Regulation of phospholipase D isoenzymes by transforming Ras and atypical protein kinase C- ι

James MWANJEWEE¹, Martin SPITALER, Maria EBNER, Michaela WINDEGGER, Markus GEIGER, Sonja KAMPFER, Johann HOFMANN, Florian ÜBERALL and Hans H. GRUNICKE²

Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Fritz-Pregl-Strasse 3/VI, A-6020 Innsbruck, Austria

The activation of phospholipase D (PLD) by transforming Ras is well documented. Although two distinct PLD isoforms, PLD1 and PLD2, have been cloned from mammalian cells, it has remained unclear whether both isoenzymes are activated by Ras and, if this is the case, whether they are stimulated by a common mechanism. In the present study we show that expression of transforming Ras in HC11 mouse mammary epithelial cells enhanced the activity of endogenous PLD. Co-expression of Ras with either PLD1b or PLD2 resulted in elevated activities of both PLD isoenzymes in HC11 cells, indicating that transforming Ras was capable of activating both PLD isoforms *in vivo*. Ras-induced activation of PLD was resistant to the protein kinase C (PKC) inhibitor GF109203X, which preferentially affects

conventional- and novel-type PKCs, but sensitive to Ro-31-8220, which inhibits atypical PKCs more effectively. Co-transfection of atypical PKC- ι with either PLD1b or PLD2 led to a selective activation of PLD2 by PKC- ι , whereas PLD1b was not affected. PLD1b, however, was found to be a potent activator of PKC- ι , whereas PLD2 was less effective in this respect. The data suggest that PKC- ι acts upstream of PLD2 and that PLD1b is implicated in the activation of PKC- ι . The data are discussed as indicating a putative signalling cascade comprising Ras $\rightarrow$ PLD1b $\rightarrow$ PKC- ι $\rightarrow$ PLD2. Evidence for the implication of this pathway in the transcriptional regulation of cyclin D1 is also presented.

Key words: PLD1b, PLD2, PKC- ι /λ, Ras, HC11, COS-7.

INTRODUCTION

Phospholipase D (EC 3.1.4.4; PLD) represents a phospholipid-specific phosphodiesterase that hydrolyses phosphatidylcholine to phosphatidic acid (PA) and choline. The enzyme is widely distributed in prokaryotes, fungi, plants and animals [1]. In mammalian cells, PLD has been shown to be stimulated by a broad variety of agonists, including hormones, neurotransmitters, cytokines, growth factors, components of the extracellular matrix, bacterial toxins and reactive oxygen species (for reviews, see [1–3]). In view of these findings, PLD is considered to represent a signal-activated phospholipase which, together with phosphoinositide-specific phospholipase C, phospholipase A₂, lipid kinases and sphingomyelinase, is involved in signal transduction by generating second messengers or effectors from lipid precursors. The stimulation of PLD by growth factors and several oncogenes [1,4–6] suggests a role of PLD in growth control. The precise intracellular function of PLD in signal transduction has, however, remained obscure. It is generally assumed that PA is the major messenger generated by PLD. While PA has been demonstrated to modulate several enzymes *in vitro* [7], the molecular targets of PA *in vivo* are still insufficiently identified.

Although advances have been made in understanding the regulation of PLD *in vitro*, the mechanisms by which PLD is controlled in intact cells is also far from clear. Cloning and characterization of isoenzymes of mammalian PLD have provided a wealth of information with respect to the putative regulators of PLD. In mammalian cells, two PLD-encoding

genes have been identified and termed *PLD1* and *PLD2*. The *PLD1* gene encodes two splice variants PLD1a and PLD1b, of which PLD1b appears to be the predominant isoform [8]. The regulatory properties of PLD1a and PLD1b have been shown to be identical, i.e. they are both stimulated *in vitro* by PtdIns(4,5) P_2 , PtdIns(3,4,5) P_3 , the small GTP-binding proteins ADP-ribosylation factor (ARF), Rho, Rac and Cdc42, as well as the conventional protein kinase C (PKC) isoforms α , β I and β II [1–3]. PKC-mediated activation of PLD1 has been shown to be ATP-independent and due to protein–protein interaction rather than phosphorylation [2]. In contrast with PLD1, the PLD2 isoform does not respond to Rho or phorbol ester-sensitive PKC isoforms, and is weakly, or not at all, stimulated by ARF [9]. PLD1 and PLD2 isoforms differ with regard to their intracellular localization. Whereas PLD1 is found predominantly in organelles and vesicles of the endosomal/lysosomal compartment, PLD2 is enriched in the plasma membrane and, to a lower, but significant extent (approx. 20%), in the cytosol [1].

Both normal and transforming Ras have been shown to mediate the activation of PLD [5,10–12]. Ras-induced stimulation of PLD has been demonstrated to be mediated by the small GTPase, RalA [11–13]. PLD1 forms a complex with GTP-charged RalA *in vitro* [14], suggesting that the PLD isoenzyme activated by Ras, via RalA, is PLD1 [15]. However, whether Ras selectively activates PLD1 and not PLD2 remains to be investigated.

Furthermore, in previous studies we have demonstrated that transforming Ras activates atypical PKC- ι /λ and -ζ and that these PKC isoforms are required for the Ras-mediated

Abbreviations used: ARF, ADP-ribosylation factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LIP, lambda-interacting protein; Ni²⁺-NTA, Ni²⁺-nitrilotriacetate; PA, phosphatidic acid; PHM, pre-hormone medium; PLD, phospholipase D; PKC, protein kinase C; RGS, Arg-Gly-Ser.

¹ Present address: Department of Biology, McMaster University, 1200 Main Street W., Hamilton L8N 3Z5, Ontario, Canada

² To whom correspondence should be addressed (e-mail hans.grunicke@uibk.ac.at).

induction of *c-fos*, reorganization of the actin cytoskeleton and the transcriptional activation of the cyclin D1 promoter [16–18]. The relevance of PKC- λ (the mouse homologue of human PKC- ζ) in Ras-mediated transformation is emphasized by the observation that expression of a dominant negative mutant of this PKC isoform reverted a Ras-transformed phenotype [19]. However, the mechanism by which Ras activates these atypical PKC isoforms has remained obscure. PA acts as an activator of PKC- ζ *in vitro* [20–22] and, in view of the close sequence homology between PKC- ι/λ and - ζ [23,24], it appeared conceivable that PA might also function as an activator of PKC- ι . Therefore we have investigated whether ectopic overexpression of either PLD1 or PLD2 is capable of activating PKC- ι in intact cells. Although PLD may activate PKC- ι , the reverse, i.e. a direct or indirect stimulation of a PLD isoform by atypical PKC- ι , can also be envisaged. The activation of PLD by PKC is well established: according to published observations, phorbol ester-responsive PKC isoforms, and in particular, conventional Ca²⁺-sensitive PKCs, act as activators of PLD [1,2]. However, studies investigating the role of atypical PKC isoforms in the regulation of PLD isoenzymes have, to our knowledge, not been reported. Therefore we have also investigated whether overexpression of PKC- ι affects the activity of a PLD isoform in intact cells, or is itself activated by PLD.

The data presented here demonstrate that the expression of transforming Ras activates ectopically-expressed PLD1b and PLD2. Furthermore, expression of PKC- ι selectively activates ectopically expressed PLD2, but has no effect on PLD1b, whereas the expression of PLD1b strongly enhanced the activity of PKC- ι . As Ras has been shown to activate PLD via RalA, and RalA binds PLD1b, the data are in accordance with a putative signal transduction pathway comprising Ras $\rightarrow$ RalA $\rightarrow$ PLD1b $\rightarrow$ PKC- ι $\rightarrow$ PLD2.

EXPERIMENTAL

Plasmids

The PKC- ι constructs were subcloned into a pEFneo expression vector as described previously [17,25]. The plasmids pCGN-hPLD1b, pCGN-mPLD2 and pCGN-mPLD2/KR were generously given by Dr Michael Frohman (Institute for Cell and Developmental Biology, Stony Brook, New York, NY, U.S.A.). The Ras-L61 plasmid was generously given by Dr Michael Karin (Department of Pharmacology, University of California, San Francisco, CA, U.S.A.). The cyclin D1-luciferase reporter construct was generously given by Dr Richard Pestell (Division of Hormone-Dependent Tumor Biology, Albert Einstein College, Bronx, NY, U.S.A.).

Cell lines

HC11 cells were cultured in growth medium [RPMI 1640 supplemented with 10% (v/v) fetal calf serum (FCS), 4 mM L-glutamine, 5 μ g/ml insulin (Sigma) and 10 ng/ml epidermal growth factor (Sigma)]. Pre-hormone medium (PHM) was RPMI 1640 supplemented with 2% (v/v) FCS, 4 mM L-glutamine and 5 μ g/ml insulin. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS. All cell-culture media and supplements were obtained from Gibco BRL (Carlsbad, CA, U.S.A.) unless otherwise stated.

PLD *in vivo* assay

The cells were seeded in 6-well plates at a density of 8×10^4 cells per well. The growth medium was replaced 24 h later by PHM,

and cells were incubated for a further 36 h. For labelling, the required amount of [³H]oleic acid (usually 1 μ Ci/ml; Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) was pipetted into a sterile tube and the solvent evaporated under a stream of N₂. For the labelling medium, the dried radiolabelled fatty acid was dissolved in the right amount of ethanol to make up 1% in the growth medium. The tube was vortex-mixed vigorously, followed by sonification for 2 min. This was repeated twice to ensure that the fatty acid was resuspended in the labelling medium.

The cells were washed twice with RPMI 1640 and 1 ml of labelling medium added to each well. If required, PKC inhibitors were added and the cells were incubated for 18–20 h. After labelling, the medium was removed, the cells washed twice with RPMI 1640 and incubated for 5 min with RPMI 1640 containing 1% ethanol. The medium was then removed, and the cells were incubated with RPMI 1640 containing 1% ethanol in the absence or presence of 20 nM PMA for 20 min. The reaction was stopped by washing the cells twice with ice-cold PBS and adding ice-cold SDS solution (0.2%).

The cells were scraped off and transferred to glass test tubes. After washing the cells with 1 ml of methanol, 500 μ l of chloroform was added and mixed vigorously. An additional 500 μ l of chloroform was added, followed by 500 μ l of 0.2% NaCl and the tubes were mixed again. The tubes were then kept at 4 °C for 10 min and centrifuged at 2000 g for 10 min. The lower organic phase was carefully transferred to a fresh tube and 250 μ l removed for the analysis of organic phosphate. Both sets of tubes were evaporated under a stream of N₂ before further analysis was undertaken.

The lipids in the first set of tubes were dissolved in 40 μ l of chloroform containing the standard reference lipids (25 μ g of phosphatidylethanol and PA). They were spotted on to a silica gel TLC sheet and the lipids separated using the upper phase of a mixture of ethyl acetate/acetic acid/2,2,4-trimethylpentane/water (13:2:3:10, by vol.) as the mobile phase. The chromatogram was developed using 3% phosphomolybdic acid and the spots corresponding to the reference lipid were cut out, put into scintillation vials and scintillation liquid (Ultima Gold; Packard, Meriden, CT, U.S.A.) was added. The radioactivity was counted using a Tri-Carb 1600 TR liquid-scintillation analyser (Packard) or LS-3801 liquid scintillation counter (Beckman Coulter, Fullerton, CA, U.S.A.).

For the determination of organic phosphate, 30 μ l of magnesium nitrate solution [10% (w/v) in ethanol] was added to each tube. The tubes were then heated over a strong flame to ash the organic compounds. The tubes were cooled and 300 μ l 0.5 M HCl was added and heated in a boiling water bath for 15 min to hydrolyse any pyrophosphate formed during ashing. Once cooled, 0.7 ml of colour developing reagent [1 part of ascorbic acid (10% in distilled water) and 6 parts of ammonium molybdate (0.42% ammonium molybdate, 4H₂O in 1 M H₂SO₄)] was added. The absorbance was read at a wavelength of 820 nm and compared with a standard curve derived from NaH₂PO₄ (0–70 nM).

Transfection using Transfast™ reagent

Cells were seeded at approx. 5×10^4 cells/ml in 6-well plates and incubated at 37 °C in a CO₂ incubator for 24 h. The cells were then transfected with 1 μ g DNA using the TransFast® reagent (Promega, Mannheim, Germany) at a lipid:DNA ratio of 1:1. After incubation for 2 h, the cells were washed with PBS and incubated for an additional 24–48 h in growth medium before analysis was carried out.

Western blotting

Immunoprecipitation of haemagglutinin-tagged PLD1b and PLD2, followed by Western blotting, was carried out using Pansorbin[®] (Calbiochem, Schwalbach, Germany) according to the manufacturer's protocol.

Purification of Arg-Gly-Ser (RGS)-His₆-tagged PKC and measurement of *in vitro* activity

Transiently transfected cells were scraped off the dishes in PBS and centrifuged at 1000 *g* for 5 min. The pellet was resuspended in 500 μ l of lysis buffer (150 mM NaCl, 50 mM Hepes, pH 7.5, 1% Nonidet P-40, 50 μ g/ml leupeptin, 50 μ g/ml aprotinin and 1 mM PMSF) and incubated on ice for 10 min. Cell lysates were centrifuged at 10000 *g* for 10 min and the supernatant was transferred to a fresh tube. Equilibrated Ni²⁺-nitrilotriacetate (Ni²⁺-NTA)-agarose (100 μ l; Qiagen, Hilden, Germany) was added and the tubes were rotated for 1 h to allow binding of Ni²⁺-NTA-agarose to the RGS-His₆-tagged PKCs. The purified PKCs were then washed four times with lysis buffer containing 50 mM imidazole and eluted with 500 mM imidazole in 20 mM Tris/HCl, pH 7.5.

Purified PKC (100 ng) was added to 100 μ l of assay buffer containing 20 mM Tris/HCl, pH 7.5, 20 mM MgCl₂, 50 μ M substrate peptide PKC- α -19–31/Ser25 (Alexis, Lausen, Switzerland), 40 μ M ATP and 1 μ Ci of [γ -³³P]ATP (NEN, Cambridge, U.K.). The reaction was incubated at 30 °C for 10 min and stopped on ice. A portion (50 μ l) of the reaction mixture was transferred to phosphocellulose sheets (Gibco BRL). The phosphocellulose sheets were washed three times with 1% phosphoric acid, twice with distilled water and then the radioactivity was measured in a liquid-scintillation counter. For standardization of the results, equal amounts of purified PKC were separated on a polyacrylamide gel and detected by Western blotting with an anti-(PKC- ϵ) antibody (Transduction Laboratories, Franklin Lakes, NJ, U.S.A.). The band intensities were quantified using ChemiImager 4400 software (Alpha Innotech; Cannock, Staffs, U.K.) and the results of the *in vitro* kinase assay corrected using the relative band intensity. When the cells were pretreated with butan-1-ol for inhibition of PLD, butan-2-ol, which cannot inhibit PLD, was used as a solvent control.

Dual luciferase assay

For the measurement of cyclin D1 transcription, a luciferase reporter construct with a firefly luciferase gene under the control of the cyclin D1 promoter was used. A constitutively expressed ptk-Renilla-luciferase construct (Promega) was co-transfected for standardization. Briefly, HC11 cells were transfected with the indicated expression vectors (0.15 μ g of Ras-L61, 0.15 μ g of PKC- ϵ -wt, 0.15 μ g of PKC- ϵ -AE, 0.15 μ g of PLD2, 0.3 μ g of PKC- ϵ -KW, 0.3 μ g of PLD2/KR), 0.33 μ g cyclin D1-luciferase reporter plasmid and 16.5 ng of ptk-Renilla-luciferase construct using the TransFast[®] reagent as described above. The total amount of plasmid DNA was adjusted to 1 μ g with empty pEFneo vector. Cells were washed 2 h post-transfection with DMEM and maintained in RPMI 1640 supplemented with 0.5% heat-inactivated FCS, 5 μ g/ml insulin and 2 mM L-glutamine. After transfection for 48 h, luciferase activity of reporter firefly luciferase and the control Renilla luciferase were measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The relative firefly luciferase activities were calculated by normalizing transfection efficiency to the Renilla luciferase activities.

RESULTS

Expression of Ras-L61 enhances the activities of endogenous PLD and of ectopically expressed PLD1b and PLD2 isoforms in HC11 cells

Expression of transforming Ras has been shown to activate basal levels of total PLD activity in some, but not all, cell lines [5]. Therefore it was investigated whether transfection of HC11 mouse mammary epithelial cells with transforming Ras-L61 altered total PLD activity. As shown in Figure 1(A), two clones of HC11 cells stably transfected with Ras-L61 (clones HC11-Ras13 and HC11-Ras15) exhibited elevated levels of basal PLD activity. Compared with non-transfected controls, PLD activity in Ras-expressing cells was increased from 150 to 1000–1500 units.

Activation of PLD by phorbol esters was found to be enhanced in Ras transformed NIH 3T3 cells [5]. Figure 1(A) demonstrates, however, that the fold activation by PMA was in the same range

Figure 1 Stimulation of PLD activity in HC11 cells stably transfected with transforming Ras-L61

(A) Two clones of HC11 cells stably transfected with Ras-L61 (HC11 Ras13 and HC11 Ras15) were used to measure PLD activity compared with HC11 wild-type (wt) cells as described in the Experimental section. GF109203X (GFX) and PMA were used at 7.5 μ M and 20 nM respectively. (B) Inhibition of Ras- or PMA-mediated PLD activation by Ro-31-8220 (Ro, 1 μ M). C, vector control. Results are means $\pm$ S.E.M. of three independent assays performed in triplicate.

Figure 2 Activation of PLD by transient co-transfection with transforming Ras-L61 in HC11 cells

HC11 cells were transiently transfected with plasmids encoding human PLD1b or PLD2 together with either the empty control vector or transforming Ras-L61. PLD assay was carried out as described in the Experimental section. C, vector control. Results are means $\pm$ S.E.M. of two experiments performed in triplicate.

in Ras-stimulated and non-stimulated cells, indicating that Ras and PMA activate PLD by independent pathways. This conclusion was supported by the observation that the PKC inhibitor GF109203X could abrogate the PMA-mediated increase in PLD activity, whereas the Ras-mediated activation of PLD was not affected by this concentration (7.5 μ M) of the inhibitor. The chemically related PKC inhibitor Ro-31-8220, which has been shown to inhibit atypical PKC isoforms [26,27], and results not shown), reduced Ras-mediated PLD activation (Figure 1B), suggesting a putative effect of atypical PKC isoforms.

In order to investigate which PLD isoform was activated by Ras-L61, HC11 cells were transiently co-transfected with the vector encoding Ras-L61 with plasmids encoding either PLD1b or PLD2 respectively. PLD activity was then measured in the whole cell population without selection for transfected cells. The results shown in Figure 2 demonstrate that transient transfection with the PLD-encoding constructs did not result in a measurable increase in total PLD activity in non-stimulated cells. This can be explained by the low transfection efficiency (3–5%), as determined by green fluorescent protein transfection markers. For the same reason, transient transfection with Ras-L61 alone did not cause a detectable activation of endogenous PLD. However, co-transfection of transforming Ras with the PLD-encoding vectors significantly enhanced the PLD activities of cultures transfected with PLD1b, an effect even more pronounced with PLD2.

Atypical PKC- ζ selectively activates PLD2 but has no effect on PLD1b

We have previously shown that transforming Ras can activate atypical PKC- ζ in intact cells [16]. This PKC isoform is relatively insensitive to the PKC inhibitor GF109203X [28], but inhibited at higher concentrations (> 1 μ M) of Ro-31-8220. Therefore it was investigated whether Ras employed PKC- ζ for the activation of either PLD1b or PLD2. The data demonstrate that transient co-transfection of PKC- ζ with PLD2 resulted in elevated PLD2 activity, whereas no effect of PKC- ζ on co-expressed PLD1b was observed (Figure 3A).

In contrast with HC11 cells, in which transient transfection

Figure 3 Activation of PLD by transient co-transfection with PKC- ζ in HC11 and COS cells

(A) HC11 cells were transiently transfected with PLD1b and PLD2 together with either the empty control vector or PKC- ζ . After 48 h of starvation, the PLD assay was carried out as described in the Experimental section. (B) The same experiment performed with COS cells. C, vector control. Results are means $\pm$ S.E.M. ($n = 3$) from a representative experiment.

with plasmids encoding the two PLD isoenzymes did not result in a detectable increase in PLD activity, enhanced PLD activities could be measured after transfection of COS-7 cells, which express the transfected constructs at much higher levels (Figure 3B). The increase in PLD activity was particularly pronounced in cells expressing PLD2, whereas overexpression of PLD1b exhibited a much smaller, but still significant, elevation of PLD activity. These differences are explained, in part, by a higher level of PLD2 protein expression compared with PLD1b as shown by Western blotting (results not shown). Furthermore, PLD2 is known to exhibit higher basal activity than PLD1. In accordance with the data obtained with HC11 cells (Figure 3A), PKC- ζ markedly enhanced PLD2 activity in COS-7 cells, but had no effect on PLD1b (Figure 3B).

Activation of PKC- ζ by PLD

The main product of PLD enzymic activity, PA, has been shown to activate atypical PKC- ζ *in vitro* [20–22]. As stated in the

Figure 4 Activation of PKC- ϵ by the PLD isoenzymes when either PLD1 or PLD2 are co-transfected with PKC- ϵ

COS cells were transiently co-transfected with a plasmid encoding wild-type human RGS-His₆-tagged PKC- ϵ and PLD1b or PLD2. Cells were lysed 48 h later, PKC- ϵ was purified by adsorption to Ni²⁺-NTA-agarose and the enzymic activity determined as described in the Experimental section. Results are means $\pm$ S.E.M. of two independent experiments performed in triplicate.

Figure 5 Inhibition of PLD1b-mediated activation of PKC- ϵ by butan-1-ol

COS cells were transiently transfected with a plasmid encoding wild-type human RGS-His₆-tagged PKC- ϵ and PLD1b or PLD2. Butan-1-ol (1-ButOH) was added to the medium 12 h prior to extraction. Cells were lysed 48 h after transfection, PKC- ϵ was purified by adsorption to Ni²⁺-NTA-agarose and the enzymic activity determined as described in the Experimental section. Results are means $\pm$ S.E.M. of two independent experiments performed in triplicate.

Introduction, it is possible that PA can also activate PKC- ϵ . Thus it was investigated whether overexpression of either PLD1b or PLD2 was capable of activating PKC- ϵ . The studies revealed that co-expression of PLD1b with PKC- ϵ resulted in a marked elevation in the kinase activity of PKC- ϵ . Co-expression of PLD2 with PKC- ϵ led to a smaller, but still significant, activation of this PKC isoform (Figure 4). In view of the observation that the activity and expression level of ectopically expressed PLD1b was even lower than PLD2 (Figure 3B and results not shown), it can be concluded that PLD1b is a very potent activator of PKC- ϵ .

In order to study whether PA activates PKC- ϵ *in vivo*, the PLD-generated PA was trapped by exogenous butan-1-ol, thus exploiting the transphosphorylation activity of PLD. As shown

Figure 6 Inhibition of Ras-mediated activation of PKC- ϵ by butan-1-ol

COS cells were transiently transfected with a plasmid encoding wild-type human RGS-His₆-tagged PKC- ϵ and Ras-L61. Butan-1-ol (1-ButOH) was added to the medium 12 h prior to extraction. Cells were lysed 48 h after transfection, PKC- ϵ was purified by adsorption to Ni²⁺-NTA-agarose and the enzymic activity determined as described in the Experimental section. Results are means $\pm$ S.E.M. performed in triplicate from two independent experiments.

Figure 7 Transcriptional activation of a cyclin D1-luciferase reporter gene by PLD2 and PKC- ϵ

HC11 cells seeded in 6-well plates were transiently co-transfected with plasmids containing luciferase reporter gene under the control of the full length cyclin D1 promoter (−1745 base pairs) together with the indicated combinations of the cDNAs for Ras-L61, wild-type PKC- ϵ (PKC- ϵ -wt), constitutively active PKC- ϵ (PKC- ϵ -AE), dominant negative PKC- ϵ (PKC- ϵ -KW), wild-type PLD2 and dominant negative PLD2 (PLD2/KR). A constitutively expressed ptk-Renilla-luciferase construct was co-transfected for standardization. After transfection for 48 h, luciferase activity of firefly luciferase and the control Renilla luciferase were measured with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. C, vector control. Results are means $\pm$ S.E.M. of two independent experiments.

in Figure 5, the primary alcohol completely inhibited the PLD1b-mediated activation of PKC- ϵ . This effect was not due to non-specific toxic effects of butan-1-ol, as butan-2-ol, which does not act as a substrate of PLD, exhibited only a marginal reduction in PKC- ϵ activity when used at the same concentrations (results not shown). Similar effects, i.e. a reduction of PKC- ϵ activation by butan-1-ol, were also observed after stimulation of endogenous PLD by transforming Ras (Figure 6).

Evidence for a role of PKC- ι -regulated PLD in cyclin D1 expression

Expression of transforming Ras has been shown to cause the transcriptional activation of cyclin D1 [29,30] and the activation of PKC- ι [16]. In view of the data indicating a PKC- ι -mediated activation of PLD2, it was investigated whether PLD2 was implicated in the regulation of cyclin D1 expression. As shown in Figure 7, overexpression of PLD2 resulted in a slight transcriptional activation of the cyclin D1-luciferase reporter. However, co-expression of PLD2 with wild-type PKC- ι (PKC- ι -wt) caused a significant induction of cyclin D1, comparable with the effect of transforming Ras (Ras-L61). This effect was completely inhibited by a kinase-deficient dominant negative PKC- ι (PKC- ι -KW) mutant. Overexpression of the constitutively active PKC- ι (PKC- ι -AE) mutant proved almost as effective as the co-expression of PKC- ι -wt and PLD2. Transcriptional activation of the cyclin D1-luciferase reporter by PKC- ι -AE was drastically reduced by co-expression of the dominant negative PLD2 (PLD2/KR) mutant. These data suggest that both PKC- ι and PLD2 are implicated in the regulation of cyclin D1 expression, and that PKC- ι requires PLD2 for transcriptional activation of cyclin D1.

DISCUSSION

Both normal as well as transforming Ras have been implicated in the regulation of PLD [5,10–12]. To date, it has remained unclear which PLD isoenzyme is affected by Ras. The data presented in this study demonstrate that expression of transforming Ras led to the activation of ectopically expressed PLD1b as well as PLD2, suggesting that both PLD isoenzymes are mediators of cellular Ras signalling. However, the mechanism by which Ras mediates the activation of PLD is still insufficiently understood. Stimulation of PLD by transforming Ras or Src has been shown to depend on the small GTPase RalA. Overexpression of RalA potentiates PLD activation by v-Src, whereas dominant negative RalA inhibits elevated PLD activity in v-Src- and v-Ras-transformed cells [11–13]. The PLD isoenzyme that can associate with RalA *in vitro* has been identified as PLD1 [15]. In the present study, PLD1b has been shown to act as a potent activator of atypical PKC- ι . Overexpression of PKC- ι in turn can selectively enhance the activity of PLD2. Furthermore, the activation of PKC- ι by transforming Ras has previously been demonstrated [16]. It is intriguing, therefore, to speculate that transforming Ras initiates a signalling cascade Ras $\rightarrow$ RalA $\rightarrow$ PLD1b $\rightarrow$ PKC- ι $\rightarrow$ PLD2, even if other mechanisms, such as a RhoA-mediated activation of PLD1b [1,2], cannot be excluded. Nevertheless, the data presented here indicate that PKC- ι acts downstream of PLD1b and upstream of PLD2.

In accordance with previous findings [5,10,31], the Ras-induced activation of PLD appears to be mediated via a different mechanism from the phorbol ester-mediated PLD stimulation. This follows from the observation that activation by Ras and PMA act in an additive fashion, and that the PMA-induced activation of PLD can be selectively abrogated by the PKC inhibitor GF109203X without affecting the Ras-mediated activation of PLD. Thus Ras activates PLD by a pathway that does not employ phorbol ester-sensitive PKC isoforms.

The model that Ras activates PLD2 via PKC- ι , however, is supported by the observation that the Ras-mediated enhancement of PLD activity is reduced by Ro-31-8220, a PKC inhibitor which has been shown to inhibit atypical PKC isoforms more efficiently than GF109203X [26,27]. Although GF109203X at the concentration used in these studies has been shown to inhibit atypical PKC- ζ by approx. 50% *in vitro* [28], the inhibitor

concentration achieved within the cell is unknown and may well be significantly lower than the *in vitro* concentration.

The observation that overexpression of PKC- ι selectively activates PLD2 without any effect on PLD1b is of particular interest. To date, PLD1 is considered to represent the PKC-responsive PLD isoenzyme [1–3]. The PKC isoforms that have been reported to activate PLD1 represent phorbol ester-responsive isotypes, predominantly PKC- α [32]. However, the role of atypical PKC isoforms in PLD activation has not been reported. Atypical PKC- ι and - ζ differ substantially from conventional or novel isoforms and fulfil different roles in cellular signalling [23,33,34]. It is, therefore, not surprising that they may also interact with different PLD isoforms. Furthermore, PLD2, which shares 50–53% sequence identity with PLD1, exhibits several structural differences when compared with PLD1, particularly with regard to putative regulatory sequences [1]. PLD2 lacks the 116-amino-acid 'loop' that is found in PLD1 following the first HKD motif. This loop is dispensable for catalytic activity and may thus serve regulatory functions [35]. In addition to the 'loop-region', PLD1 and PLD2 differ predominantly with respect to their N- and C-termini. The N-terminal sequence of PLD2, comprising amino acids 1–308, is not required for catalytic activity, but may serve important regulatory functions, as its removal confers responsiveness to ARF [35]. Thus with regard to the structural differences between atypical PKCs and conventional- or novel-type PKCs, and also between PLD1 and PLD2, it is conceivable that PKC- ι exhibits a PLD isoenzyme specificity that differs from conventional- or novel-type PKCs.

The mechanism by which PKC- ι is regulated *in vivo* is still poorly understood. In contrast with conventional- and novel-type PKCs, atypical PKCs appear to be regulated, at least in part, by protein–protein interactions. A PKC- λ -interacting protein, termed LIP, has been described which acts as a PKC- λ -specific activator *in vitro* [36]. However, the extent to which LIP is implicated in PKC- ι / λ regulation *in vivo* remains unclear. Another protein, PAR4, that binds to and inhibits both PKC- ι / λ and PKC- ζ , has also been identified [37]. *In vitro* PA acts as an activator of atypical PKC- ζ [20–22]. As PKC- ζ is homologous with PKC- ι / λ [23,24], the role of PA in the regulation of PKC- ι appears conceivable. Our findings that overexpression of PLD1b and, to a lesser extent, PLD2 result in the activation of PKC- ι are in accordance with the assumption that PA, generated by PLD, acts as an activator of this atypical PKC isoform *in vivo*. The conclusion that activation of PKC- ι is mediated by PLD-generated PA is supported by our data demonstrating that butan-1-ol, which sequesters PA, inhibits PLD1b-mediated activation of PKC- ι . Although PA may be necessary for PKC- ι activation, it is probably not sufficient for maximal activity. In addition to PA, full activation of PKC- ι may require phosphorylation of the enzyme, probably catalysed by phosphoinositide-dependent kinase ('PDK') [34,38], and other co-factors, including regulatory proteins.

The evaluation of the biological significance of the data presented here is hampered by the fact that they have all been obtained in overexpression systems. Nevertheless, the observation that co-expression of PLD2 with PKC- ι induced cyclin D1 to a similar degree as transforming Ras supports their potential role in mitogenic signalling. This effect was blocked by expressing a dominant negative mutant of either PKC- ι or PLD2, suggesting that both enzymes are involved in cyclin D1 regulation and that PKC- ι requires PLD2 for transcriptional activation of cyclin D1.

We thank Dr M. Frohman for providing the PLD1 and PLD2 plasmids, Dr R. Pestell for the cyclin D1-luciferase reporter construct and Dr M. Karin for the Ras-L61 plasmid. This work was supported in part by a grant from the Austrian Science Fund

(Fonds zur Förderung der wissenschaftlichen Forschung) project F008 as part of the Special Research Centre (SFB) F002 'Biological communications systems'.

REFERENCES

- Liscovitch, M., Czarny, M., Fiucci, G. and Tang, X. (2000) Phospholipase D: molecular and cell biology of a novel gene family. *Biochem. J.* **345**, 401–415
- Exton, J. H. (1999) Regulation of phospholipase D. *Biochim. Biophys. Acta* **1439**, 121–133
- Frohman, M. A., Sung, T. C. and Morris, A. J. (1999) Mammalian phospholipase D structure and regulation. *Biochim. Biophys. Acta* **1439**, 175–186
- Carnero, A., Cuadrado, A., del Peso, L. and Lacal, J. C. (1994) Activation of type D phospholipase by serum stimulation and ras-induced transformation in NIH3T3 cells. *Oncogene* **9**, 1387–1395
- del Peso, L., Lucas, L., Esteve, P. and Lacal, J. C. (1997) Activation of phospholipase D by growth factors and oncogenes in murine fibroblasts follow alternative but cross-talking pathways. *Biochem. J.* **322**, 519–528
- Thompson, N. T., Garland, L. G. and Bonser, R. W. (1993) Phospholipase D: regulation and functional significance. *Adv. Pharmacol.* **24**, 199–238
- English, D., Cui, Y. and Siddiqui, R. A. (1996) Messenger functions of phosphatidic acid. *Chem. Phys. Lipids* **80**, 117–132
- Meier, K. E., Gibbs, T. C., Knoepf, S. M. and Ella, K. M. (1999) Expression of phospholipase D isoforms in mammalian cells. *Biochim. Biophys. Acta* **1439**, 199–213
- Exton, J. H. (2000) Phospholipase D. *Ann. N. Y. Acad. Sci.* **905**, 61–68
- Lucas, L., del Peso, L., Rodriguez, P., Penalba, V. and Lacal, J. C. (2000) Ras protein is involved in the physiological regulation of phospholipase D by platelet derived growth factor. *Oncogene* **19**, 431–437
- Voss, M., Weernink, P. A., Haupenthal, S., Moller, U., Cool, R. H., Bauer, B., Camonis, J. H., Jakobs, K. H. and Schmidt, M. (1999) Phospholipase D stimulation by receptor tyrosine kinases mediated by protein kinase C and a Ras/Ral signaling cascade. *J. Biol. Chem.* **274**, 34691–34698
- Lu, Z., Hornia, A., Joseph, T., Sukezane, T., Frankel, P., Zhong, M., Bychenok, S., Xu, L., Feig, L. A. and Foster, D. A. (2000) Phospholipase D and RalA cooperate with the epidermal growth factor receptor to transform 3Y1 rat fibroblasts. *Mol. Cell Biol.* **20**, 462–467
- Aguirre-Ghiso, J. A., Frankel, P., Farias, E. F., Lu, Z., Jiang, H., Olsen, A., Feig, L. A., Kier Joffe, E. B. and Foster, D. A. (1999) RalA requirement for v-Src- and v-Ras-induced tumorigenicity and overproduction of urokinase-type plasminogen activator: involvement of metalloproteases. *Oncogene* **18**, 4718–4725
- Kim, J. H., Lee, S. D., Han, J. M., Lee, T. G., Kim, Y., Park, J. B., Lambeth, J. D., Suh, P. G. and Ryu, S. H. (1998) Activation of phospholipase D1 by direct interaction with ADP-ribosylation factor 1 and RalA. *FEBS Lett.* **430**, 231–235
- Luo, J. Q., Liu, X., Hammond, S. M., Colley, W. C., Feig, L. A., Frohman, M. A., Morris, A. J. and Foster, D. A. (1997) RalA interacts directly with the Arf-responsive, PIP2-dependent phospholipase D1. *Biochem. Res. Commun.* **235**, 854–859
- Ueberall, F., Hellbert, K., Kampfer, S., Maly, K., Villunger, A., Spitaler, M., Mwanjewe, J., Baier-Bitterlich, G., Baier, G. and Grunicke, H. H. (1999) Evidence that atypical protein kinase C-lambda and atypical protein kinase C-zeta participate in Ras-mediated reorganization of the F-actin cytoskeleton. *J. Cell Biol.* **144**, 413–425
- Kampfer, S., Hellbert, K., Villunger, A., Doppler, W., Baier, G., Grunicke, H. H. and Ueberall, F. (1998) Transcriptional activation of c-fos by oncogenic Ha-Ras in mouse mammary epithelial cells requires the combined activities of PKC-lambda, epsilon and zeta. *EMBO J.* **17**, 4046–4055
- Hellbert, K., Kampfer, S., Maly, K., Hochholdinger, F., Mwanjewe, J., Baier, G., Ueberall, F. and Grunicke, H. H. (2000) Implication of atypical protein kinase C isoenzymes lambda and zeta in Ras mediated reorganization of the actin cytoskeleton and cyclin D1-induction. *Adv. Enzyme Regul.* **40**, 49–62
- Bjorkoy, G., Perander, M., Overvatn, A. and Johansen, T. (1997) Reversion of Ras- and phosphatidylcholine-hydrolyzing phospholipase C-mediated transformation of NIH 3T3 cells by a dominant interfering mutant of protein kinase C lambda is accompanied by the loss of constitutive nuclear mitogen-activated protein kinase/extracellular signal-regulated kinase activity. *J. Biol. Chem.* **272**, 11557–11565
- Nakanishi, H. and Exton, J. H. (1992) Purification and characterization of the zeta isoform of protein kinase C from bovine kidney. *J. Biol. Chem.* **267**, 16347–16354
- Limatola, C., Schaap, D., Moolenaar, W. H. and van Blitterswijk, W. J. (1994) Phosphatidic acid activation of protein kinase C-zeta overexpressed in COS cells: comparison with other protein kinase C isoforms and other acidic lipids. *Biochem. J.* **304**, 1001–1008
- Limatola, C., Barabino, B., Nista, A. and Santoni, A. (1997) Interleukin 1-beta-induced protein kinase C-zeta activation is mimicked by exogenous phospholipase D. *Biochem. J.* **321**, 497–501
- Akimoto, K., Mizuno, K., Osada, S., Hirai, S., Tanuma, S., Suzuki, K. and Ohno, S. (1994) A new member of the third class in the protein kinase C family, PKC lambda, expressed dominantly in an undifferentiated mouse embryonal carcinoma cell line and also in many tissues and cells. *J. Biol. Chem.* **269**, 12677–12683
- Selbie, L. A., Schmitz-Peiffer, C., Sheng, Y. and Biden, T. J. (1993) Molecular cloning and characterization of PKC iota, an atypical isoform of protein kinase C derived from insulin-secreting cells. *J. Biol. Chem.* **268**, 24296–24302
- Edwards, A. S., Faux, M. C., Scott, J. D. and Newton, A. C. (1999) Carboxyl-terminal phosphorylation regulates the function and subcellular localization of protein kinase C beta11. *J. Biol. Chem.* **274**, 6461–6468
- van Dijk, M., Muriana, F. J., Der Hoeven, P. C., de Widt, J., Schaap, D., Moolenaar, W. H. and van Blitterswijk, W. J. (1997) Diacylglycerol generated by exogenous phospholipase C activates the mitogen-activated protein kinase pathway independent of Ras- and phorbol ester-sensitive protein kinase C: dependence on protein kinase C-zeta. *Biochem. J.* **323**, 693–699
- van Dijk, M. C., Hilkman, H. and van Blitterswijk, W. J. (1997) Platelet-derived growth factor activation of mitogen-activated protein kinase depends on the sequential activation of phosphatidylcholine-specific phospholipase C, protein kinase C-zeta and Raf-1. *Biochem. J.* **325**, 303–307
- Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D. and Schachtele, C. (1993) Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J. Biol. Chem.* **268**, 9194–9197
- Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A. and Pestell, R. G. (1995) Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J. Biol. Chem.* **270**, 23589–23597
- Matsumura, I., Kitamura, T., Wakao, H., Tanaka, H., Hashimoto, K., Albanese, C., Downward, J., Pestell, R. G. and Kanakura, Y. (1999) Transcriptional regulation of the cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. *EMBO J.* **18**, 1367–1377
- del Peso, L., Hernandez, R., Esteve, P. and Lacal, J. C. (1996) Activation of phospholipase D by ras proteins is independent of protein kinase C. *J. Cell. Biochem.* **61**, 599–608
- Conricode, K. M., Smith, J. L., Burns, D. J. and Exton, J. H. (1994) Phospholipase D activation in fibroblast membranes by the alpha and beta isoforms of protein kinase C. *FEBS Lett.* **342**, 149–153
- Mellor, H. and Parker, P. J. (1998) The extended protein kinase C superfamily. *Biochem. J.* **332**, 281–292
- Ron, D. and Kazanietz, M. G. (1999) New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J.* **13**, 1658–1676
- Sung, T. C., Zhang, Y., Morris, A. J. and Frohman, M. A. (1999) Structural analysis of human phospholipase D1. *J. Biol. Chem.* **274**, 3659–3666
- Diaz-Meco, M. T., Municio, M. M., Sanchez, P., Lozano, J. and Moscat, J. (1996) Lambda-interacting protein, a novel protein that specifically interacts with the zinc finger domain of the atypical protein kinase C isoform lambda/iota and stimulates its kinase activity in vitro and in vivo. *Mol. Cell Biol.* **16**, 105–114
- Diaz-Meco, M. T., Municio, M. M., Frutos, S., Sanchez, P., Lozano, J., Sanz, L. and Moscat, J. (1996) The product of par-4, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C. *Cell (Cambridge, Mass)* **86**, 777–786
- Chou, M., Hou, W., Johnson, J., Graham, L. K., Lee, M. H., Chen, C. S., Newton, A. C., Schaffhausen, B. and Toker, A. (1998) Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. *Curr. Biol.* **8**, 1069–1077

Received 22 January 2001/30 May 2001; accepted 17 July 2001