent with such a model and suggest that besides its regulatory actions on the GTP/GDP bound state of *ras*, GAP is actively participating in the transduction of mitogenic signals.

Microinjection of normal *ras* p21 produces little or no effect on the biochemical parameters controlling oocyte maturation (not shown). Furthermore, microinjection of GAP does not synergistically interact with normal *ras* p21 in the oocyte (not shown). Therefore, it is difficult to compare the effects of GAP in the oocyte with those detected in fibroblast cell lines (Zhang *et al.*, 1991) with regard to the mechanism of action of c-*ras*. However, we analysed

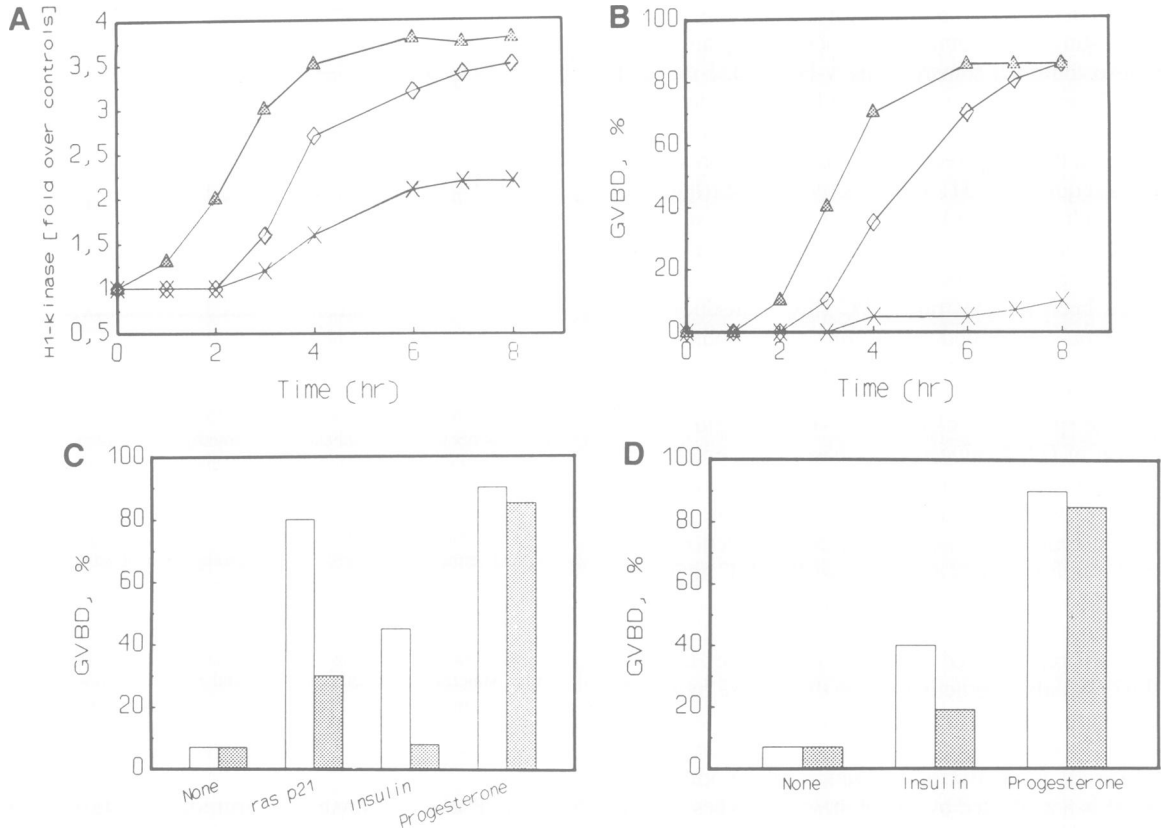

Fig. 4. Functional interaction of GAP and transforming *ras* p21 in oocyte maturation. (**A**) oocytes were microinjected with either bovine recombinant GAP (100 pg) (×), transforming v-H-*ras* p21 (10 ng) (◇), or with GAP plus transforming v-H-*ras* p21 (△), and H1-kinase activity was determined at different times thereafter, as described in the legend to Figure 2A. (**B**) oocytes were treated as described above and the breakdown of the germinal vesicle was determined. (**C**) oocytes either control (empty bars) or microinjected with neutralizing anti-GAP antibody (60 ng) (filled bars), were stimulated with either insulin (1 μM) or progesterone (1 μM), or microinjected with *ras* p21 (10 ng) and the breakdown of the germinal vesicle was determined by 6 h. (**D**) oocytes either control (empty bars) or microinjected with bovine recombinant GAP (100 pg) (filled bars), were stimulated with either insulin (1 μM) or progesterone (1 μM), and the breakdown of the germinal vesicle was determined by 6 h. Essentially identical results were obtained in three other experiments.

how microinjection of GAP affected the ability of insulin to promote maturation. Results from Figure 4D demonstrate that GAP dramatically inhibits insulin-induced GVBD with little or not effect on progesterone-activated GVBD. These results can be interpreted as that GAP is negatively modulating the activation of c-*ras* by insulin-triggered signals, probably by increasing its GTPase activity. If it is assumed that v-*src* transfectants permanently activate the normal mitogenic cascades in fibroblasts, our results will be in agreement with those of Nori *et al.* (1991) and DeClue *et al.* (1991) demonstrating that GAP inhibits transformation by *src* oncogene.

These apparently contradictory results could be explained if it is considered that GAP not only down-modulates c-*ras* but also actively participates in the transmission of mitogenic signals. The lack of effect of GAP on the GTPase activity of *ras* once this is oncogenically mutated unveils the important function of GAP as a transmitter of mitogenic signals. The fact that overexpression in fibroblasts or microinjection of GAP in oocytes down-modulates normal mitogenic pathways indicates that the GAP effector properties are tightly controlled and are quantitatively less important than its down-modulating actions. However, this latter function seems to play a critical role at least in transformation events mediated by mutated *ras* p21. In this regard, it is tempting to speculate that mutations in the GAP molecule that would lead to the loss of its down-modulatory properties will make of GAP an oncogene. In any event, the results from Figure 4C clearly indicate that GAP is necessary for induction of maturation both by transforming *ras* and insulin which stresses its importance as a downstream mitogenic transmitter.

A number of recent studies are unveiling the mechanisms of activation as well as the function of PLC-mediated breakdown of PC. Our recent data are consistent with the idea that the stimulation of PC-PLC is important and analogous to the action of *ras* p21 (Lacal *et al.*, 1987a; Price *et al.*, 1989; Diaz-Laviada *et al.*, 1990; Lopez-Barahona *et al.*, 1990; Garcia de Herreros *et al.*, 1991). According to the results presented here, GAP appears to be a further step necessary in the chain of mitogenic signals channelled by PC-PLC.

Materials and methods

Oocyte culture and labelling

Oocytes were prepared following standard procedures (Garcia de Herreros *et al.*, 1991). Briefly, ovaries from *Xenopus laevis* frogs (Blades Biologicals, UK) were incubated with 2 mg/ml of collagenase (Boehringer Mannheim, Germany) for 45 min in modified Barth solution (MBS) without Ca^{2+} (110 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM HEPES, pH 7.8). After extensively washing, stage VI oocytes were selected and incubated overnight at 20°C. Selected oocytes

were labelled with 12 μCi/ml of [methyl-^{14}C]choline (Amersham International; specific radioactivity = 55 mCi/mmol) for 24 h in modified Barth solution, after which medium was removed and fresh, label-free medium was added and experiments were carried out after a 30 min equilibration period.

Analysis of products of phospholipid metabolism

Labelled oocytes were treated or not with the corresponding agonists, or were microinjected with *B.cereus* PC-PLC, recombinant GAP, or with transforming v-H-*ras* p21. At different times, reactions were stopped by adding ice-cold methanol. Methanolic cell extracts were fractionated into chloroform and aqueous phases as previously described (Garcia de Herreros *et al.*, 1991). The presence and levels of water-solule choline metabolites were evaluated in the aqueous phases by thin layer chromatography (Garcia de Herreros *et al.*, 1991), followed by autoradiography of plates in which standards corresponding to the different water-soluble choline metabolites were included.

Isolation of PC-PLC from Bacillus cereus and preparation of affinity-purified antibody

PtdCho-PLC was isolated from cultures of *Bacillus cereus* SE-1 essentially as described previously (Larrodera *et al.*, 1990; Garcia de Herreros *et al.*, 1991). Following this protocol the enzyme preparation was purified to complete homogeneity as confirmed by SDS−PAGE followed by silver-staining. The specific activity of the purified enzyme was 1.5 U/μg. A rabbit antiserum was raised against this *B.cereus* PC-PLC by multiple intradermal injections with 75 μg of this enzyme. Serum was diluted 1:3 in phosphate-buffered saline (PBS) and applied to an Affigel 10 (Bio-Rad) column containing immobilized *B.cereus* PC-PLC. The column was washed with PBS, with PBS with increasing salt (up to 1 M NaCl), and with PBS containing 3 M urea before elution with 4 M urea, 0.5 M NaCl adjusted to pH 3.0 with acetic acid. The affinity-purified antibody was eluted directly into 1 M glycine−NaOH, pH 10.5 and dialysed extensively against a suitable buffer. The sole presence of heavy and light antibody chains in the final preparation was confirmed by SDS−PAGE followed by silver-staining (Larrodera *et al.*, 1990; García de Herreros *et al.*, 1991).

Preparation of ras p21 proteins

Transforming and normal *ras* p21 proteins were expressed in bacteria as previously described (Lacal *et al.*, 1984). A final step of purification consisted in a gel filtration chromatography through a 2.5×90 cm Sephadex G-100 column; fractions containing the purified protein were pooled and dialysed extensively against 20 mM Tris−HCl pH 7.5 to remove urea and kept at −70°C until utilized.

Preparation of recombinant GAP

Bovine recombinant GAP was expressed and purified from *E.coli* as described (Marshall *et al.*, 1989). Human recombinant GAP was purified from Sf9 cells infected with baculovirus vectors expressing GAP cDNA as described previously (Trahey *et al.*, 1988).

Preparation of neutralizing anti-GAP antibody

Affinity-purified neutralizing polyclonal anti-GAP antibody was made by injecting rabbits with human recombinant GAP purified from insect cells (Trahey *et al.*, 1988).

Analysis of oocyte maturation

Groups of 20 oocytes were cultured at 20°C in modified Barth solution, and germinal vesicle (nuclear) breakdown (GVBD) was assessed by the appearance of a white spot in the animal pole. In some cases, nuclear breakdown was confirmed by dissection of oocytes fixed in 10% trichloroacetic acid (Garcia de Herreros *et al.*, 1991).

Maturation promoting factor histone 1 kinase assay

Twenty oocytes were homogenized in a buffer containing 20 mM HEPES (pH 7.0), 10 mM β-glycerophosphate, 5 mM EGTA, 5 mM $MgCl_2$, 50 mM NaF, 2 mM dithiothreitol, 100 μg of leupeptin/ml, and 100 μM PMSF. Following centrifugation at 13 000 *g* for 15 min, extracts (1−2 mg/assay) were assayed for 10 min at 30°C in a final reaction volume of 50 μl containing 20 mM HEPES (pH 7.0), 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, 100 μM γ-^{32}P (2−5 d.p.m./fmol), 0.2 μg of heat-stable inhibitor of cAMP-dependent protein kinase, and 0.6 mg/ml of Sigma type III-S calf thymus histone. Reactions were terminated, spotted onto Whatman p81 phosphocellulose paper, washed and quantified as described (Garcia de Herreros *et al.*, 1991). In some experiments, extracts were incubated with p13^{suc1} linked to agarose beads, and histone 1 kinase activity was determined in the precipitates followed by separation in SDS−PAGE.

Phosphorylation of p34$^{CDC28/cdc2+}$

The tyrosine phosphorylation state of p34$^{CDC28/cdc2+}$ was determined by immunoblotting with antiphosphotyrosine antibodies as described previously (Morla *et al.*, 1989; Ferrel *et al.*, 1991).

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