Biochem. J. (1997) **322**, 519–528 (Printed in Great Britain)

Activation of phospholipase D by growth factors and oncogenes in murine fibroblasts follow alternative but cross-talking pathways

Luis DEL PESO, Luisa LUCAS, Pilar ESTEVE and Juan Carlos LACAL*
Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid, Spain

Phospholipase D (PLD) is activated by a variety of stimuli, including mitogenic stimulation by growth factors and oncogene transformation. Activation of PLD by growth factors requires protein kinase C (PKC) since depletion of the enzyme by down-regulation or direct inhibition by specific drugs completely abrogates this effect. Transformation by the *ras* and *src* oncogenes is also associated with an increase in basal PLD activity. However, this effect is not dependent on PKC, suggesting that growth factors and oncogenes may activate PLD by two independent mechanisms. Here we demonstrate that activation of PLD by phorbol esters is greatly enhanced in *ras*-transformed cells, suggesting synergistic activation of PLD by *ras* oncogenes and PKC. Also, *ras*-transformed cells showed a dramatic attenuation of the PLD activation induced by growth factors, although receptor function was still detectable. This attenuation paralleled the specific uncoupling of the phosphatidylinositol-specific phospholipase C (PI-PLC) pathway, indicating that activation of PLD by growth factors may be mediated by PI-PLC and PKC activation. Attenuation of PLD activation by platelet-derived growth factor was also observed in several oncogene-transformed cells, as well as the uncoupling of the PI-PLC pathway. Neither the co-operation with PKC activation nor the attenuation of the PLD response to growth factors in *ras*-transformed cells was a general consequence of cell transformation, since cells transformed by other oncogenes showed a normal response to either treatment. These results support the existence of at least two alternative signalling routes for the activation of PLD, one mediated by the PI-PLC/diacylglycerol/PKC pathway and a second one mediated by several oncogenes, independent of the PKC pathway, which synergizes with the PI-PLC/PKC-dependent pathway.

INTRODUCTION

Several lines of evidence implicate the involvement of phospholipase D (PLD) in signal-transduction processes in a variety of cellular systems. Besides the overwhelming evidence that growth factors activate PLD [1], it is well established that PLD mediates signal-transduction processes such as the action of gonadotrophin-releasing hormone on ovary cells [2] and granule secretion in human neutrophils [3]. Also, a PLD activity is involved in sporulation in *Saccharomyces* [4]. We have also recently demonstrated that (1) maturation of *Xenopus laevis* oocytes induced by microinjection of the activated Ras protein is accompanied by activation of PLD [5], (2) germinal vesicle breakdown induced by Ras proteins is inhibited by neomycin at concentrations reported to be inhibitory for PLD and (3) PLD-derived products mimic the biological effect of Ras proteins in this system [5]. In addition, several reports demonstrate that exogenously added PLD has mitogenic activity [6,7].

In support of a role for PLD in signal transduction, growth factor stimulation of murine fibroblasts induces a rapid and transient activation of PLD, which has been demonstrated to be dependent on protein kinase C (PKC) by down-regulation of the enzyme [8,9]. Overexpression of PKCα enhances PLD activation by growth factors, further supporting the involvement of a diacylglycerol (DAG)-sensitive PKC isoenzyme in this process [10,11]. Although the precise mechanism for activation of PLD induced by growth factors is still not known, strong evidence suggests that it is mediated by the activation of phospholipase Cγ (PLCγ) that follows growth factor receptor activation [12,13]. Thus, on growth factor stimulation, PLCγ becomes activated, resulting in phosphatidylinositol (PI) hydrolysis and the generation of DAG and inositol trisphosphate (IP_3). IP_3 is responsible for the opening of endoplasmic reticulum Ca^{2+} channels and the increase in free cytosolic Ca^{2+}. Since classical PKCs are activated by DAG and Ca^{2+}, PLCγ may act as a link between tyrosine kinase receptors and the PKC stimulation that leads to PLD activation. In keeping with this hypothesis, activation of GTPase-coupled receptors also induces activation of PLD [12,13], which may be mediated by activation of the PI-PLCβ isoenzyme with the generation of DAG and IP_3 and the further activation of PKC.

In addition to PI-PLC and PKC, some GTPases have also been implicated in PLD activation. Treatment of membranes isolated from several cell lines with the non-hydrolysable GTP analogue, guanosine 5′-[γ-thio]triphosphate (GTP[S]), results in PLD activation. Several GTPases that belong to both the classical G-protein family as well as the Ras superfamily have been proposed as being responsible for this effect [1]. Whereas ADP ribosylation factor has been shown to induce PLD activation in membranes from HL-60 cells [14–16], the Rho protein is responsible for this effect in neutrophils and HL-60 cells [17–20]. Also Ras proteins have been implicated in the induction of PLD

Abbreviations used: PLCγ, phospholipase Cγ; PLD, phospholipase D; PKC, protein kinase C; PI-PLC, phosphatidylinositol-specific phospholipase ; PDGF, platelet-derived growth factor; DAG, diacylglycerol; IP_3, inositol trisphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; PI3K, phosphatidylinositol 3-kinase; DMEM, Dulbecco's modified Eagle's medium; TGFα, transforming growth factor α; PDBu, phorbol 12,13-dibutyrate; bFGF, basic fibroblast growth factor; FCS, fetal calf serum; PtdBut, phosphatidylbutanol; MAPK, mitogen-activated protein kinase; IP, inositol phosphate.

* To whom correspondence should be addressed.

activation, since *ras* transformation is associated with increased PLD activity in fibroblasts [6,21] as well as in *Xenopus laevis* oocytes [5]. We have recently demonstrated that, like other well-established Ras effectors such as Raf-1 and phosphatidylinositol 3-kinase (PI3K), stable *ras*-transformed cells do not always show a constitutive activated PLD, suggesting that other factors may be also involved in this activation [22].

It has been reported that, like *ras*-transformed fibroblasts, *src*-transformed fibroblasts show constitutive elevated PLD activity [23]. This effect is also observed in membranes isolated from *src*-transformed cells and it is dependent on the addition of GTP[S], suggesting that it is mediated by a GTPase [24]. It has been suggested that this GTPase corresponds to the Ras protein, since preincubation of membranes isolated from *src*-transformed cells with the Ras-neutralizing monoclonal antibody Y13-259 abrogates PLD activation [25]. Furthermore PLD activation induced by *src* involves not only Ras but also Ral, another GTPase of the Ras superfamily, through the Ras-dependent activation of Ral-GDS [21].

These results imply that, although PLD activation is a frequent event associated with growth stimulation, our actual knowledge of both the mechanism involved and its relevance remains very scant. In this study, we have further investigated the mechanisms involved in the regulation of PLD by growth factors and Ras proteins. The results presented demonstrate the existence of at least two alternative mechanisms for PLD activation, one of them involving Ras proteins.

MATERIALS AND METHODS

Cell culture and reagents

Normal NIH 3T3 mouse fibroblasts as well as *ras*-transformed derivatives were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (Gibco) under standard conditions of temperature (37 °C), humidity (95%) and CO_2 (5%). The *ras*-transformed cell lines used here were obtained from independent transfections and have been described previously [6,22], or were freshly generated using an LTR-driven vector (LP14-8) or simian virus 40 early promoter (710RAS) and present a transformed morphology as well as high expression of exogenous Ras protein. Cell lines transformed by transforming growth factor α (TGFα) (611-20), v-*src* (SRC), c-*sis* (704-42), *trk* (C51-36), v-*fgr* (C26-41) and v-*fms* (C51-64) were obtained from A. Cuadrado and S. A. Aaronson (The Derald H. Ruttenberg Cancer Center, New York, NY, U.S.A.). All *ras*-transformed cells and those transformed by the different oncogenes were generated using the same parental NIH 3T3 cell line. The cell line transformed by the *met* (12b) oncogene was obtained from M. Park (McGill University, Montreal, Canada).

[2-^{3}H]Glycerol (1 Ci/mmol) and [*methyl*-^{3}H]thymidine (45 Ci/mmol) were from Amersham, [U-^{14}C]glycerol (148 mCi/mmol) was from Moravek Biochemicals, *myo*-[2-^{3}H]inositol was from Amersham (22.8 Ci/mmol) or NEN (15.2 Ci/mmol), and butan-1-ol was from Merck. Bisindolylmaleimide was purchased from Calbiochem. Phorbol 12,13-dibutyrate (PDBu) was purchased from Sigma and platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) were from UBI.

Assay of PLD activity

Activation of PLD can be measured accurately by its trans-phosphatidylating activity on butanol to generate phosphatidyl-butanol (PtdBut) [23,26]. Cells were grown in six-well plates and labelled for 48–72 h in the presence of 10 μCi/ml [2-^{3}H]glycerol (0.5–1 Ci/mmol) or 1 μCi/ml [U-^{14}C]glycerol. In some experiments cells were labelled for 24 h in DMEM supplemented with 0.2% fetal calf serum (FCS). In down-regulation experiments, 350 nM PDBu was added to the culture medium during the labelling period. To assay PLD activity, labelling medium was discarded, and cells were washed with TD buffer (137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 20 mM Tris/HCl, pH 7.4) and incubated for 30–45 min in serum-free DMEM supplemented with 0.5% butan-1-ol in the presence of the indicated agonists or inhibitors. The cells were then scraped into 1 ml of methanol, and plates washed once with 1 ml of methanol. The two methanol samples were collected and mixed with 2.5 ml of chloroform and 1.25 ml of water. The organic phases were dried in a 37 °C heating block under a nitrogen stream and the lipids separated by TLC using silica gel 60A plates (Whatman; LK6D). The plates were developed with the upper phase of a mixture of ethyl acetate/iso-octane/acetic acid/water (90:50:20:100, by vol.) plus 1 ml of acetic acid. They were then sprayed with enhancer (Dupont) and exposed to X-ray-sensitive films. Bands corresponding to PtdBut were scraped off and counted for radioactivity. PtdBut was identified using a standard generated *in vitro* using purified PLD and [^{14}C]phosphatidylcholine as substrate. Counts in PtdBut were normalized for the radioactivity incorporated into total lipid. Where indicated, plates were visualized and quantified using an electronic autoradiography system (InstantImager; Packard).

Determination of tyrosine phosphorylation and mitogen-activated protein kinase (MAPK) activation

The experiments were performed using confluent cells that had been serum-starved for 12–24 h. The medium was replaced with serum-free DMEM containing the indicated amounts of PDGF-BB (human recombinant). Stimulation proceeded at 37 °C for 5–10 min, and was terminated by aspirating off the medium, washing twice with ice-cold TD buffer and adding 300 μl of ice-cold lysis buffer (50 mM Tris/HCl, pH 7.4, 0.25% Nonidet P40, 0.25% SDS, 150 mM NaCl, 15 mM 2-glycerophosphate, 10 mM sodium pyrophosphate, 50 mM NaF, 10 μg/ml aprotinin, 1 mM PMSF) or RIPA buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P40, 0.5% deoxycholate, 0.1% SDS, 15 mM 2-glycerophosphate, 10 mM sodium pyrophosphate, 50 mM NaF, 10 μg/ml aprotinin, 1 mM PMSF). Nuclei and detergent-insoluble material were removed by centrifugation at 11000 ***g*** (13000 rev./min) in a Microfuge for 20 min. The resulting supernatants were assayed for total cell protein (Bio-Rad), and equal amounts of cell lysates (30–100 μg) were boiled at 95 °C for 5 min in SDS/PAGE sample buffer. For Western-blot analysis, proteins were subjected to SDS/PAGE (8% gels for phosphotyrosine analysis and 10% gels for MAPK analysis poured on 20 cm × 20 cm glass plates). Resolved proteins were transferred to nitrocellulose, and blots were blocked for 1 h in 3% non-fat dried milk in T-TBS (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) plus phosphotyrosine phosphatase inhibitors (15 mM 2-glycerophosphate, 10 mM sodium pyrophosphate, 50 mM NaF) for the anti-phosphotyrosine Western blots. Blots were washed once in T-TBS and incubated for 2 h with an anti-phosphotyrosine-specific monoclonal antibody (RPN 138; Amersham) or a polyclonal serum raised against a C-terminal peptide of MAPK and developed by enhanced chemiluminescence (Amersham).

Analysis of PI3K activity

Cells were serum-starved overnight and stimulated with 10 ng/ml PDGF for 5 min. They were then washed with cold PBS, rinsed with buffer A (137 mM NaCl, 20 mM Tris/HCl, pH 8,

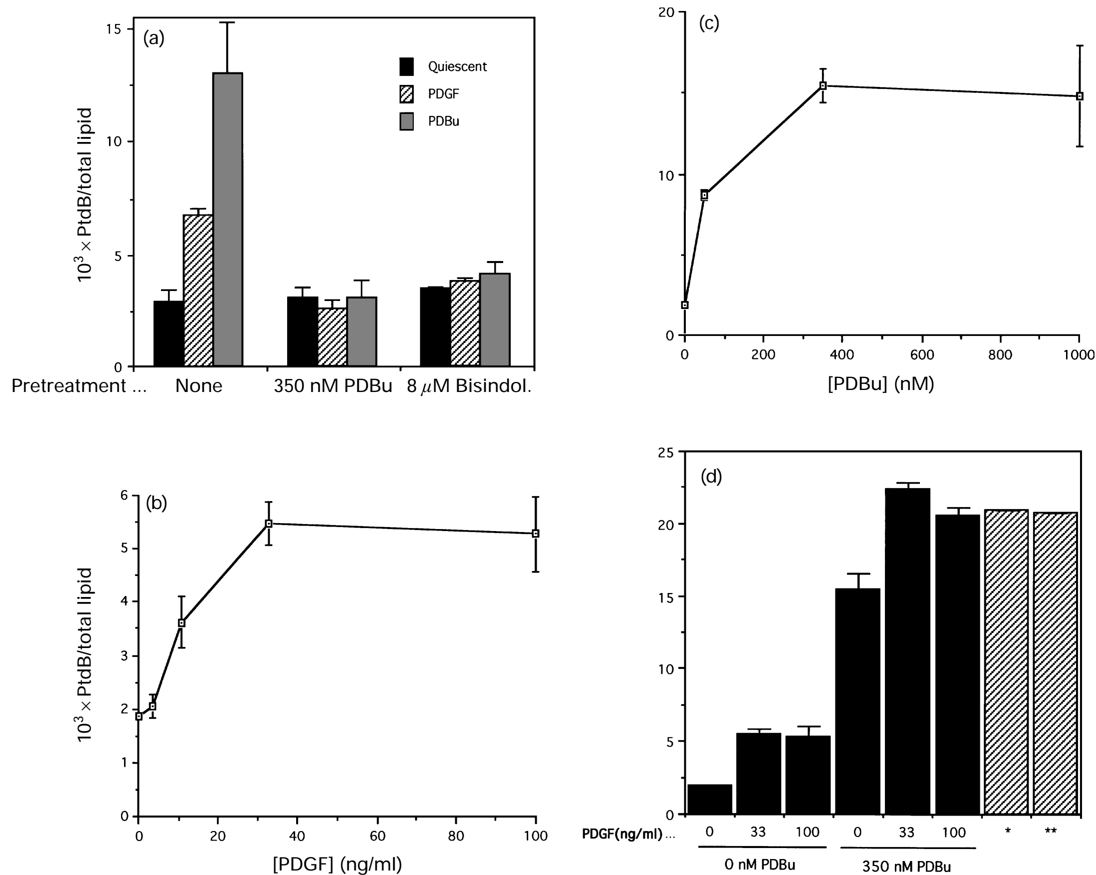

Figure 1 PDGF activates PLD in a PKC-dependent manner that is additive with PDBu treatment

(**a**) NIH 3T3 cells were grown and labelled with 1 μCi/ml [^{14}C]glycerol in DMEM supplemented with 10% newborn calf serum for 48 h. Where indicated, cells were pretreated with 350 nM PDBu during the labelling period. After labelling, cells were washed and incubated for 30 min in serum-free DMEM alone or supplemented with 8 μM bisindolylmaleimide (Bisindol). Then cells were stimulated with PDGF (20 ng/ml) or PDBu (350 nM) for 20–30 min and processed as indicated in the Materials and methods section for PLD activation using an electronic autoradiography system (InstantImager). Data represent means ± variation for duplicate determinations. The experiment shown is a representative of four (down-regulation) or two (bisindolylmaleimide) with similar results. (**b–d**) NIH 3T3 cells were grown and labelled as indicated above and then washed and stimulated for 30 min in serum-free DMEM supplemented with the indicated amounts of PDGF (**b**) or PDBu (**c**) to saturation. The effect of a combination of the two treatments, PDGF (33 or 100 ng/ml) alone or plus PDBu (350 nM), is shown in (**d**). Both the observed experimental results (black bars) and those expected for the additional independent treatment with 350 nM PDBu plus either 33 ng/ml (*) or 100 ng/ml PDGF (**) are shown. Data are means ± variation for duplicate determinations quantified by an electronic autoradiography system. The experiment shown is representative of at least three independent experiments.

1 mM MgCl, 1 mM $CaCl_2$), and lysed in buffer containing 1 % Nonidet P40 and 1 mM PMSF at 4 °C for 20 min. Insoluble material was removed by centrifugation. The supernatants (1 ml) were incubated with a mouse monclonal anti-phosphotyrosine antibody overnight at 4 °C. Equivalent amounts of Protein A–Sepharose CL4B (Sigma) were added, and incubation was continued for an additional hour. The immunoprecipitates were collected and washed once with PBS, twice with 0.5 M LiCl/0.1 M Tris/HCl, pH 7.4, and finally with 10 mM Tris/100 mM NaCl/1 mM EDTA. Immunoprecipitates were then assayed for PI phosphorylation activity, as described by Varticovski et al. [27].

Analysis of PI-PLC activity

Cells were grown in six-well plates in DMEM supplemented with 10 % newborn calf serum and labelled with 50 μCi/ml *myo*-[2-^{3}H]inositol for 48–72 h. After being radiolabelled, cultures were washed with TD buffer, and cells were incubated for 15–20 min in serum-free medium in the presence of 22 mM LiCl. Where indicated, cells were stimulated with mitogens in the presence of 22 mM LiCl. Culture media were discarded and reactions were terminated by the addition of 0.8 ml of ice-cold 5 % $HClO_4$. Wells were kept at 4 °C for 15–20 min, then the $HClO_4$ fractions were collected and the wells washed with an additional 1 ml of water. $HClO_4$ and water washes were diluted to a final volume of 5 ml with water, and total inositol phosphates (IPs) were eluted from Dowex 1 (X8) columns with 1.2 M ammonium formate/0.1 M formic acid as described previously [12]. The resulting counts were normalized for the radioactivity content in the $HClO_4$ insoluble material.

Growth in soft agar

Equivalent numbers of cells from each cell line were plated in duplicate in 60 mm dishes. Either 5×10^3 or 20×10^3 cells in 0.5 ml were mixed with 1.5 ml of DMEM + Hepes supplemented with 10 % FCS and 0.5 % noble agar warmed at 42 °C. The mixture was then plated on a layer of DMEM + Hepes supplemented with 10 % FCS and 0.5 % noble agar. Once a week 500 μl of DMEM supplemented with 10 % FCS was added to

each dish. Colonies were photographed and quantified 2–3 weeks after plating.

Mitogenic assays

Cells were seeded on 24-well plates and grown to confluence, serum-starved for 24 h in 0.2 % newborn calf serum and then stimulated for an additional 24 h with the indicated mitogens. During the last 6 h, 1 μCi/ml [*methyl*-^{3}H]thymidine (45 Ci/mmol; Amersham) was added and cells were incubated under standard conditions of temperature, humidity and CO_2 atmosphere. [^{3}H]Thymidine incorporation into DNA was determined as the amount of radioactivity present in trichloroacetic acid-insoluble material. The cells were rinsed twice with PBS and twice with ice-cold 16 % trichloroacetate. Then trichloroacetate-insoluble material was solubilized in 1 ml of 0.25 M NaOH for 1 h. The solubilized solution was scintillation counted.

RESULTS

Growth factors activate PLD in a PKC-dependent manner that is additive with PDBu treatment

Suppression of PKC by either down-regulation by chronic treatment with PDBu or direct inhibition with bisindolylmaleimide completely abrogates PLD activation by growth factors such as PDGF or bFGF in NIH 3T3 cells (Figure 1a). These results are consistent with previous observations by other investigators using other cell systems such as Swiss 3T3 cells [9]. Thus mitogenic stimulation of NIH 3T3 fibroblasts with growth factors is associated with a PKC-dependent activation of PLD. The addition of PDGF or PDBu to NIH 3T3 cells induced a dose-dependent activation of PLD with saturation at 30 ng/ml for PDGF (Figure 1b) and 350 nM for PDBu (Figure 1c). Furthermore treatment of cells with saturating concentrations of PDGF and PDBu at the same time caused an additive effect on PLD activity when compared with PLD stimulation by each agonist alone (Figure 1d), since the increase in PLD activity matched that of the addition of each independent treatment (Figure 1d). These results are in agreement with the hypothesis that activation of PLD induced by growth factors is mediated, at least in part, by activation of PI-PLC through the production of DAG and Ca^{2+}, resulting in the subsequent activation of PKC [12,13]. However, this seems not to be the only mechanism for activation of PLD, since there is still some co-operation when both growth factors and phorbol esters are added together. These results are in keeping with those recently reported by Kim and colleagues and others showing that growth factors can induce activation of PLD by a mechanism that may be independent of PI-PLC [28–30].

ras transformation synergizes with PKC stimulation of PLD activation

When *ras*-transformed NIH 3T3 cells were stimulated with PDBu, PLD activity was synergistically enhanced as compared with PLD activation induced by PDBu in control cells (Figure 2). PKC down-regulation by chronic treatment with PDBu completely eliminated the stimulation of PLD by PDBu in both *ras*-transformed and control cells. Thus the co-operative effects of *ras* and PDBu on PLD activity were mediated by PKC. A similar result was obtained by direct inhibition of PKC by bisindolylmaleimide, a specific inhibitor of the PKC family (results not shown; [22]).

As we have previously reported, some of the *ras*-transformed cells show a constitutive elevated PLD activity [22]. The above

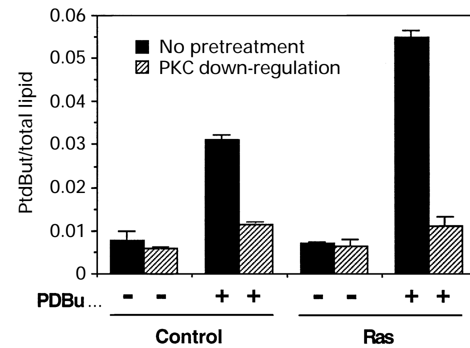

Figure 2 Ras co-operates with PKC in PLD activation

Cells were grown to confluence and then serum-starved and labelled with [^{3}H]glycerol as described in the Materials and methods section. Where indicated, PKC was down-regulated by chronic treatment with 350 nM PDBu during the labelling period. The cells were then washed and stimulated (+) with 350 nM PDBu for 35 min or left untreated (−). Data represent means ± variation for duplicate determinations. Cell lines used correspond to normal NIH 3T3 cells (Control) and the LP8-3A cell line (Ras) generated as previously described [22]. The experiment was repeated five times with similar results, using either the LP8-3A cell line or distinct *ras*-transformed cell lines (H-*ras*^{V12} as well as v-*K-ras*) generated as previously described [22].

effect of co-operative activation by PDBu and *ras* was observed in all the *ras*-transformed NIH 3T3 cell lines analysed, regardless of the constitutive increase in PLD activity (Table 1). This PLD activation induced by PDBu was significantly different in all *ras*-transformed cells from that induced in control cells when analysed by Student's *t* test ($P \leqslant 0.001$; $n = 15$).

Attenuation of growth-factor-induced PLD activation by *ras* transformation

As indicated above, growth factors activate PLD by a PKC-dependent mechanism, which probably implies activation of PI-PLC and generation of the PKC-activating molecules, DAG and Ca^{2+}. This process is mediated by the activation of the PI-specific PLCγ after stimulation of the receptor. Figure 2 shows that *ras* co-operates synergistically with PKC stimulation by PDBu treatment in PLD activation. In contrast, as shown above (Figure 1), growth factors do not co-operate synergistically with PKC in PLD activation but instead have an additive effect. Thus we next investigated if growth factor stimulation, which is able to activate PKC, co-operated synergistically or additively with *ras* in PLD activation. As shown in Table 2, the PLD response to PDGF-BB and bFGF treatment was much lower (or even abolished) in the *ras*-transformed cells than in normal NIH 3T3 cells. This result implies that growth factors and *ras* do not co-operate in PLD activation, but rather *ras* transformation attenuates the PLD response induced by growth factors. Attenuation of the PLD response was highly significant when analysed by Student's *t* test for PDGF ($P \leqslant 0.001$; $n = 15$) and FGF ($P = 0.036$; $n = 4$).

In order to identify the mechanism by which *ras* transformation abrogated the activation of PLD by growth factors, we studied whether receptor function was affected in *ras*-transformed cells by investigating receptor autophosphorylation or activation of PI3K, one of the best characterized substrates for the PDGF receptor. Neither receptor autophosphorylation (Figure 3a) nor receptor activity towards endogenous substrates such as PI3K (Figure 3b) were significantly affected in the *ras*-transformed cells, an indication that the effect on PLD activation was quite specific. Furthermore activation of the MAPK pathway was also

Table 1 Synergistic effect of PDBu and *ras* transformation on PLD activity

Cells were grown under standard conditions and, when confluent, serum-starved in DMEM supplemented with 0.2% newborn calf serum and labelled for 24 h in the presence of 10 μCi/ml [2-^{3}H]glycerol. To assay PLD activity, labelling medium was discarded, cells were washed with TD buffer and incubated for 30 or 45 min in DMEM supplemented with 0.2% newborn calf serum and 0.5% butan-1-ol in the presence or absence of 350 nM PDBu. PLD activity was quantified as described in the Materials and methods section. Data represent the mean $\pm$ variation for duplicate determinations. Fold increase represents the ratio between the PDBu-treated cells and the untreated controll cells. Net increase is the data for the PDBu-treated cells minus the background levels in untreated control cells.

Expt. no.	Cell line	Gene	PtdBut/total lipid		Fold increase	Net increase
			Control	+ PDBu		
1	NIH 3T3	None	3.92 ± 0.53	26.72 ± 2.16	6.81	22.80
	H-*ras*	H-*ras*^{V12}	6.85 ± 0.73	96.77 ± 1.52	14.12	89.92
2	NIH 3T3	None	3.03 ± 1.34	16.77 ± 0.22	5.53	13.74
	K2	v-K-*ras*	6.34 ± 0.49	80.23 ± 0.07	12.65	73.89
3	NIH 3T3	None	3.39 ± 0.88	25.06 ± 0.06	7.39	21.67
	710RAS	H-*ras*^{V12}	2.14 ± 0.18	55.48 ± 6.96	25.88	53.34
4	NIH 3T3	None	3.89 ± 0.20	35.66 ± 1.24	9.15	31.77
	LP8-1	None	4.27 ± 0.52	36.47 ± 0.92	8.53	32.20
	LP8-3	H-*ras*^{V12}	3.74 ± 0.46	59.73 ± 2.67	15.96	55.99

observed in *ras*-transformed cells after stimulation with specific growth factors such as PDGF or bFGF, a further indication of the conservation of an intact signalling cascade in the *ras*-transformed cells even though, in some of the cell lines, a partial activation of MAPK by *ras* transformation could be observed (Figure 3c). Finally, analysis of [^{3}H]thymidine uptake in response to PDGF or bFGF stimulation showed the same maximum level in both normal and *ras*-transformed cells besides the higher basal level of the *ras*-transformed cells (Figure 3d). These results further strengthen the conclusion that the mitogenic signalling for PDGF and FGF is functional alongside *ras* transformation.

We also tested whether *ras* transformation affected the generation of PI-derived metabolites in response to growth factors as an indication of PI-PLC activation. To this end, cells were labelled with *myo*-[^{3}H]inositol and then stimulated with PDGF-BB, bFGF or serum, and the release of total IPs was quantified. As shown in Figure 4, cells transformed by the *ras* oncogene lost their ability to generate PI-derived metabolites in response to growth factors such as PDGF or bFGF or even 20% FCS. A similar attenuation of PI-PLC activation in response to PDGF was observed in other *ras*-transformed cell lines (Table 3), an indication that this effect is not dependent on any specific *ras* clone. All of these results indicate that *ras* transformation specifically affects the pathway by which growth factors induce PLD activation, probably by abrogating the activation of PI-PLC, but has no detectable effect on other functions triggered by receptor activation.

Not all transformed cells show co-operation with PDBu or PDGF attenuation in the PLD response

We next determined whether the synergism between *ras* and PDBu in PLD activation and the attenuation of PLD activation induced by PDGF resulted from a non-specific effect resulting from cell transformation. To this end, we studied the induction of PLD activity by PDBu and PDGF treatment in a number of cell lines transformed by oncogenes other than *ras*. NIH 3T3 cells transformed by the c-*sis*, v-*fgr*, v-*fms*, *trk*, v-*src* and v-*met* oncogenes as well as those transformed by TGFα were investigated. The transforming capacity of each cell line was taken as its ability to grow in soft agar and compared in relative terms among the different cell lines (Table 4). Thus cells overexpressing TGFα showed weak transforming activity in this assay, whereas those expressing the *trk* or c-*sis* oncogenes were moderately transforming, those expressing the v-*fgr* or v-*fms* oncogenes were highly transforming, and those expressing the v-*met*, v-*src* or *ras* oncogenes were very highly transforming.

As shown in Table 4, there is no correlation between cell transformation and synergism in PLD stimulation with PKC activation by PDBu, since the cells transformed by oncogenes such as *trk*, v-*fgr* or v-*fms*, which showed moderate to high transforming activity, and those transformed by TGFα, which showed weak transforming activity, responded to PDBu treatment with a similar level of PLD activation to control untrans-

Table 2 Attenuation of the growth-factor-induced PLD activity in *ras* transformed cell lines

Cells were grown under standard conditions and, when confluent, serum-starved in DMEM supplemented with 0.2% newborn calf serum and labelled for 24 h in the presence of 10 μCi/ml [2-^{3}H]glycerol. To assay PLD activity, labelling medium was discarded, cells were washed with TD buffer and incubated for 30 or 45 min in DMEM supplemented with 0.2% newborn calf serum and 0.05% butan-1-ol in the presence or absence of 10 ng/ml PDGF-BB or bFGF. PLD activity was quantified as described in the Materials and methods section. Data represent the mean $\pm$ variation for duplicate determinations. Fold increase represents the ratio between the PDGF- or FGF-treated cells and the untreated control cells. Net increase is the data for the PDGF- or FGF-treated cells minus the background levels in the untreated control cells. ND, not determined.

Expt. no.	Cell line	Gene	PtdBut/total lipid			Fold increase		Net increase	
			Control	+ PDGF	+ bFGF	+ PDGF	+ bFGF	+ PDGF	+ bFGF
1	NIH 3T3	None	8.59 ± 3.69	20.95 ± 3.95	ND	2.43	ND	12.36	ND
	H-*ras*	H-*ras*^{V12}	11.78 ± 0.87	14.19 ± 1.08	ND	1.20	ND	2.41	ND
2	NIH 3T3	None	3.39 ± 0.88	16.15 ± 0.40	ND	4.76	ND	12.76	ND
	710RAS	H-*ras*^{V12}	2.14 ± 0.18	5.40 ± 0.71	ND	2.51	ND	3.26	ND
3	NIH 3T3	None	6.12 ± 0.82	14.67 ± 0.92	10.99 ± 0.57	2.39	1.79	8.55	4.87
	LP8-3A	H-*ras*^{V12}	6.26 ± 0.63	7.43 ± 0.43	7.29 ± 0.61	1.18	1.16	1.17	1.03
	H-*ras*	H-*ras*^{V12}	10.81 ± 0.12	14.28 ± 1.33	13.97 ± 0.15	1.32	1.29	3.47	3.16
4	LP8-1	None	4.14 ± 1.18	9.74 ± 0.00	7.79 ± 0.42	2.35	1.88	5.60	3.65
	LP8-3	H-*ras*^{V12}	5.02 ± 0.03	6.46 ± 0.82	7.88 ± 0.01	1.28	1.57	1.44	2.86
	H-*ras*	H-*ras*^{V12}	14.66 ± 0.75	14.39 ± 0.95	14.33 ± 0.41	0.98	0.97	−0.27	−0.33

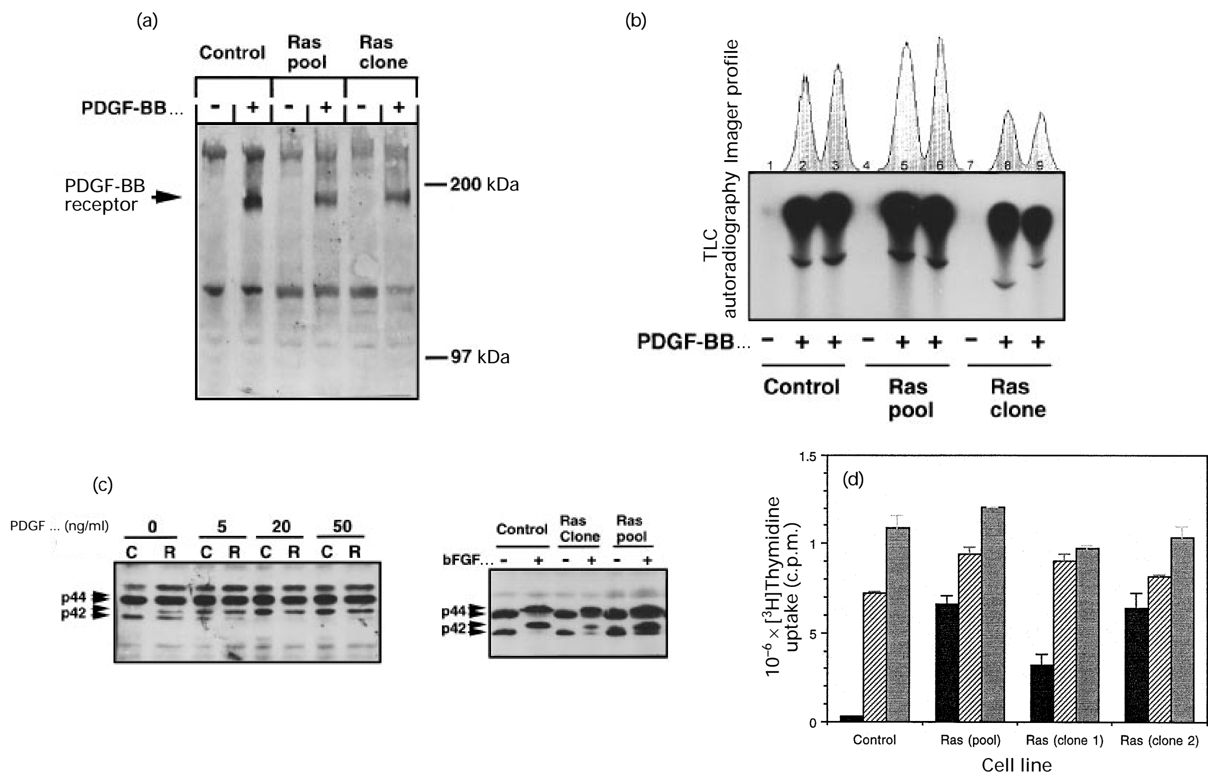

Figure 3 Lack of effect on PDGF receptor by *ras*-induced transformation

(**a**) Western blot using an anti-phoshotyrosine (αP-Tyr) antibody. Cells were grown to confluence, serum-starved for 20 h and then stimulated with 20 ng/ml PDGF-BB (+) for 10 min or left untreated (−). After incubation, cells were lysed, and equivalent amounts of cell extracts (100 μg) resolved by SDS/PAGE, transferred to nitrocellulose paper and blotted against an αP-Tyr antibody as described in the Materials and methods section. Molecular-mass markers used as standards are indicated. Control cells correspond to the LP8-1 cell line, Ras pool corresponds to the LP8-3 cell line, and Ras clone corresponds to the LP8-3A cell line [22]. The experiment was repeated once with the same result. (**b**) PI3K activity. Quiescent cells were stimulated with 20 ng/ml PDGF-BB (+) or left untreated (−) for 5 min. After incubation, cells were processed as described in the Materials and methods section for immunoprecipitation of the PI3K using an anti-P-Tyr antibody, and the immunoprecipitates assayed for PI3K activity as described by Varticovsky et al. [27]. The results shown are an autoradiograph of the products resolved by TLC and the profile (quantification) of the bands obtained in an electronic autoradiography system (InstantImager). The cell lines are described above. The experiment was repeated twice using the same and other *ras*-transformed cell lines (H-*ras*) with similar results. (**c**) Western blot using an anti-MAPK (p42 and p44) antibody. Cells were grown to confluence, serum-starved overnight and then stimulated with the indicated amounts of PDGF or 30 ng/ml bFGF for 10 min. After stimulation, cells were washed twice with ice-cold TD buffer and lysed with lysis or RIPA buffer. Equivalent amounts of protein (30–40 μg) were resolved by SDS/PAGE (10% gels), transferred to nitrocellulose and blotted against an anti-MAPK polyclonal antibody capable of recognizing both $p42^{MAPK}$ and $p44^{MAPK}$. Activation of $p42^{MAPK}$ and $p44^{MAPK}$ is followed by their shift in electrophoretic mobility. C and Control, represent the control LP8-1 cell line; R and Ras pool represent the *ras*-transformed LP8-3 cell line; Ras clone represents the *ras*-transformed LP8-3A cell line. (**d**) Thymidine incorporation induced by mitogens in *ras*-transformed cells. Cells were grown to confluence and serum-starved for 24 h in 0.2% FCS, and then stimulated with the indicated growth factors (10 ng/ml) or 20% FCS and incubated in the presence of [^{3}H]thymidine. Cells were processed as described in the Materials and methods section for thymidine incorporation. Data represent means ± variation for duplicate determinations. The experiment was repeated once with similar results. The cell lines used correspond to normal NIH 3T3 cells (control), the LP8-3 cell line [Ras (pool)], the LP8-3A clone [Ras (clone 1)] or the H-ras^{V12} [Ras (clone 2)]. ■, Quiescent; ▨, PDGF-BB; ▩, bFGF.

formed cells. In contrast, in cells transformed by v-*src*, c-*sis* or v-*met*, which showed either moderate or very high transforming activity, PDBu treatment activated PLD to an extent similar to that found for the *ras*-transformed cells. Thus no general correlation of the aggressiveness of each cell line to the synergistic effect of PDBu on PLD activation was observed. Therefore the synergistic PLD stimulation is not a result of non-specific effects of transformation, but seems rather to be specific for the signal-transduction mechanisms involved for each oncogene.

We also investigated whether the attenuation of PLD stimulation in response to PDGF treatment was a consequence of unspecific cell transformation. As shown in Table 5, cells transformed by TGFα, c-*sis* or v-*fgr* responded to PDGF treatment with a similar level of PLD activation to control untransformed cells, an indication that there was no attenuation of the PDGF response. In contrast, cells transformed by the v-*met* or v-*src* oncogenes showed a similar attenuation to that observed in the *ras*-transformed cells.

Finally, in order to analysis whether there was any correlation between attenuation of PDGF-induced PLD activation and uncoupling of the activation of the PI-PLC enzyme in response to PDGF treatment, we determined IP generation after PDGF treatment in different oncogene-transformed cells as a measure of the PI-specific PLC enzyme. As shown in Figure 5, transformation by TGFα or v-*fgr* with either weak or high transforming activity in the soft-agar assay had no effect on PI-PLC activation by PDGF. Transformation by c-*sis* showed a dramatic but not complete reduction. However, transformation by v-*met*, *ras* and v-*src*, which showed very high transforming activity in the soft-agar assay, completely abrogated the effect. These results indicate that, although there was not a perfect correlation between attenuated PLD response to PDGF and cell transformation, in all the cases where dramatic uncoupling of the PI-PLC activation by PDGF treatment was observed, it was accompanied by PLD attenuation in response to PDGF treatment. These results suggest that attenuation of the PLD response may be due to uncoupling of the activation of PI-PLC by PDGF treatment.

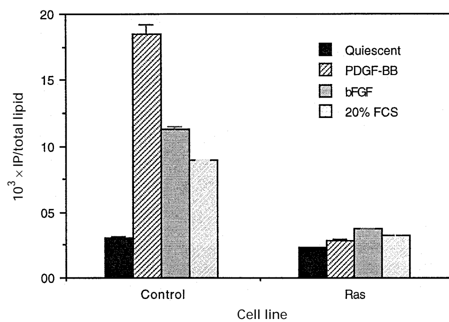

Figure 4 Effects of *ras* transformation on PDGF-, FGF- and serum-induced PI-PLC activation

Cells were grown and labelled with [^{3}H]inositol as described. Labelling medium was discarded, and cells were washed with TD buffer and preincubated with serum-free DMEM supplemented with 22 mM LiCl for 20 min. They were then stimulated with growth factors (20 ng/ml) or 20% FCS in the presence of 22 mM LiCl for 30 min. Cells were processed for IP determination as described in the Materials and methods section. Data represent means $\pm$ variation for duplicate determinations. The experiment was repeated once with similar results. Cell lines correspond to normal NIH 3T3 cells (Control) and the LP8-3 cell line (Ras) as previously described [22].

Table 3 PI-PLC activation in *ras* transformed cell lines

Cells were grown and labelled with [^{3}H]inositol as described in the Materials and methods section. Labelling medium was discarded, cells were washed with TD buffer and preincubated with serum-free DMEM supplemented with 22 mM LiCl for 20 min. Then the cells were stimulated with 20 ng/ml PDGF in the presence of 22 mM LiCl for 30 min. Cells were processed for IP determination as described in the Materials and methods section. Data are means $\pm$ range for duplicate determinations. Fold increase is the ratio between the data in PDGF column and the data in Basal column.

Expt. no.	Cell line	Gene	IP/total lipid		Fold increase
			Basal	PDGF	
1	LP8-1	None	5.89 ± 0.14	37.40 ± 1.28	6.34
	LP8-3	H-*ras*^{V12}	4.80 ± 0.07	5.61 ± 0.04	1.16
2	LP14-7	None	3.65 ± 0.10	17.31 ± 0.45	4.74
	LP14-8	H-*ras*^{R12T59}	2.62 ± 0.11	3.82 ± 0.14	1.45
3	LP14-7	None	3.28 ± 0.19	8.29 ± 2.18	2.52
	LP14-8	H-*ras*^{R12T59}	3.09 ± 0.53	3.59 ± 0.23	1.16

DISCUSSION

Alteration of signal-transduction mechanisms at diverse steps between the membrane and the nucleus is the major target for cell transformation and the acquisition of oncogenic potential. Signal transduction is critical for communication between cells and the regulation of cell growth, differentiation and apoptosis, the three major events which, if altered, can lead to the generation of transformed cells. A large number of components involved in the regulation of such transduction pathways have been characterized since the discovery of the first human oncogene. However, we may be far from uncovering all the relevant components for the regulation of these biological processes. Indeed, it is now generally accepted that cross-talk between different signalling pathways is a common phenomenon frequently observed in cell regulation.

Cancer cells are the result of an accumulation of genome alterations [31]. At least two events, competence and promotion, are required [32]. Although most oncogenes are thought to be potent initiators of this process, phorbol esters, which directly activate PKC, are among the most potent tumour promoters known [32]. A similar scenario is seen in normal cell proliferation, since both competence and progression factors are required for full mitogenic response [33]. Both competence and progression factors use signalling pathways that complement each other for full mitogenic stimulation. One such pathway frequently used in cell growth induction involves the mobilization of lipid-derived second messengers [34].

Oncogenes such as *src* and *ras* are capable of inducing the constitutive activation of PLD, an enzyme that is also activated by stimulation with growth factors in normal cells [6,22,23,35]. There is a functional connection between growth factors, PKC

Table 4 Synergism in PLD activation by PDBu in different oncogene-transformed cells

Cells were grown and labelled in DMEM supplemented with 10% newborn calf serum and 1 μCi/ml [^{14}C]glycerol for 48 h. PLD activation was analysed as indicated in the Materials and methods section after 45 min of incubation in serum-free DMEM supplemented with 0.5% butan-1-ol, in the presence or absence of 350 nM PDBu. Quantification was performed by an electronic autoradiography system. Fold increase represents the increase in PLD activity in the PDBu-treated cells over that in untreated control cells. Data shown are the means $\pm$ S.D. obtained from four independent experiments (each one carried out in duplicate). The relative number and size of colonies grown in soft agar quantified after 3 weeks of plating as described in the Materials and methods section are shown. (—), No colonies.

Cell line	Gene	Colonies	Fold increase caused by PDBu
NIH 3T3	None	–	4.57 ± 1.09
611-20	TGFα	+	5.31 ± 2.00
C51-36	*trk*	++	6.30 ± 1.25
704-42	c-*sis*	++	8.84 ± 1.92*
C26-41	v-*fgr*	+++	6.15 ± 1.95
C51-64	v-*fms*	+++	6.02 ± 1.85
12b	v-*met*	++++	12.03 ± 1.87*
LP8-3	H-*ras*^{V12}	++++	9.39 ± 2.61*
SRC	v-*src*	++++	10.14 ± 3.18*

* Significantly different from control NIH 3T3 cells, $P \leqslant 0.05$ (Student's *t* test).

Table 5 Attenuation of PLD activation induced by PDGF in different oncogene-transformed cells

Cells were grown and labelled as indicated in the legend to Table 4. PLD activation was analysed as indicated in Table 4 after 30 min incubation in the presence of 20 ng/ml PDGF.

Cell line	Gene	NIH 3T3*	Oncogenes†	Decrease (%)	*n*
611-20	TGFα	2.58 ± 0.57	2.17 ± 0.63	16	5
704-42	c-*sis*	2.82 ± 0.36	2.51 ± 0.31	11	3
C26-41	v-*fgr*	2.58 ± 0.57	2.80 ± 0.89	-9	5
12b	v-*met*	2.55 ± 0.61	1.57 ± 0.37‡	38	4
LP8-3	H-*ras*^{V12}	2.20 ± 0.65	1.32 ± 0.18‡	40	7
SRC	v-*src*	2.37 ± 0.67	1.26 ± 0.46‡	47	5

* Represents the ratio between the PDGF-treated cells and the untreated control cells. This column represents the values obtained for the control cell line (NIH 3T3 cells) as mean $\pm$ S.D. for the indicated number of determinations.

† This column represents the fold induction in PLD activity in the different oncogene-transformed cells after treatment with PDGF over unstimulated control cells. Data correspond to means $\pm$ S.D. for the number of independent experiments (each one performed in duplicate) indicated.

‡ Significantly different from the results obtained in control NIH 3T3 cells, $P \leqslant 0.05$ (Student's *t* test).

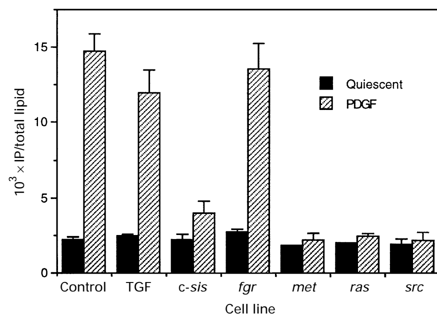

Figure 5 Effect of transformation by different oncogenes on PI-PLC activation induced by PDGF

Cells were grown and labelled with [^{3}H]inositol as described in the Materials and methods section. Labelling medium was discarded, and cells were washed with TD buffer and then preincubated with serum-free DMEM supplemented with 22 mM LiCl for 20 min. They were then stimulated with 20 ng/ml PDGF in the presence of 22 mM LiCl for 30 min. Cells were processed for IP determination as described in the Materials and methods section. Data represent mean $\pm$ S.D. for three independent experiments each one performed in duplicate. The control cell line was normal NIH 3T3 cells. Transformed cells were those described in Tables 4 and 5.

and PLD activation, since inhibition of PKC abrogates the PLD activation in response to growth factors ([9]; this report). Moreover, direct activation of PKC by phorbol esters induces a large and transient increase in PLD activity [1,36], consistent with the direct involvement of PKC in PLD regulation. In agreement with previous publications using PDGF receptor mutants [13], we report here that this activation is probably mediated through the generation of PI-PLC-derived metabolites which ends in the activation of PKC. This mechanism would also provide a common pathway for the effects observed in both growth factor stimulation with tyrosine kinase receptors and those related to G-protein-coupled receptors by activation of the PI-PLCγ or PI-PLCβ isoenzymes respectively. However, we also show here that PDBu and growth factors have an additive effect on PLD activation, since simultaneous stimulation of NIH 3T3 cells with saturating concentrations of both PDGF and PDBu gives rise to a PLD activation equivalent to the addition of either one of the stimuli alone. Since all reactive PKC must be activated by the concentrations of PDBu used, growth factors must affect PLD by a different mechanism from that of generation of DAG and PKC activation. Thus, although PKC is necessary for the activation of PLD by growth factors, it may not be sufficient for complete PLD activation. Also, it can be concluded that growth factors require the simultaneous induction of a PKC-dependent and a PKC-independent mechanism for complete activation of PLD.

We have further studied whether activation of PLD by growth factors and oncogenes follow similar pathways. To this end, we investigated whether the direct activation of PKC by phorbol esters or mitogenic stimulation by growth factors in the *ras*-transformed cells affected PLD activity. Our previous results demonstrate that *ras* utilizes a PKC-independent mechanism for constitutive PLD activation [22]. These results are in keeping with those published by Song and Foster for *src*-transformed cells, demonstrating that activation of PLD by *src* is PKC-independent [35]. Ras proteins have been suggested to mediate the activation of PLD by *src* [25]. Therefore activation of PLD by *ras* should also be PKC-independent, as we have demonstrated [22]. Unexpectedly, we found that PDBu treatment and *ras* transformation have a synergistic effect on PLD activation, the increase in PLD activity by PDBu treatment being much higher in *ras*-transformed cells than in the parental cells under the same conditions. We demonstrate that this effect is also observed for the *src* oncogene, as expected from previous studies where PLD activation induced by *src* was proposed to be mediated by the Ras proteins [25]. In an effort to uncover the mechanism involved in this synergism, we observed that PKC levels (detected by Western blot using a monoclonal antibody specific for PKCα/β) were increased in the *ras*-transformed cells (results not shown) in agreement with previous reports [37,38]. However, this does not seem to be the likely explanation, as the same effect was not observed in either *src*- [38] or *met*-transformed cells, whereas both these cell lines exhibited strong synergism with phorbol esters in PLD activation.

In contrast with our observations, a previous report by Martin et al. [39] showed that in rat 2 fibroblasts transformed by *ras*, the PLD response to treatment with phorbol esters was attenuated. However, consistent with our findings, the constitutive basal levels of PLD activity were elevated by 100 % in the *ras*-transformed cells. Besides the different cell lines used in the two studies, Martin et al. used [^{3}H]myristate for PLD determination, whereas we used [^{14}C]glycerol, a precursor that will report all putative PLD isoenzymes, rather than the specific PLD that uses myristate-containing phosphatidylcholine as the preferred substrate. This is an important technical difference, which would explain the results observed, and has been previously demonstrated to be the correct explanation for *src*-transformed cells [23].

In keeping with our observations, other authors have demonstrated that GTP[S] synergizes with phorbol esters in PLD activation [40,41], indicating that a GTPase is involved in this activation, which mimics the effects observed in the *ras*-transformed cells. Both Rho and ARF, two members of the monomeric superfamily of GTPases, have been reported to activate PLD [15,17]. Furthermore, the two proteins act synergistically in the activation of rat brain PLD [42], suggesting the existence of alternative pathways for the activation of PLD. Finally, it has been recently reported [21] that activation of PLD in *ras*-transformed NIH 3T3 cells is mediated by GTP loading of Ral through activation of Ral-GDS. This finding is consistent with our results, and suggests that more than one PLD isoenzyme may exist, each being regulated by specific mechanisms. The fact that the *ras*-mediated co-operative activation of PLD by phorbol esters is dependent on a phorbol-ester-sensitive PKC isoenzyme indicates that the effect observed involves a functional PKC of the classical or novel type, which is responsive to DAG and phorbol esters [43]. This therefore excluded the atypical PKC isoenzymes (ι/λ and ζ), which are not sensitive to phorbol ester activation [43].

Unlike the PLD activation induced by PDBu, activation of PLD by growth factors was significantly attenuated in *ras*-transformed cells. This effect was not mediated by non-specific impairment of PDGF signalling pathways, as other receptor functions were not uncoupled in the *ras*-transformed cells. In contrast, PI-derived metabolites were not generated in response to serum, bFGF or PDGF-BB in the *ras*-transformed cells, an indication that the PI-specific PLC response was completely abrogated. These results support the hypothesis that activation of PLD by growth factors is mediated by PKC through the generation of PI-PLC-derived metabolites. In contrast with these results, Alam et al. [44] have reported that, in a Kirsten-*ras*-transformed cell line, DT, the lack of response of PI-PLC activation by PDGF was associated with the loss of the PDGF receptor. However, the authors only tested a single *ras* clone, in contrast with our study which uses both mass cultures and

isolated clones, with similar results. Also, Vaziri and Faller [45] have recently reported a decreased number of PDGF receptors in the surface of Kirsten-*ras*- and *src*-transformed Balb/c-3T3 cells. Although we have not determined the actual levels of PDGF receptor in our Harvey-*ras*-transformed cells, the fact that we observed similar levels of receptor autophosphorylation, and PI3K and MAPK activation after PDGF and FGF treatment suggests that a decrease in the levels of PDGF receptors is not a sufficient explanation for the complete abrogation of the PI-PLC response. Furthermore, we have also observed the same PI-PLC effect when using PDGF, FGF or serum, which strongly supports the existence of an alternative explanation. Thus our results suggest that the attenuation of PLD activation in *ras*-transformed cells is probably a consequence of the specific uncoupling of PI-PLC activation by a still unknown mechanism, preventing generation of the second messengers, DAG and Ca^{2+}, required for PKC regulation and subsequent PLD activation.

To investigate whether the effects observed on PLD activation were a consequence of non-specific events caused by cell transformation, we studied the activation of PLD by PDBu and PDGF treatment in several transformed cell lines. A complete range of weak to very highly transformed cells was selected to study the putative correlation between PLD activation and tumorigenicity. The results indicate that there is no general correlation between transformation and synergism or attenuation of PLD response to PDBu and growth factors respectively. Different situations were found among the different oncogene-transformed cells such as synergism with PDBu and attenuation of PDGF treatment (*ras*, *src*, *met*), co-operation with PDBu but no attenuation of PDGF (*sis*), and no co-operation and no attenuation (TGFα, *fgr*). These results indicate that activation of PLD by oncogenes is specific and depends on the particular pathway activated in each case. Besides the involvement of PLD in signalling by growth factors and oncogenes, it can also be concluded that PLD activation is a redundant or non-essential effect which may be dispensable for cell proliferation and transformation. This important conclusion is also based on the finding that abrogation of PLD activation by growth factors, accomplished by either PKC down-regulation or direct inhibition, does not affect DNA synthesis in response to growth factors ([45a]; this report). A similar situation has been described for other signalling molecules activated after growth stimulation such as PI-PLC [46,47], PI3K [47] and MAPK [48]. However, it is important to realize that the most highly transforming cell lines analysed consistently showed both effects, synergism with PDBu and attenuation of PLD activation by growth factors. Whether these events are functionally related requires further investigation.

Thus PLD activity is synergisticaly enhanced in some of the oncogene-transformed cells in reponse to PKC activation, and it is dramatically impaired in response to growth factor stimulation. This attenuation is, in most cases investigated, paralleled by complete uncoupling of the PI-PLC pathway. We have further demonstrated the existence of at least two alternative mechanisms, PKC-dependent and PKC-independent, for the activation of PLD, and provide evidence that suggests the involvement of Ras proteins in the regulation of PLD. Although we do not yet have a complete understanding of the mechanism involved, this effect could be a result of the interaction of the Ras protein with the PLD enzyme and its translocation to the appropriate membrane environment. Once in the membrane, it may be accessible to full activation by PKC. This model is similar to that of the Raf-1 kinase, which becomes translocated to the membrane by association with Ras proteins, where it is further activated by either direct phosphorylation [49–52] or by interaction with other proteins such as the 14-3-3 proteins, by still unresolved mechanisms [53–57]. Consistent with our findings, a recent report indicates that Ras proteins can activate PLD through Ral-GDS, the exchange factor of Ral, another GTPase of the Ras superfamily [21]. Other oncogenes also affect the activation of the PLD enzyme in a similar way to *ras*. Although we have not determined whether this effect depends on *ras*, this is a possibility that deserves further investigation. Finally, we show that activation of PLD by growth factors is more complex than expected, requiring molecules other than those involved in the PI-PLC/PKC pathway. All the results presented strongly indicate that PLD is an important molecule in signal transduction and may be involved in signalling in cell transformation induced by several oncogenes.

We greatly appreciate the excellent technical assistance of M. A. Ramos and M. Carmen Paje. We also thank N. Embade and S. Montaner, who were responsible for the generation of some of the *ras*-transformed cells used in this study. This work was supported by Grant 93/0293 from Fondo de Investigación Sanitaria (FIS) of the Spanish Department of Health, Grant PB94-0009 from DGICYT, and Grant AE 00387/95 from the Consejería de educación of Communidad de Madrid. L.P. is a fellow of DGICYT, and P.E. is a fellow of FIS.

REFERENCES

1 Exton, J. H. (1994) Biochim. Biophys. Acta **1212**, 26–42
2 Liscovitch, M. and Amsterdam, A. (1989) J. Biol. Chem. **264**, 11762–11767
3 Suchard, S. J., Nakamura, T., Abe, A., Shayman, J. A. and Boxer, L. A. (1994) J. Biol. Chem. **269**, 8063–8068
4 Ella, K. M., Dolan, J. W. and Meier, K. E. (1995) Biochem. J. **307**, 799–805
5 Carnero, A. and Lacal, J. C. (1995) Mol. Cell. Biol. **15**, 1094–1101
6 Carnero, A., Cuadrado, A., del Peso, L. and Lacal, J. C. (1994) Oncogene **9**, 1387–1395
7 Kondo, T., Hiroshi, I., Fumiko, K. and Inagami, T. (1992) J. Biol. Chem. **267**, 23609–23616
8 Lacal, J. C., Moscat, J. and Aaronson, S. A. (1987) Nature (London) **330**, 269–272
9 Plevin, R., Cook, S. J., Palmer, S. and Wakelam, M. J. O. (1991) Biochem. J. **279**, 559–565
10 Divecha, N. and Irvine, R. F. (1995) Cell **80**, 269–278
11 Eldar, H., Ben-av, P., Schmidt, U.-S., Livneh, E. and Liscovitch, M. (1993) J. Biol. Chem. **268**, 12560–12564
12 Lee, Y. H., Kim, H. S., Pai, J.-K., Ryo, S. H. and Suh, P.-G. (1994) J. Biol. Chem. **269**, 26842–26847
13 Yeo, E.-J., Kazlauskas, A. and Exton, J. H. (1994) J. Biol. Chem. **269**, 27823–27826
14 Bourgoin, S., Harbour, D., Desmarais, Y., Takai, Y. and Beaulieu, A. (1995) J. Biol. Chem. **270**, 3172–3178
15 Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C. and Sternweis, P. (1993) Cell **75**, 1137–1144
16 Lambeth, J. D., Kwak, J. Y., Bowman, E. P., Perry, D., Uhlinger, D. J. and Lopez, I. (1995) J. Biol. Chem. **270**, 2431–2434
17 Bowman, E. P., Uhlinger, D. J. and Lambeth, J. D. (1993) J. Biol. Chem. **268**, 21509–21512
18 Malcolm, K. C., Ross, A. H., Qiu, R. G., Symons, M. and Exton, J. H. (1994) J. Biol. Chem. **269**, 25951–25954
19 Ohguchi, K., Banno, Y., Nakashima, S. and Nozawa, Y. (1995) Biochem. Biophys. Res. Commun. **211**, 306–311
20 Siddiqi, A. R., Smith, J. L., Ross, A. H., Qiu, R. G, Symons, M. and Exton, J. H. (1995) J. Biol. Chem. **270**, 8466–8473
21 Jiang, H., Luo, J.-Q., Urano, T., Frankel, P., Lu, Z., Foster, D. A. and Freig, L. A. (1995) Nature (London) **378**, 409–412
22 del Peso, L., Esteve, P., Hernandez, R. and Lacal, J. C. (1996) J. Cell. Biochem. **61**, 599–608
23 Song, J., Pfeffer, L. M. and Foster, D. A. (1991) Mol. Cell. Biol. **11**, 4903–4908
24 Jiang, H., Alexandropoulos, K., Song, J. and Foster, D. A. (1994) Mol. Cell. Biol. **14**, 3676–3682
25 Jiang, H., Lu, Z., Luo, J.-Q., Wolfman, A. and Foster, D. A. (1995) J. Biol. Chem. **270**, 6006–6009
26 Pazit, B.-A. and Liscovitch, M. (1989) FEBS Lett. **259**, 64–66
27 Varticovski, L., Daley, G. F., Jackson, P., Baltimor, D. and Cantley, L. (1991) Mol. Cell. Biol. **1**, 1107–1113
28 Kim, B. Y., Ahn, S. C., Oh, H. K., Lee, H. S., Mheen, T. I., Rho, H. M. and Ahn, J. S. (1995) Biochem. Biophys. Res. Commun. **212**, 1061–1067

29 Kim, B. Y., Ahn, S. C., Kang, D. O., Ko, H. R., Oh, W. K., Lee, H. S., Mheen, T. I., Rho, H. M. and Ahn, J. S. (1996) Biochim. Biophys. Acta **1311**, 33–36
30 Catz, S. D. and Sterin-Speziale, N. B. (1996) J. Leukocyte Biol. **59**, 591–597
31 Sugimura, T. (1992) Science **258**, 603–607
32 Hunter, T. (1991) Cell **64**, 249–270
33 Aaronson, S. A. (1991) Science **254**, 1146–1153
34 Berridge, M. J. (1993) Nature (London) **361**, 315–325
35 Song, J. and Foster, D. A. (1993) Biochem. J. **294**, 711–717
36 Huang, C. and Cabot, M. C. (1990) J. Biol. Chem. **265**, 14848–14863
37 Borner, C., Guadagno, S. N., Hsieh, L. L., Hsiao, W. L. and Weinstein, I. B. (1990) Growth Differ. **1**, 653–660
38 Borner, C., Guadagno, S. N., Hsiao, W. W.-L., Fabbro, D., Barr, M. and Weinstein, I. B. (1992) J. Biol. Chem. **267**, 12900–12910
39 Martin, A., Gomez-Muñoz, A., Waggoner, D. A., Stone, J. C. and Brindley, D. N. (1993) J. Biol. Chem. **268**, 23924–23932
40 Coorssen, J. R. and Halam, R. J. (1993) FEBS Lett. **316**, 170–174
41 Geny, B. and Cockcroft, S. (1992) Biochem. J. **284**, 531–538
42 Kuribara, H., Tago, K., Yokozeki, T., Sasaki, T., Takai, Y., Marii, N., Narumiya, S., Katada, T. and Kanaho, Y. (1995) J. Biol. Chem. **270**, 25667–25671
43 Nishizuka, Y. (1992) Science **258**, 607–614
44 Alam, M. S., Banno, Y., Naiashima, S. and Nozawa, Y. (1995) Biochem. Biophys. Res. Commun. **207**, 460–466
45 Vaziri, C. and Faller, D. V. (1995) Mol. Cell. Biol. **15**, 1244–1253
45a Lacal, J. C., Fleming, T. P., Warren, B. S., Blumberg, P. M. and Aaronson, S. A. (1987) Mol. Cell. Biol. **7**, 4146–4149
46 Peters, K. G., Marie, J., Wilson, E., Ives, H. E., Escobedo, J., Del Rosario, M., Mirda, D. and Williams, L. T. (1992) Nature (London) **358**, 678–681
47 Valius, M. and Kazlauskas, A. (1993) Cell **73**, 321–334
48 Cowley, S., Paterson, H., Kemp, P. and Marshall, C. (1994) Cell **77**, 841–852
49 Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marmé, D. and Rapp, U. R. (1993) Nature (London) **364**, 249–252
50 Leevers, S. J., Paterson, H. F. and Marshall, C. J. (1994) Nature (London) **369**, 411–414
51 Sözeri, O., Vollmer, K., Liyanage, M., Frith, D., Kour, G., Mark, III, G. E. and Sabel, S. (1992) Oncogene **7**, 2259–2262
52 Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M. and Handcock, J. F. (1994) Science **264**, 1463–1466
53 Fantl, W. J., Muslin, A. J., Kikuchi, A., Martin, J. J., MacNicol, A. M., Gross, R. W. and Williams, L. T. (1994) Nature (London) **371**, 612–614
54 Freed, E., Symons, M., Macdonald, S. G., McCormick, F. and Ruggieri, R. (1994) Science **265**, 1713–1716
55 Irie, K., Gotoh, Y., Yashar, B. M., Errede, B., Nishida, E. and Matsumoto, K. (1994) Science **265**, 1716–1719
56 Li, S., Janosch, P., Tanji, M., Rosenfeld, G. C., Waymire, J. C., Mischak, H., Kolch, W. and Sedivy, J. (1995) EMBO J. **14**, 685–696
57 Michaud, N. R., Fabian, J. R., Mathes, K. D. and Morrison, D. K. (1995) Mol. Cell. Biol. **15**, 3390–3397

Received 9 July 1996/3 October 1996; accepted 28 October 1996