# Phospholipase D2 stimulates integrin-mediated adhesion via phosphatidylinositol 4-phosphate 5-kinase Iγ*b*

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## Summary

**Cellular adhesion can be regulated by, as yet, poorly defined intracellular signalling events. Phospholipase D enzymes generate the messenger lipid phosphatidate and here we demonstrate that suppression of this reaction inhibits cellular adhesion. This effect was reversed by the addition of cell-permeable analogues of either phosphatidate or phosphatidylinositol 4,5-bisphosphate. By contrast, neither diacylglycerol nor lysophosphatidic acid were able to reverse this effect suggesting that phosphatidate itself acts directly on a target protein(s) to regulate adhesion rather than as the result of its conversion to either of these metabolite lipids. Antibodies that block $\beta_1$ and $\beta_2$ integrin-substrate interactions inhibited adhesion stimulated by both phosphatidate and phosphatidylinositol 4,5-bisphosphate indicating that these lipids regulate $\beta_1$ and $\beta_2$ integrin-mediated adhesion. In vivo, these lipids can be generated by phospholipase D2 and phosphatidylinositol 4-phosphate 5-kinase Iγ*b*, respectively, and over-expression of catalytically-functional forms of these enzymes dose-dependently stimulated adhesion while siRNA depletion of PLD2 levels inhibited adhesion. Furthermore the ability of over-expressed phospholipase D2 to stimulate adhesion was inhibited by a dominant-negative version of phosphatidylinositol 4-phosphate 5-kinase Iγ*b*. Consistent with this, phosphatidylinositol 4-phosphate 5-kinase Iγ*b*-mediated adhesion was dependent upon phospholipase D2's product, phosphatidate indicating that phosphatidylinositol 4-phosphate 5-kinase Iγ*b* is downstream of, and necessary for, phospholipase D2's regulation of adhesion. It is likely that this phospholipase D2-generated phosphatidate directly stimulates phosphatidylinositol 4-phosphate 5-kinase Iγ*b* to generate phosphatidylinositol 4,5-bisphosphate as this mechanism has previously been demonstrated in vitro. Thus, our data indicates that during the initial stages of adhesion, phospholipase D2-derived phosphatidate stimulates phosphatidylinositol 4-phosphate 5-kinase Iγ*b* to generate phosphatidylinositol 4,5-bisphosphate and that consequently this inositol phospholipid promotes adhesion through its regulation of cell-surface integrins.**

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## Introduction

The control of cellular adhesion involves the activation of many pathways and signalling enzymes. The major mechanism by which cells anchor themselves to their extracellular environment is mediated by the integrin family of proteins (Carman and Springer, 2003; Plow et al., 2000). These heterodimeric receptors consist of an α and a β subunit, and are generally classified into families based on the identity of the β subunit. Following the activation of diverse intracellular signalling pathways, integrin heterodimers exist in either high or low affinity binding states for their extracellular substrates (Calderwood et al., 2000; Carman and Springer, 2003; Garcia-Alvarez et al., 2003; Woods and Couchman, 2000). Rapid changes in the adhesion of the whole cell, or specific areas of the cell, are important in cellular mechanisms such as migration, phagocytosis and cytokinesis and diseases such as metastasis, deregulated inflammation and allergy (Felding-Habermann, 2003; Harris et al., 2000; Marshall and Haskard, 2002). Thus, the characterisation of the intracellular signalling pathways regulating integrin-mediated adhesion may be critical to both the understanding and future treatment of such diseases.

Prior to engagement, the high affinity, substrate-binding conformation of an integrin can be induced by the interaction of talin with the cytoplasmic tail of the integrin β subunit (Calderwood et al., 2002; Garcia-Alvarez et al., 2003; Tadokoro et al., 2003). This interaction is facilitated by unmasking the band 4.1 ezrin, radixin, moesin (FERM) domain of talin, brought about by the binding of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)$P_2$] to an adjacent region within the FERM domain (Martel et al., 2001). Currently, the mechanism and enzyme(s) responsible for generating this lipid are undetermined.

The major route of PtdIns(4,5)$P_2$ generation within cells is the phosphorylation of phosphatidylinositol 4-phosphate catalysed by members of the phosphatidylinositol 4-phosphate 5-kinase type I (PIPkin I) family, of which there are three isoforms α, β and γ (Ishihara et al., 1998). The PIPkin Iγ sub-family exists as three splice variants, *a*, *b* and *c* (Giudici et al., 2004) [in humans alternatively named 661 (90 kDa), 635 (87 kDa) and 688 as defined by their numbers of amino acids] and subsequent to integrin-ligand engagement at nascent sites of adhesion, talin dimers complexed with the β subunit of the engaged integrin are believed to recruit and activate the *a* splice variant of PIPkin Iγ (Barsukov et al., 2003; Di Paolo et al., 2002; Ling et al., 2002; Ling et al., 2003). The consequent generation of PtdIns(4,5)$P_2$ following integrin-ligand engagement would appear to be essential to the complete formation of focal complexes/adhesions necessary to strengthen adhesion through the recruitment of other PtdIns(4,5)$P_2$-binding proteins, such as vinculin, α-actinin and syndecan-4 (Fukami et al., 1992; Giannone et al., 2003; Gilmore and Burridge, 1996; Greene et al., 2003; Horowitz et al., 1999).

The hydrolysis of phosphatidylcholine (PtdCho) catalysed by phospholipase D1 (PLD1) and PLD2 generates the messenger lipid phosphatidate (PtdOH) (Hodgkin et al., 1998). The activation of PLD1 is believed to be predominantly agonist-dependent (Powner and Wakelam, 2002). In the basophil/mast cell line rat basophil leukaemia (RBL)-2H3, PLD1 can be activated downstream of FcεRI cross-linking in a phosphatidylinositol 3-kinase-dependent manner by ARF6, Rac1 and PKCα (Powner et al., 2002). By contrast, little is known about the regulation of PLD2 although its activity is dependent upon PtdIns(4,5)$P_2$ and it has been hypothesised that availability of this lipid may be a major factor in its regulation (Divecha et al., 2000). Furthermore, in contrast to PLD1 which also requires PtdIns(4,5)$P_2$ for activation, PLD2 has been shown to have high basal activity in the presence of PtdIns(4,5)$P_2$ and may therefore be subject to negative regulation through direct protein interaction as has been demonstrated through interaction with proteins such as actin, α-actinin, α-synuclein and tubulin (Lee et al., 2001; Park et al., 2000; Payton et al., 2004; Chae et al., 2005).

The activities of both PLD1 and PLD2 have been demonstrated to regulate mechanisms that require the activation of integrins, such as cellular spreading and migration (McDermott et al., 2004; Powner et al., 2002; Santy and Casanova, 2001; Zhou et al., 1995). However no molecular targets for PtdOH within these processes have been identified. In this report we demonstrate that PLD2-derived PtdOH stimulates PIPkin I*γb* and the subsequent generation of PtdIns(4,5)$P_2$ drives the initial stages of cellular adhesion by enhancing $\beta_1$ and $\beta_2$ integrin binding to their cognate substrates.

## Materials and Methods

### Cell culture and transfection

Rat basophilic leukemic (RBL)-2H3 cells, human epithelial kidney (HEK)293 cells, human KS1.6 fibroblast cells and rat L6 myoblast cells were cultured at 37°C in 5% $CO_2$ in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% foetal calf serum. PLD1 and PLD2 (and their mutants) were cloned from human sources. PIPkin Iα was cloned from a murine source. PIPkin Iγ was cloned from a *rattus* source. For RBL-2H3 cells, $5\times10^6$ cells were transfected by electroporation with 6 pmol of each construct in a Bio-Rad Gene Pulser II (270 V, 1500 μF). Experiments were performed 16 hours post transfection. For L6 cells, one well of a 6-well plate was transfected with 240 pmol of siRNA complexed to 8 μl of Lipofectamine 2000 (Invitrogen). PLD1 siRNA oligonucleotides 5′-GCACUUCCAAGAGUUUCAUdTdT-3′ and 5′-AUGAAACU-CUUGGAAGUGCdTdT (Eurogentec S.A.). PLD2 siRNA oligonucleotides 5′-GACACAAAGUCUUGAUGAGdTdT-3′ and 5′-CUCAUCAAGACUUUGUGUCdTdT-3′ (Eurogentec S.A.). Scrambled oligonucleotides were also supplied. Experiments were performed 48-72 hours post transfection.

### Quantitative PCR

Total RNAs were extracted 48 hours following siRNA transfection of 6-well plates using Nucleobond II kit (Clontech) and cDNAs were generated from made using the Ambion Retroscript kit (oligodT primers) and the primer-probe mixtures ('Assay-on-Demand' mixtures) (Applied Biosystems). Quantitative PCR was performed on ABI Prism 7700 Sequence Detection System. Standard curves were generated using fivefold dilutions of cDNA from non-siRNA treated cells. β-Galactosidase mRNA levels were determined as controls.

### PLD activity assay

Cells were grown for 16 hours in the presence of 5 μCi $ml^{-1}$ [$^3$H]-palmitate (Amersham PharmaciaBiotech) and incubated in assay medium (125 mM NaCl, 5 mM KCl, 1.8 mM $MgCl_2$, 0.5 mM $Na_2PO_4$, 5 mM $NaHCO_3$, 10 mM glucose, 0.1% BSA and 10 mM HEPES pH 7.2) containing 0.3% (v/v) butan-1-ol, for 5 minutes prior to the application of an experimental condition. If required cells were detached from substrate using a cell lifter (Sarstedt). Lipids were extracted with chloroform-methanol and $^3$[H]-PtdBut isolated by thin layer chromatography as previously described (Wakelam et al., 1995).

### Lipid preparation

Relevant amounts of organic solvent-dissolved lipids (Avanti Polar Lipids, A.G. Scientific and Sigma) were dried down under $N_2$(g) in a glass vial. HEPES-buffered saline (HBS) was added to 90% of the volume required to give 1 mM stock solution. This solution was subsequently probe-sonicated twice for 15 seconds at 10 Watts (RMS). The extra 10% HBS supplemented with 10 mg $ml^{-1}$ fatty acid-free bovine serum albumin (Sigma) was added before freezing at –80°C. After partial thawing, complete thawing was facilitated by a further round of probe-sonication. Aqueous-soluble lipids were then added directly to media.

### Adhesion assays

Lipids, antibodies and butan-1-ol were pre-incubated with the cells for 5-15 minutes. Dose response and time of incubation experiments were performed for each lipid and antibodies. Conditions used thereof, gave maximal stimulation of adhesion. Anti-rat $\beta_1$ (CD29) clone HA2/5 and anti-rat $\beta_2$ (CD18) clone WT3 (both BD Biosciences). Substrates were added to plates for 16 hours at 4°C. Substrates were as follows in 96-well plates: 50 μl foetal calf serum; 0.5 μg per well of FN (Sigma); 0.33 μg per well of ICAM1 (R and D Systems). Excess substrate was removed and 50 μl of heat-inactivated goat serum was added to each well for 3 hours at 4°C. [Cells were able to bind to heat-inactivated goat serum alone but not to the extent that they bound to plastic also treated with serum, FN or ICAM1 (data not shown). This indicates that adhesion was predominantly dependent upon either serum, FN or ICAM1.]

Untransfected cells: cells were pre-incubated as necessary and subsequently released into suspension using a cell lifter (Sarstedt).

Cells were seeded at around 40,000 cells per well in substrate-coated 96-well plates, sedimented at 17 ***g*** and allowed to adhere for 10 minutes. Non-adhered cells were removed before the plate was frozen overnight at –80°C. Cell number was quantified by the addition of CyQuant (Molecular Probes) and subsequent analysis using a fluorimeter (Labsystems).

GFP transfected cells: cells were pre-incubated as necessary and subsequently released into suspension using a cell lifter (Sarstedt). Half the cell population was analysed by flow cytometry (Coulter XL-MCL) to determine the proportion of GFP-transfected cells. The other half of the cell population was seeded into foetal calf serum-treated wells, sedimented at 17 ***g*** and allowed to adhere for 5 minutes. Not adhered cells were removed and subsequently analysed by flow cytometry to determine the proportion of GFP-transfected cells. The change in proportion of GFP-transfected cells between the two samples was used to calculate the effect of transfection on adhesion. At least 50,000 gated, single, live (not apoptosing) cells were analysed per sample and where transfection efficiency was between 25-70%.

### Lipid analysis

$7\times10^6$ cells were incubated with 80 nmol of lipid for 5 minutes at 37°C. Cells were subsequently pelleted and washed 2× in PBS. Lipids were extracted from the cells by the addition of 1 ml $CHCl_3$, 0.5 ml of $CH_3OH$ and 0.375 ml of 0.88% KCl. To determine the maximum level of lipid recoverable by the extraction procedure 80 nmol of each lipid was subjected to the same extraction conditions. The $CHCl_3$ phase was dried and lipids were resuspended in 15 μl of $CHCl_3$ in the presence of 500 ng of each of diC12-1,2 diacylglycerol (DAG), diC12-PtdOH, diC12-phosphatidylcholine (PtdCho), diC12-phosphatidylethanolamine, diC12-phosphatidylglycerol and diC12-phosphatidylserine as internal standards. For PtdOH and PtdCho analysis, samples were separated by HPLC on a silica column (Phenomenex) with a gradient of 100% $CHCl_3$:$CH_3OH$:$H_2O$ (90:9.5:0.5) changing to 100% acetonitrile:$CHCl_3$:$CH_3OH$:$H_2O$ (30:30:35:5) over 40 minutes at 0.1 ml minute$^{-1}$. Detection was by negative electrospray ionisation on a Shimadzu QP8000α Mass Spectrometer. For DAG analysis, samples were separated by HPLC using $CHCl_3$:hexane:isopropanol:$H_2O$ (30:70:1.5:0.025) as the first solvent and then as above. Detection was by positive electrospray ionisation.

### Confocal immunofluorescence microscopy

Cells in suspension were sedimented at 17 ***g*** on to FCS-coated glass coverslips and fixed for 7 minutes with ice-cold 4% paraformaldehyde and permeabilised using 0.5% CHAPS (Sigma) for 2 minutes. After blocking for 1 hour with 20% heat-inactivated goat serum in PBS the HA-tag was detected with clone Y11 antibody (Santa Cruz), the FLAG-tag was detected with clone M2 antibody (Sigma), talin was detected with clone 8D4 antibody (Sigma) and filamentous actin was detected using phalloidin conjugated to Alexa 546 (Molecular Probes) each for 1 hour. Subsequently, subclass-specific Alexa 546 conjugated secondary antibodies (Molecular Probes) were added for 1 hour to label fluorescently primary antibody-bound proteins. All antibodies were diluted in 0.2% saponin 20% heat-inactivated goat serum. Coverslips were mounted onto slides in Prolong (Molecular Probes). All images are confocal sections acquired using a Nikon TE 300 microscope/PCM2000 system and the accompanying EZ2000 software.

## Results

### PLD activation stimulates adhesion

High shear forces within blood flow or tissue injury can result in complete or partial loss of cellular attachment to substratum

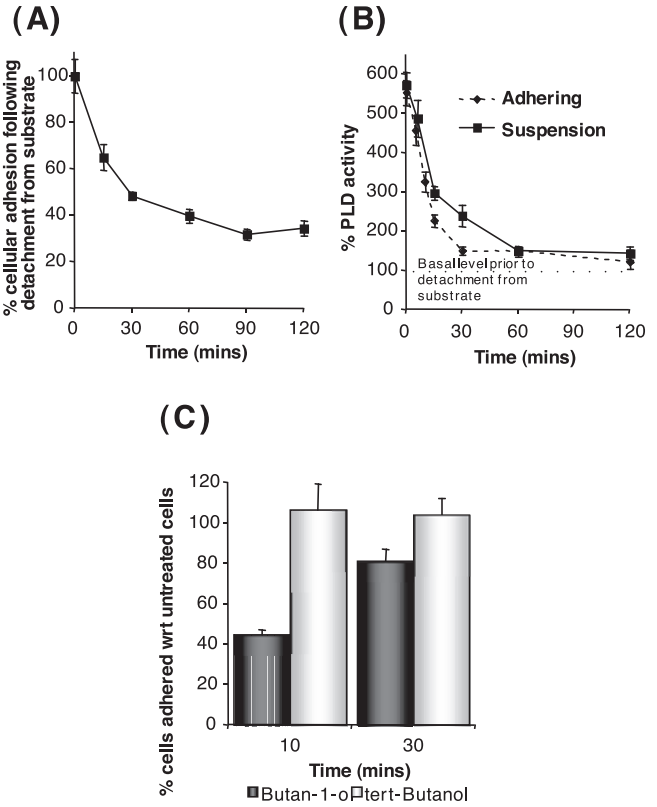


**Fig. 1.** PLD activation regulates adhesion. (A) At each time point following detachment from substrate ($t$=0), the number of cells able to adhere to serum-coated plastic over a 5 minute period was measured. (B) At each time point following detachment from substrate ($t$=0), PtdBut accumulation was quantified over a 5 minute period as a measure of PLD activity in either cells allowed to immediately re-adhere (dotted line) or cells maintained in suspension (solid line). (C) The level of adhesion after 10 or 30 minutes on serum-coated plastic was quantified for cells that had previously been detached from substrate and treated for 5 minutes with 0.5% butan-1-ol/*tert*-butanol. Results are expressed as a percentage of the number of untreated cells adhered after 10 or 30 minutes. At 10 minutes, the level of untreated cells adhered were approximately 70% of total cells added to each well. At 30 minutes, the level of untreated cells adhered were approximately 90% of total cells added to each well (data not shown). Similar results were obtained in at least three separate experiments and error bars represent the standard deviation from the mean.

(Fukuda and Schimid-Schonbein, 2002; Fukuda and Schimid-Schonbein, 2003; Luchetti et al., 2004). Generally, for cells to survive these events this is followed by their rapid re-attachment (Fukuda and Schimid-Schonbein, 2002; Fukuda and Schimid-Schonbein, 2003; Grinnell, 1992; Luchetti et al., 2004; Oliver et al., 1997; Strater et al., 1996). To mimic these processes we mechanically detached RBL-2H3 cells from serum-coated plastic (resulting in less than 5% cell death) and analysed the signalling cascades involved in their subsequent re-adhesion. Following detachment and immediate re-addition to substrate, >70% of viable cells re-adhered within 10 minutes rising to >90% within 30 minutes (data not shown). However, these levels of adherence declined the longer that the cells were maintained in suspension (Fig. 1A). As this time in suspension had little effect upon cell viability (supplementary material Fig.

S1) it is likely that the signals and mechanisms required to regulate adhesion became progressively down-regulated over the period of time that the cells were left in suspension.

PLD activity can be stimulated in RBL-2H3 cells by a variety of agonists and cell-permeable compounds (McDermott et al., 2004; Powner and Wakelam, 2002). The mechanical detachment of RBL-2H3 cells from serum-coated plastic stimulated an immediate, 5-6-fold increase in PLD activity in the absence of such additional stimuli (Fig. 1B). With the maintenance of these cells in suspension this activity declined to the basal level found in adhered cells. This reduction in activity correlated with the decline in the ability of these cells to re-adhere, suggesting that PLD activity may provide a signal necessary for the re-adhesion of these cells. Accordingly, when cells were not maintained in suspension but added back to substrate immediately following detachment, PLD activity declined at a greater rate than when left in suspension (Fig. 1B). This may suggest that PLD activity is specifically stimulated to regulate adhesion and that intracellular signals initiated following cell-substrate interaction provide inhibitory mechanisms for down-regulating PLD activity once it is not required.

Selective chemical inhibitors of PLD are not available, but signalling via PLD can be suppressed by the presence of short chain, primary aliphatic alcohols, for example butan-1-ol, as non-signalling phosphatidylalcohols are generated by PLD rather than PtdOH (Yang et al., 1967). By contrast, related alcohols, such as butan-2-ol or *tert*-butanol, do not mediate this effect and can be used as controls for the non-PLD-dependent effects of alcohols upon cells. Treatment of RBL-2H3 cells for 5 minutes with 0.5% butan-1-ol reduced the numbers of cells adhered to serum-coated plastic over 10 or 30 minutes periods when compared with untreated or *tert*-butanol-treated cells (Fig. 1C). Hence, the correlation between PLD activity and adhesion demonstrated in Fig. 1A,B probably results from PLD functioning as an upstream regulator of adhesion. A similar pattern of results, albeit to different extents, was also obtained for cell lines from different tissue origins (fibroblast (KS1.6), epithelial (HEK293) and myoblast (L6) origin), indicating that this is not simply a leukocyte (RBL-2H3) restricted phenomenon (supplementary material Fig. S2).

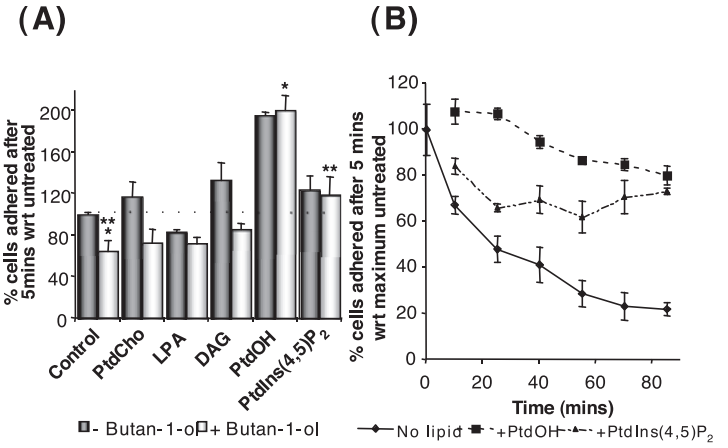


**Fig. 2.** PtdIns(4,5)$P_2$ regulates adhesion downstream of PLD activation. (A) Both in the absence or presence of 0.5% butan-1-ol and following treatment with either 100 μM PtdCho, 100 μM PtdOH, 50 μM DAG, 100 μM PtdIns(4,5)$P_2$ or vehicle (control) the level of cellular adhesion to serum-coated plastic was quantified. Optimal concentrations of each lipid were determined in dose-response experiments. The dotted line represents the level of adhesion observed in control cells not treated with butan-1-ol. In the presence of butan-1-ol, treatment with lipid was considered to have completely recovered the loss of adhesion due to PLD inhibition if the level of adhesion was above this line (B) Adhesion to serum-coated plastic was quantified after cells had been maintained in suspension for the indicated time points and subsequently treated with PtdOH, PtdIns(4,5)$P_2$ or vehicle (no lipid). Using a Student's *t*-test, *P* values of <0.05% were obtained (* and **). The level of adhesion for each individual lipid was obtained from an average of at least three experiments and error bars represent the standard deviation from the mean.

## PtdOH and PtdIns(4,5)$P_2$ stimulate adhesion downstream of PLD activation

PtdOH can function as a signalling molecule by directly interacting with specific protein targets or indirectly, following its conversion to 1,2 diacylglycerol (DAG) or lysophosphatidic acid (LPA). Lipids with acyl chains of no more than eight carbons (diC8) are soluble in aqueous solutions and many have been shown to be cell permeable and to mimic the effects of their endogenously synthesised counterparts when added to cells (Davis et al., 1985; Pagano et al., 1983; Pitcher et al., 1995). To determine the pathway(s) downstream of PLD activation required to regulate adhesion, we attempted to reconstitute the adhesion of cells in the absence of endogenous PLD activity using diC8 analogues of PtdOH and its immediate metabolites. We also treated cells with diC8-PtdIns(4,5)$P_2$, a lipid previously demonstrated to regulate adhesion (Di Paolo et al., 2002; Ling et al., 2002; Ling et al., 2003; Martel et al., 2001).

In the absence of butan-1-ol we found that DAG, PtdOH and PtdIns(4,5)$P_2$ significantly increased adhesion above that of control cells (Fig. 2A). However, in the presence of butan-1-ol, only diC8-PtdOH and diC8-PtdIns(4,5)$P_2$ were able to completely recover the inhibition of adhesion mediated by the loss of endogenous PtdOH production. Similarly to treatment with butan-1-ol, the maintenance of cells in suspension resulted in a reduction in endogenous PLD-generated PtdOH and a concurrent decrease in adhesion (Fig. 1A,B). Under these conditions, adhesion was also recovered by the addition of either diC8-PtdOH or diC8-PtdIns(4,5)$P_2$ (Fig. 2B).

Mass spectroscopic analysis of RBL-2H3 cells treated with the most potent concentration of diC8-PtdOH with respect to the stimulation of adhesion, revealed that approximately 1% of the added lipid was incorporated into cellular membranes (Table 1). This corresponds to approximately $50\times10^{-18}$ moles of diC8-PtdOH per cell (1% of $32728\times10^{-12}$ in $7\times10^{6}$ cells). Resting levels of PtdOH in a single cell are estimated to be less than $30\times10^{-18}$ mol (Yorek, 1993) indicating that the incorporated level of diC8-PtdOH resulted in greater than a 2.5-fold increase in total cellular PtdOH levels. This is of similar magnitude to the increases in PLD activity detected following agonist stimulation of cells (Cross et al., 1996; Powner et al., 2002). As diC8-PtdCho was also detected in these cells, it was apparent that additional diC8-PtdOH had

**Table 1. DiC8-lipids become incorporated into intracellular membranes**

| | Recovered lipid | | | | | |
|---|---|---|---|---|---|---|
| | diC 8-DAG | | diC 8-PtdOH | | diC 8-PtdCho | |
| | pmol | Recoverable (%) | pmol | Recoverable (%) | pmol | Recoverable (%) |
| Cells alone | 11 | – | 142 | – | 9 | – |
| Cells+80 nmol DAG | 1853 | 18 | 136 | 0 | 265 | 3 |
| Cells+80 nmol PtdOH | 825 | 2 | 523 | 1 | 474 | 1 |
| 80 nmol DAG alone | 10,364 | 100 | 189 | – | 4 | – |
| 80 nmol PtdOH alone | 260 | – | 32,728 | 100 | 50 | – |

$7\times10^6$ cells were incubated with 80 nmol of lipid for 5 minutes at 37°C. Lipids were recovered and subjected to mass spectroscopic analysis. Cellular incorporated diC8-lipid is expressed as a percentage of the total recoverable diC8-lipid where the total recoverable diC8-lipid is set to 100%. Total recoverable diC8-lipid was determined by the level of diC8-lipid recovered in the absence of cells but when subjected to the same extraction procedure.

become incorporated into cellular membranes and was subsequently metabolised into other diC8 lipids. The metabolism of PtdOH species into PtdCho species is an intracellular process and illustrated that diC8-PtdOH was distributed within the cytoplasmic leaflet(s) of cellular membranes (Yorek, 1993). Thus treatment of cells with diC8-PtdOH clearly elevated the intracellular, signalling relevant, concentration of PtdOH and this therefore probably accounts for its ability to recover the adhesion of cells, lost due to the absence of endogenous PtdOH generation (Fig. 2).

While diC8-PtdOH presumably restores the PLD signal in cells lacking this endogenous activity, these data additionally indicate that PtdIns(4,5)$P_2$ may also regulate adhesion and that this may occur downstream of PLD activation. However, although diC8-DAG was incorporated into cellular membranes (Table 1), the failure of either this or LPA to recover adhesion in the absence of PLD-generated PtdOH demonstrated that the effect of PtdOH was not due to its conversion to either of these lipids but presumably resulted from its direct regulation of a component of the adhesion pathway.

## PtdOH and PtdIns(4,5)$P_2$ stimulate integrin-mediated adhesion

Intracellular signalling pathways are essential to the potentiation of adhesion through the regulation of cell-surface integrins (Calderwood et al., 2000; Carman and Springer, 2003; Garcia-Alvarez et al., 2003; Woods and Couchman, 2000). Using function-blocking antibodies directed against specific families of integrins, we investigated the contribution of such integrins to the adhesion of these cells. In the presence of function-blocking antibodies to $\beta_1$ and/or $\beta_2$ integrins, the increased adhesion stimulated by either cell-permeable PtdOH or PtdIns(4,5)$P_2$ was inhibited indicating that these lipid signals regulate adhesion to serum-coated plastic via both of these integrins (Fig. 3A).

As well as expressing the almost universal $\beta_1$ family of integrins (CD29), leukocytes such as RBL-2H3 cells are unique in their expression of $\beta_2$ family integrins (CD18). Fibronectin (FN) is a component of serum that has been identified as a cognate receptor for many $\beta_1$ family integrins, while intercellular adhesion molecule 1 (ICAM1) is a component of serum that has been identified as a cognate receptor for most $\beta_2$ family integrins. Both PtdOH and PtdIns(4,5)$P_2$ were able to stimulate adhesion of RBL-2H3 cells to these substrates (supplementary material Fig. S3).

Following a reduction in PLD activity to basal levels, adhesion to FN was inhibited by antibodies directed against $\beta_1$ integrins (no lipid, Fig. 3B). Thus, the residual adhesion to FN, found in cells with basal PLD activity, is at least partially mediated through $\beta_1$ integrins, their cognate receptors. Furthermore, increased adhesion to FN stimulated by cell-permeable PtdOH and PtdIns(4,5)$P_2$ was also inhibited by this antibody indicating that nascent $\beta_1$ integrin-FN interaction is

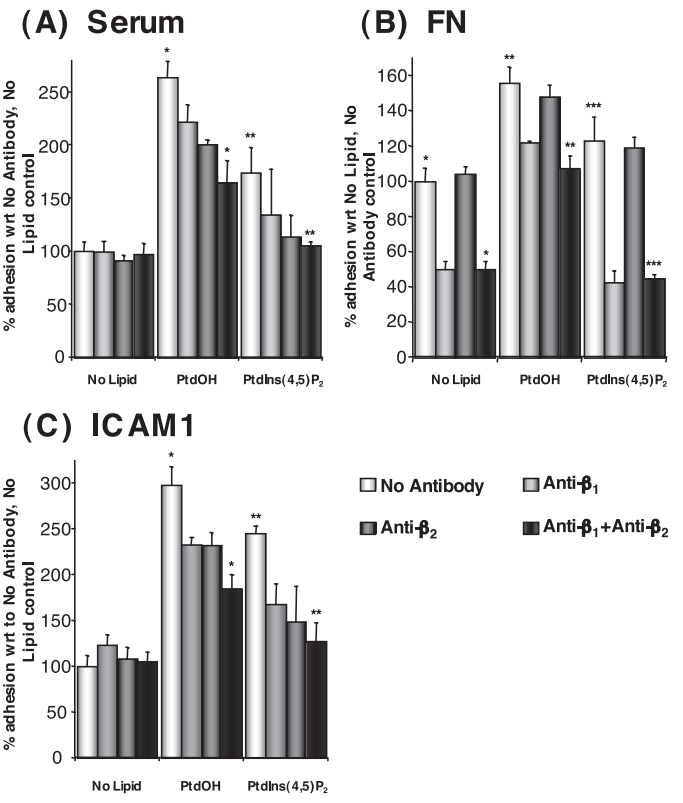


**Fig. 3.** PtdOH and PtdIns(4,5)$P_2$ regulate integrin-mediated adhesion. Following the maintenance of cells in suspension for 60 minutes to reduce PLD activity to basal levels, cells were treated with 10 mg $ml^{-1}$ of function-blocking antibodies to either $\beta_1$ or $\beta_2$ integrins or vehicle (no antibody) and either 100 μM PtdOH, 100 μM PtdIns(4,5)$P_2$ or vehicle (no lipid) before their adhesion was quantified to wells coated with (A) serum, (B) FN or (C) ICAM1. Using a Student's *t*-test, *P* values of <0.01% were obtained (*, ** and ***). Similar results were obtained in at least three separate experiments and error bars represent the standard deviation from the mean.

regulated by the generation of these lipids (Fig. 3B). By contrast, anti-$\beta_2$ integrin antibodies had no effect indicating that these integrins are not required for adhesion to FN.

While $\beta_1$ and $\beta_2$ antibodies had no effect upon the adhesion of cells to ICAM1 following a reduction in PLD activity to basal levels, anti-$\beta_2$ integrin antibodies were able to inhibit adhesion stimulated by the addition of cell-permeable PtdOH or PtdIns(4,5)$P_2$ (Fig. 3C). This indicates that adhesion to ICAM1 stimulated by PtdOH and PtdIns(4,5)$P_2$, is mediated by the cognate $\beta_2$ integrins.

The antibody to $\beta_1$ integrins also partially blocked PtdOH and PtdIns(4,5)$P_2$-mediated adhesion to ICAM1. However as ICAM1 is not believed to be a substrate for $\beta_1$ integrins, this possibly reflects binding to $\beta_1$ integrin substrates contained within the heat-inactivated goat serum that was used to 'block' plastic, not coated by ICAM1.

In all Fig. 3 indicates that the regulation of $\beta_1$ and $\beta_2$ integrin-mediated adhesion to serum by PtdOH and PtdIns(4,5)$P_2$ may be determined by the interaction of these integrins with their cognate receptors, FN and ICAM1, contained within the serum.

## PLD2 activation stimulates adhesion

Both the mammalian isoforms of PLD are expressed in RBL-2H3 cells (R.M.P., D.J.P. and M.J.O.W., unpublished). To determine if either of these isoforms can generate the PtdOH required to regulate integrin-mediated adhesion, we compared the adhesion of cells transfected with green fluorescent protein (GFP)-tagged wild type and catalytically inactive versions of each isoform, with that of control, GFP-transfected cells and non-transfected cells. Over-expression of wild-type PLD2 induced more adhesion than non-transfected, GFP-transfected or PLD1-transfected cells (Fig. 4A). Furthermore, as an inactive mutant of PLD2, containing mechanism-disrupting substitutions within both catalytic motifs (H442D/K758E), did not mediate increased adhesion, but localised identically to the wild-type enzyme (data not shown), the enhancement of adhesion mediated by wild-type PLD2 transfection must result from increased local production of PtdOH. Accordingly, increasing levels of PLD2 expression (determined by GFP-fluorescence) correlated with increased adhesion (supplementary material Fig. S4). However, this was not true for PLD1, indicating that the adhesion process is specifically regulated by PLD2 activation and is not simply the result of over-expressing any PtdOH-generating enzyme. Consistent with this, following detachment from substrate, PLD2-transfected cells exhibited higher PLD activity than non-transfected cells in contrast to GFP- or PLD1-transfected cells (Fig. 4B).

Inhibition of PLD activity in L6 myoblast cells also resulted in reduced adhesion to serum-coated plastic (supplementary material Fig. S2). To determine if gene knockdown of PLD2 had the complementary effect to its over-expression, the L6 myoblast cells were transfected with a PLD2-specific siRNA or a PLD1-specific siRNA. PLD1 mRNA levels were reduced by up to 70% (Fig. 4C) and PLD2 mRNA levels were reduced by up to 90% (Fig. 4D) 72 hours post-transfection. Measurement of the PLD activity

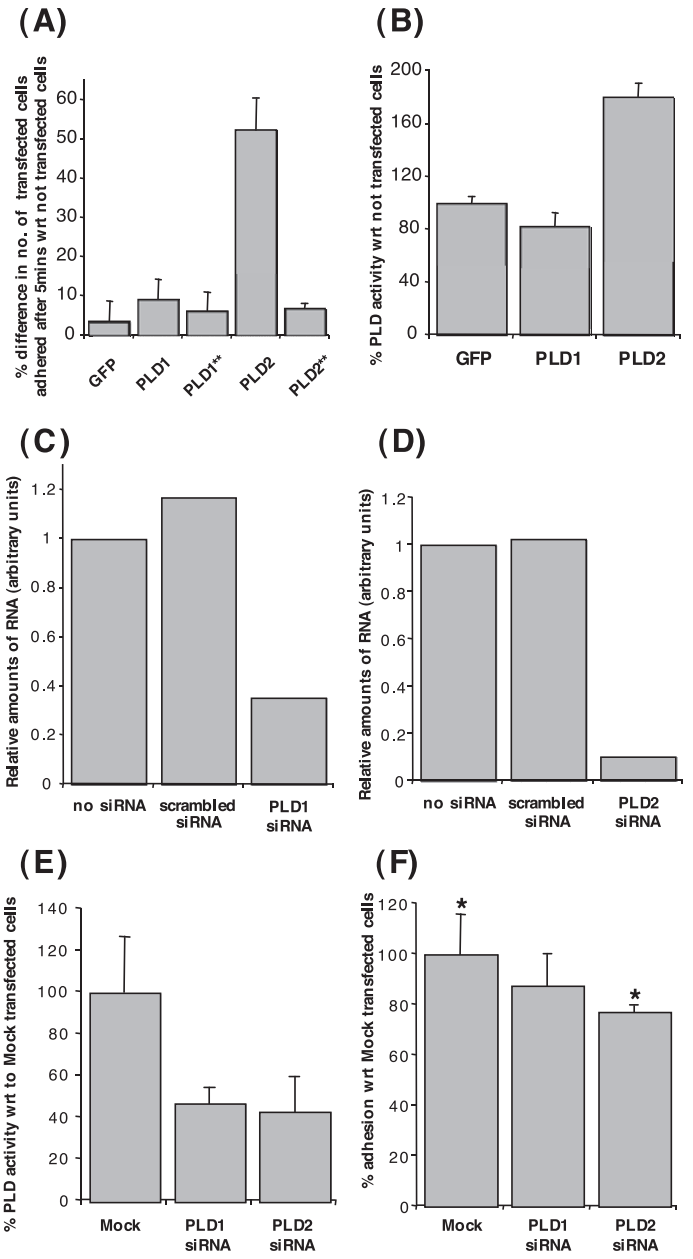


**Fig. 4.** PLD2 stimulates adhesion. (A) Following transfection of GFP-tagged protein constructs, the level of adhesion to serum-coated plastic was measured. The level of adhesion for each individual transfectant was obtained from an average of three experiments and is expressed as a percentage difference to non-transfected cells. (B) The PtdBut accumulation over a 5-minute period was used as a measure of PLD activity of transfected cells following detachment from substrate. PLD1** corresponds to PLD1(H464D/K860E) and PLD2** corresponds to PLD2(H442D/K758E). (C) Following siRNA transfection, the levels of PLD1 mRNA was analysed by quantitative PCR. (D) Following siRNA transfection, the levels of PLD2 mRNA was analysed by quantitative PCR. (E) Following siRNA transfection, the level of PtdBut accumulation over a 5-minute period was quantified as a measure of PLD activity. (F) Following siRNA transfection, the level of adhesion to serum-coated plastic was quantified. Using a Student's *t*-test, *P* values of <0.001% were obtained (*) between these samples. Similar results were obtained in at least three separate experiments and error bars represent the standard deviation from the mean.

in these cells revealed approximately a 50% decrease in activity for cells transfected with siRNAs for either PLD1 or PLD2, indicating decreased levels of each protein (Fig. 4E). Consistent with the ability of PLD2 to regulate adhesion in the RBL-2H3 cells, the adhesion of PLD2-depleted L6 cells was reduced by more than 20% in comparison to mock-transfected cells (Fig. 4F). This is equivalent to the loss of adhesion induced by the treatment of L6 cells with butan-1-ol (supplementary material Fig. S2). PLD1 mRNA-depleted cells also exhibited reduced adhesion however this was to a lesser extent than for PLD2 mRNA depletion. As over-expression of PLD2, but not PLD1, in RBL-2H3 cells induced increased cell adhesion, it is possible that the ability of PLD1 to regulate adhesion is specific to other cell types such as L6 cells. Despite employing multiple transfection methodologies we were unable to reduce PLD1 or PLD2 mRNA levels in RBL-2H3 cells using the same siRNAs.

## PIPkin Iγ*b*-generated PtdIns(4,5)$P_2$ stimulates adhesion

The major route for signal-mediated PtdIns(4,5)$P_2$ generation in cells is via the phosphorylation of phosphatidylinositol 4-phosphate by members of the PIPkin I family (Ishihara et al., 1998). Thus the ability of PtdIns(4,5)$P_2$ to stimulate adhesion demonstrated in Fig. 2 suggests that the generation of this lipid by a PIPkin I family member may also regulate adhesion. As observed with PLD2, cells transfected with either PIPkin Iγ*b* or Iγ*c* exhibited increased adhesion in comparison to GFP-transfected or non-transfected cells (Fig. 5A). By contrast, neither PIPkin Iγ*a*, PIPkin Iα nor mutants of PIPkin Iγ*b* or Iγ*c* with reduced kinase activity (D316K) (Giudici et al., 2004) promoted this increased level of adhesion. As D316K mutation had no effect upon localisation of these enzymes (data not shown) it is probable that the enhancement of adhesion mediated by wild-type PIPkin Iγ*b* and Iγ*c* was due to their generation of PtdIns(4,5)$P_2$ at the plasma membrane. Accordingly, higher levels of PIPkin Iγ*b* and Iγ*c* expression correlated with increased adhesion (Fig. 5B). However, this was not observed in cells transfected with PIPkin Iγ*a* or PIPkin Iα, indicating that the adhesion process is regulated by the specific activation of PIPkin Iγ*b* and Iγ*c* and is not simply an artefact of over-expressing any PtdIns(4,5)$P_2$-generating enzyme. Indeed, high levels of PIPkin Iγ*a* transfection caused a small, but significant, decrease in adhesion (see Discussion).

Over-expression of the PtdIns(4,5)$P_2$-binding PH domain from PLCδ1 (De Vos et al., 2003; Garcia et al., 1998) has previously been shown to inhibit the formation of cellular adhesion complexes (Martel et al., 2001) and consistent with this, over-expression of this PH domain in the RBL-2H3 cells lead to approximately a 15% decrease in the numbers of cells adhered when compared with GFP transfected cells (Fig. 5A). This presumably results from the PH domain sequestering PtdIns(4,5)$P_2$ generated at the plasma membrane to regulate the formation of such adhesion complexes and is therefore consistent with the regulation of adhesion by PIPkin Iγ*b* or Iγ*c* activation.

## PLD2-derived PtdOH stimulates adhesion via PIPkin Iγ*b*

The ability of PtdIns(4,5)$P_2$ to regulate adhesion in the absence of PLD activity suggests that the generation of this lipid could be downstream of PLD2 activation (Fig. 2). Furthermore, the ability of PtdOH, but not its metabolites, to regulate adhesion in the absence of PLD activity suggests that PtdOH directly stimulates a target protein essential to adhesion. Pertinently, PtdIns(4,5)$P_2$ can be generated in vitro following the activation

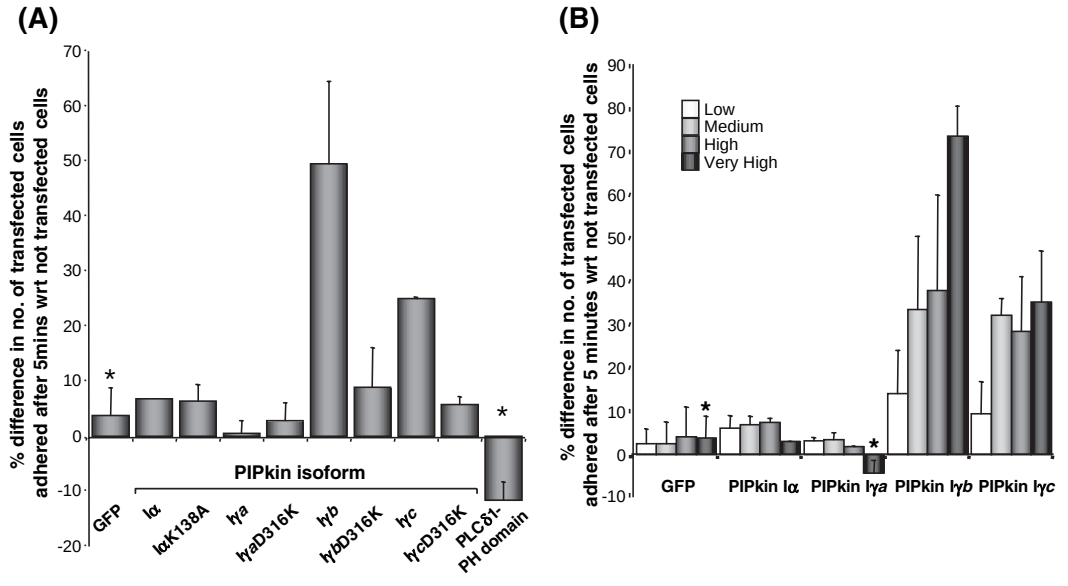


**Fig. 5.** PIPkin Iγ*b* stimulates adhesion. (A) Following transfection with GFP-tagged PIPkin Iα and γ (and derivatives) and the GFP-tagged PH domain for PLCδ1, the level of adhesion to serum-coated plastic was quantified and is expressed as a percentage difference to non-transfected cells. Using a Student's *t*-test, a *P* value of <0.01% was obtained (*) between these transfectants. (B) The dose-response for increasing levels of PIPkin Iγ transfection derived from the total levels observed in (A). 'Low', 'medium', 'high' and 'very high' correspond to arbitrary divisions in the level of expression. The levels of expression were determined by the quantity of GFP fluorescence detected by a FACs instrument. The level of adhesion for each individual transfectant was obtained from an average of three experiments and error bars represent the standard deviation from the mean. Using a Student's *t*-test, a *P* value of <0.01% was obtained (*) between these transfectants.

of PIPkin I family members by PLD-derived PtdOH (Ishihara et al., 1998). This is also consistent with results in vivo, where the activity of PIPkin I family members have been demonstrated to be ARF GTPase family-dependent (Aikawa and Martin, 2003; Oude Weernink et al., 2004) and ARF GTPases directly regulate the activation of PLD2 (Honda et al., 1999; Kim et al., 2003; Koch et al., 2003; Lopez et al., 1998; Powner and Wakelam, 2002).

Examination of RBL-2H3 cells by confocal microscopy, demonstrated a high degree of co-localisation between PLD2, but not PLD1, and both PIPkin I$\gamma b$ and I$\gamma c$ at the plasma membrane of cells in suspension or during the initial stages of

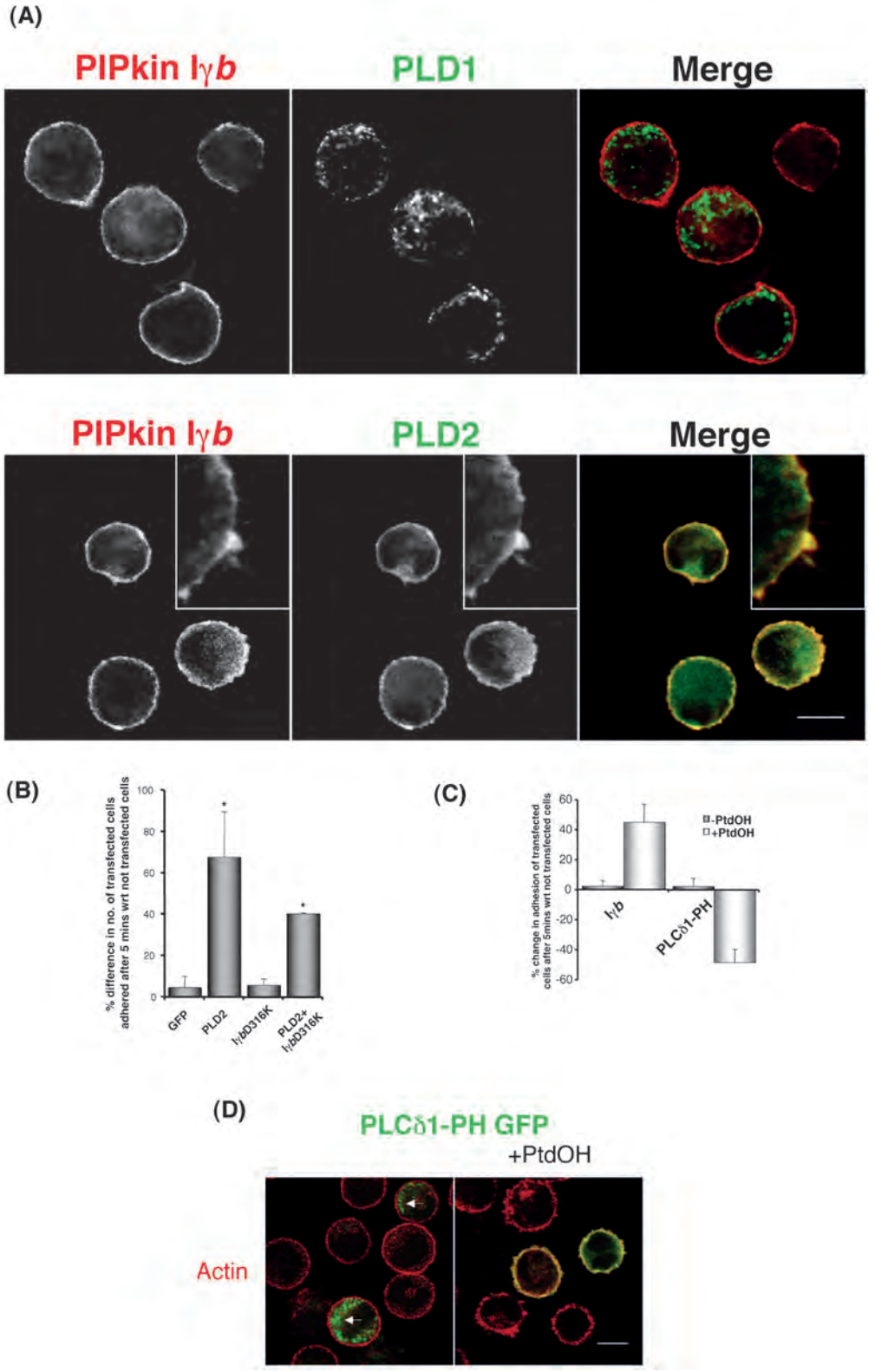


**Fig. 6.** PLD2 regulates activation of PIPkin I$\gamma b$. (A) The localisations of GFP-tagged PLD1 and 2 with FLAG-tagged PIPkin I$\gamma$ were determined for cells in suspension (spun down on to a cover-slip). The data shown is that of PIPkin I$\gamma b$, but I$\gamma a$ and I$\gamma c$ gave identical results (supplementary material Fig. S5A). The inset image represents an image at a further 10× magnification. (B) Following transfection with GFP, GFP-tagged PLD2, FLAG-tagged PIPkin I$\gamma b$D316K and co-transfection of GFP-tagged PLD2 and FLAG-tagged PIPkin I$\gamma b$D316K adhesion to serum-coated plastic was quantified. Using a Student's *t*-test, a *P* values of <0.05% was obtained (*) between these transfectants. (C) Following maintenance in suspension for 60 minutes, the level of adhesion to serum-coated plastic was quantified for cells transfected with GFP-tagged PIPkin I$\gamma b$ or the GFP-tagged PH domain from PLC$\delta$1, in the presence of diC8-PtdOH or vehicle. The level of adhesion for each individual transfectant was obtained from an average of three experiments and error bars represent the standard deviation from the mean. Similar results were obtained when PLD-activity was suppressed in the presence of butan-1-ol (data not shown). (D) Following maintenance in suspension for 60 minutes, the localisation of GFP-tagged PLC$\delta$1 was analysed in the presence of diC8-PtdOH or vehicle. Similar results were obtained when PLD-activity was suppressed in the presence of butan-1-ol (data not shown). All images are confocal and similar results were obtained in at least three separate experiments. Bar, 10 μm.

adhesion (Fig. 6A and supplementary material Fig. S5A). This close proximity in both space and time therefore correlates with their potential to cooperate within the same signalling cascade(s). Similarly, the PLCδ1-PH domain was also located in close proximity to PLD2 at the plasma membrane and as such, presumably inhibits adhesion by sequestering PtdIns(4,5)$P_2$ generated within this vicinity (supplementary material Fig. S5B).

Thus, to determine if the regulation of adhesion by PLD2 requires the production of PtdIns(4,5)$P_2$ by PIPkin I*γb* (or I*γc*) we co-expressed PLD2 with a dominant-negative version of PIPkin I*γb* (D316K) (Fig. 6B). The decreased level of adhesion observed in these cells in comparison to cells transfected with PLD2 alone, indicates that the ability of PLD2 to regulate adhesion is at least partially dependent upon the activity of PIPkin I*γb*.

To determine the sequence in which these two enzymes regulate the adhesion process, i.e. if the synthesis of this adhesion-stimulating PtdIns(4,5)$P_2$ by PIPkin I*γb* (or I*γc*) was dependent upon the prior generation of PtdOH by PLD2 as hypothesised, the levels of adhesion of PIPkin I*γb*-transfected cells and PLCδ1 PH domain-transfected cells were quantified following a reduction in PLD activity to basal levels. Neither the enhanced adhesion of PIPkin I*γb*-transfected cells nor the inhibited adhesion of the PH domain-transfected cells demonstrated in Fig. 5 was observed following the reduction in PLD activity (Fig. 6C). Additionally, the PLCδ1-PH domain re-localised from the plasma membrane to an intracellular vesicular compartment (Fig. 6D in comparison to supplementary material Fig. S5B). However, reconstitution of the PLD signal in these cells by the addition of diC8-PtdOH restored both the enhanced adhesion of PIPkin I*γb*-transfected cells and the inhibited adhesion of PtdIns(4,5)$P_2$-specific PH domain-transfected cells (Fig. 6C). Concurrently, this PH domain also re-localised back to the plasma membrane (Fig. 6D). Thus the generation of adhesion-stimulating PtdIns(4,5)$P_2$ at the plasma membrane (by PIPkin I*γb*) is dependent upon the availability of PtdOH.

In conclusion, these data indicate that PLD2-derived PtdOH regulates integrin-mediated adhesion as a result of stimulating PIPkin I*γb* to generate PtdIns(4,5)$P_2$.

## Discussion

The data presented in this paper demonstrate that PLD2-derived PtdOH can stimulate the initial stages of cellular adhesion via $\beta_1$ and $\beta_2$ integrins. This mechanism requires the PtdOH-dependent generation of PtdIns(4,5)$P_2$ at the plasma membrane by PIPkin I*γb* (or I*γc*). These data provide some of the first descriptions of cellular functions for both PLD2 and PIPkin I*γb* (or I*γc*).

Previously it has been suggested that the activation of PLD is important to the regulation of spreading and migration, processes that both require the modulation of plasma membrane adhesion (McDermott et al., 2004; O'Luanaigh et al., 2002; Powner et al., 2002; Santy and Casanova, 2001; Zhou et al., 1995). By depleting endogenous PLD activity from RBL-2H3 cells we demonstrated that the adhesive capacity of the cell was reduced indicating a role for PLD in the adhesion process, which may therefore, be a reason for its regulation of spreading and adhesion (Fig. 1). Following depletion of endogenous PLD activity, our data showed that the adhesive capacity of these cells could be restored by the addition of cell-permeable analogues of both PtdOH and PtdIns(4,5)$P_2$ (Fig. 2). Using mass spectroscopic analysis, we found that the incorporation of such cell-permeable lipids into intracellular membranes was to signalling-significant levels and as such, demonstrated that treatment of cells with these lipids may be used to supplement specific endogenous lipid levels (Table 1) (Cross et al., 1996; Powner et al., 2002). Our data indicates that PLD2 is responsible for the generation of this PtdOH in cells as over-expression of catalytically-functional PLD2 dose-dependently enhanced cellular adhesion and conversely, siRNA reduction of PLD2 levels reduced cellular adhesion. Under the same conditions PLD1 had no effect (Fig. 4). In contrast to PtdOH, its immediate metabolites, DAG and LPA, were unable to recover adhesion in the absence of endogenous PLD activity indicating that PtdOH regulates this process through direct interaction with a protein target. A lipid-kinase-dead version of PIPkin I*γb* inhibited PLD2's stimulation of adhesion and PLD-derived PtdOH was required for PIPkin I*γb* to regulate adhesion. Furthermore, as PtdOH has been shown to directly stimulate PIPkin I*γb* in vitro (Ishihara et al., 1998), we identify PIPkin I*γb* as the target protein downstream of PLD2-derived PtdOH in the regulation of adhesion (Fig. 6). This is consistent with the ability of diC8-PtdIns(4,5)$P_2$ to stimulate adhesion in the absence of PLD activity (Fig. 2).

The treatment of cells with diC8-PtdOH resulted in a more convoluted, villous cortical plasma membrane morphology than untreated cells (Fig. 6D). A similar effect was also induced by the over-expression of PLD2 (but not PLD1) (supplementary material Fig. S6). This morphology is indicative of cellular spreading or migration, both of which are dependent upon actin cytoskeletal reorganisation and the formation new integrin-substrate adhesion complexes. Accordingly, our data demonstrates that PLD2's product, PtdOH, and its downstream effector, PtdIns(4,5)$P_2$, were both able to regulate adhesion through the stimulation of cell surface $\beta_1$ and $\beta_2$ integrins (Fig. 3). By contrast, inhibition of actin polymerisation by the addition of cytochalasin D up to 30 μg $ml^{-1}$ did not reduce the level of cellular adhesion during the first 10 minutes after addition of cells to substrate indicating that stimulation of actin polymerisation is not a reason for PLD2-derived PtdOH's regulation of the initial stages of cellular adhesion (data not shown). However subsequent to this, it is possible that cellular spreading and migration may require the regulation of actin polymerisation by PLD2-derived PtdOH as has previously been suggested, but we did not investigate this here (McDermott et al., 2004; O'Luanaigh et al., 2002; Powner et al., 2002; Santy and Casanova, 2001; Zhou et al., 1995).

The inhibition of adhesion by depletion of PLD activity with butan-1-ol was not complete and became less potent the longer that the cells were allowed to adhere, even though PLD activity was shown to decline during this time period (Fig. 1). Thus, as adhesion can proceed in the absence of PLD activity, it is likely that PLD activation is not absolutely essential to the adhesion process but regulates an additional adhesion mechanism, most prevalent during the initial stages of substrate-plasma membrane interaction. The formation of integrin-based contacts with substratum is essential to the adhesion, spreading and migration of all cell types, but in particular those of

haematopoietic origin (such as RBL-2H3 cells) whose function often requires rapid and highly regulated recruitment and homing to specific sites of inflammation/immune reaction (Harris et al., 2000). In comparison to most other cell types, PLD2 transcription is elevated in haematopoietic cells and is far in excess of PLD1 transcription (K. M. Saqib, R.M.P., D.J.P. and M.J.O.W., unpublished). This is therefore consistent with the activation of PLD2 being more critical to the regulation of haematopoietic cell specific functions, such as rapid adhesion, and thus why other, generally more static cell types such as myoblasts, may be less dependent upon PLD activity for their adhesion – inhibition of PLD activation with butan-1-ol in RBL-2H3 leukocyte cells has twice the effect on adhesion that it has in L6 myoblasts (supplementary material Fig. S2). It is possible that in such cell types where PLD activation has a smaller effect on adhesion, the activation of PIPkin I$\gamma b$ is still necessary but is predominantly regulated via a PLD-independent mechanism.

Together with haematopoietic cells, in many tumour samples, PLD2 transcription is also elevated (K. M. Saqib and M.J.O.W., unpublished). As tissue invasion requires dynamic regulation of integrin-mediated adhesion and PLD activation has been identified as a regulator of migration (Santy and Casanova, 2001), hyper-PLD2 activity may also be a contributing factor to the metastatic potential of some cancers (Felding-Habermann, 2003; Uchida et al., 1999; Uchida et al., 1997).

A combination of function-blocking antibodies to $\beta_1$ and $\beta_2$ integrins had no effect upon the residual level of adhesion of cells to serum or ICAM1 observed following depletion of endogenous PLD activity and only a partial effect upon adhesion to FN. Furthermore, these antibodies were only partially able to inhibit the enhanced level of adhesion stimulated by treatment with diC8-PtdOH (Fig. 3). These data suggest that adhesion to these surfaces can be mediated by mechanisms excluding these integrins and that may be either dependent or independent of PLD activation. These pathways may include adhesion via other β integrin families or other adhesion molecules such as syndecans, selectins or other proteoglycans. In contrast to diC8-PtdOH, the effect of diC8-PtdIns(4,5)$P_2$ on adhesion to serum or ICAM1 could be ablated in the presence of antibodies to $\beta_1$ and $\beta_2$ integrins while its effect on adhesion to FN could be ablated in the presence of the antibody to $\beta_1$ integrins alone. The regulation of adhesion to these surfaces by PtdIns(4,5)$P_2$ therefore appears to be predominantly mediated through these integrin families. As PLD2-derived PtdOH is essential to the generation of adhesion-stimulating PtdIns(4,5)$P_2$ (Fig. 6C) (Ishihara et al., 1998) these data may suggest that PtdOH regulates adhesion through at least two mechanisms, but that a major mechanism is through generation of PtdIns(4,5)$P_2$ and subsequent stimulation of cell-surface integrins. Furthermore the regulation of adhesion by PtdOH through two or more mechanisms, one of which involves the generation of PtdIns(4,5)$P_2$, may be one reason that diC8-PtdOH has a greater maximum effect on adhesion than diC8-PtdIns(4,5)$P_2$ (Fig. 2) and why a dominant-negative version of PIPkin I$\gamma b$ was unable to completely ablate adhesion enhanced by PLD2 over-expression (Fig. 6B).

Induction of the high affinity conformation of cell-surface integrins following the addition of exogenous $Mn^{2+}$ was able to overcome the loss of adhesion induced by the inhibition of PLD suggesting that PLD activation may regulate integrin-mediated adhesion by increasing $\beta_1$ and $\beta_2$ integrin affinity for their cognate substrates (supplementary material Fig. S7A). The binding of talin to the cytoplasmic tail of the β integrin subunit has been shown to directly mediate the conformational changes required to increase integrin affinity (Garcia-Alvarez et al., 2003; Martel et al., 2001; Tadokoro et al., 2003). The unmasking of the integrin interaction site within the FERM domain of talin is facilitated through its binding to PtdIns(4,5)$P_2$ (Martel et al., 2001). Thus during the initial stages of RBL-2H3 cell adhesion, PtdIns(4,5)$P_2$ generated by PIPkin I$\gamma b$ as a result of PLD2 activation may induce the high affinity conformation of integrins by stimulating an interaction with talin. Consistent with this, talin and PLD2, but not PLD1, co-localised at the plasma membrane of RBL-2H3 cells in suspension and during the initial stages of adhesion as would be expected for them to be able to cooperate in the induction of the high affinity conformation of an integrin (supplementary material Fig. S7B).

Although the plasma membrane localisation of PIPkin I$\gamma a$ and I$\gamma b$ in adhering cells was indistinguishable (Fig. 6A and supplementary material Fig. S5A), they were found to have opposing effects up on adhesion (Fig. 5B). The difference between these two splice variants is a 26 amino acid extension to the C-terminal of PIPkin I$\gamma a$, which has been shown to be responsible for its interaction with talin (Barsukov et al., 2003; Di Paolo et al., 2002; Ling et al., 2002). Di Paolo et al. (Di Paolo et al., 2002), Ling et al. (Ling et al., 2002; Ling et al., 2003), and Calderwood et al. (Calderwood et al., 2004) showed that due to this interaction, high over-expression of PIPkin I$\gamma a$ resulted in the loss of talin from integrin-based adhesions, while PIPkin I$\gamma b$ had no effect. Therefore the small but significant, PIPkin I$\gamma a$ dose-dependent decrease in adhesion shown in Fig. 5B may result from this over-expressed protein sequestering talin and as such, rendering it unable to stimulate integrin activation.

However, while our data indicate that prior to integrin engagement PIPkin I$\gamma b$-generated PtdIns(4,5)$P_2$ may regulate the recruitment of talin to integrins, it has been postulated that subsequent to integrin engagement, endogenous PIPkin I$\gamma a$ is recruited to these nascent sites of adhesion by its interaction with integrin-bound talin dimers (Barsukov et al., 2003; Di Paolo et al., 2002; Ling et al., 2002; Ling et al., 2003). As PIPkin I$\gamma a$ activity is also stimulated by its interaction with talin, it is probable that this complex mediates the generation of PtdIns(4,5)$P_2$ within these nascent adhesion complexes (Di Paolo et al., 2002; Ling et al., 2002; Ling et al., 2003). The localised generation of PtdIns(4,5)$P_2$ by PIPkin I$\gamma a$ at these sites is proposed to recruit further integrins and other PtdIns(4,5)$P_2$-binding proteins necessary to the formation of stronger adhesions such as focal complexes/adhesions (Fukami et al., 1992; Giannone et al., 2003; Gilmore and Burridge, 1996; Greene et al., 2003; Horowitz et al., 1999). Consequently, PtdOH stimulation of PIPkin I$\gamma b$ may not be required to mediate PtdIns(4,5)$P_2$ generation within engaged-integrin complexes consistent with the down-regulation of PLD activity following adhesion (Fig. 1B).

In conclusion, we propose that the initial stage of cellular adhesion mediated by integrin activation is dependent upon PLD2-generated PtdOH through the stimulation of PIPkin I$\gamma b$, whereas the subsequent strengthening of adhesion required for

focal complex/adhesion formation may be independent of PtdOH generation and be mediated by talin stimulation of PIPkin I*γa* (Di Paolo et al., 2002; Ling et al., 2003).

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