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**JOURNAL OF CELL SCIENCE**

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PLCγ1 is essential for early events in integrin signalling required for cell motility

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Summary

Cell motility is a critical event in many processes and is underlined by complex signalling interactions. Although many components have been implicated in different forms of cell migration, identification of early key mediators of these events has proved difficult. One potential signalling intermediate, PLCγ1, has previously been implicated in growth-factor-mediated chemotaxis but its position and role in more-complex motility events remain poorly understood. This study links PLCγ1 to early, integrin-regulated changes leading to cell motility. The key role of PLCγ1 was supported by findings that specific depletion of PLCγ1 by small interfering (si)RNA, or by pharmacological inhibition, or the absence of this isoform in PLCγ1/−/− cells resulted in the failure to form cell protrusions and undergo cell spreading and elongation in response to integrin engagement. This integrin-PLCγ1 pathway was shown to underlie motility processes involved in morphogenesis of endothelial cells on basement membranes and invasion of cancer cells into such three-dimensional matrices. By combining cellular and biochemical approaches, we have further characterized this signalling pathway. Upstream of PLCγ1 activity, β1 integrin and Src kinase are demonstrated to be essential for phosphorylation of PLCγ1, formation of protein complexes and accumulation of intracellular calcium. Cancer cell invasion and the early morphological changes associated with cell motility were abolished by inhibition of β1 integrin or Src. Our findings establish PLCγ1 as a key player in integrin-mediated cell motility processes and identify other critical components of the signalling pathway involved in establishing a motile phenotype. This suggests a more general role for PLCγ1 in cell motility, functioning as a mediator of both growth factor and integrin-initiated signals.

Movies and supplementary material available online at http://jcs.biologists.org/cgi/content/full/118/12/2695/DC1

Key words: PLCγ1, Signalling, Motility, Extracellular matrix

Introduction

Cell motility is essential for a range of physiological processes, as well as in tumour invasion, angiogenesis and metastasis (Friedl and Wolf, 2003; Lauffenburger and Horwitz, 1996; Ridley et al., 2003). Motility of tumour cells and certain non-neoplastic cells, such as endothelial cells during angiogenesis, involves cell migration into and within tissues consisting of three-dimensional extracellular matrices (ECMs) (Davis et al., 2002; Friedl and Wolf, 2003). In addition to the structural role of the ECM as both a barrier to cell motility and a substratum for generating traction forces, interactions between ECM ligands and cell-surface receptors such as integrins can also trigger signalling events. This interface, together with signals by soluble factors, regulates not only cell motility but also many aspects of cell morphology and behaviour (Miranti and Brugge, 2002). In the case of endothelia, cell-ECM interactions are required for most processes of angiogenesis including ‘invasion’ of capillary sprouts and formation of stable blood vessel structures (Davis et al., 2002). For many cancer types, the cell-ECM interactions underlie one of the main mechanisms of migration of an invading cell, the ‘mesenchymal’ mechanism, which is dependent on integrin-mediated adhesion dynamics and traction (Friedl and Wolf, 2003).

Phosphoinositide-specific phospholipase C (PI-PLC) enzymes have been established as crucial signalling molecules involved in the regulation of a variety of cellular functions (Katan, 1998; Rebecchi and Pentyala, 2000; Rhee, 2001). The evidence also suggests a critical involvement of members of the PLCγ family (PLCγ1 and PLCγ2) in several aspects of motility regulation. A distinct regulatory feature of PLCγ enzymes is that their activation is linked to an increase in phosphorylation of specific tyrosine residues. As a direct substrate for a number of receptors with intrinsic tyrosine kinase activity, including epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors, PLCγ1 has been shown to mediate chemotaxis towards these growth factors as a rate-limiting component (reviewed by Kassis et al., 2001; Wells, 2000). This role of PLCγ1 has been demonstrated in cells engineered to express wild-type and mutant PDGF or EGF receptors and variants of PLCγ1 (Chen et al., 1994; Kundra et al., 1994; Ronnstrand et al., 1999) as well as in several tumour cell lines, particularly those characterized by high levels of EGF receptor (Kassis et al., 1999; Price et al.,
1999; Thomas et al., 2003). It has also been suggested that EGF-driven and PLCγ1-mediated chemotaxis could have an important role in invasion in several human tumour xenograft models in vivo (Turner et al., 1997). However, possible contributions of ECM receptor signalling to haptotaxis or motility phenotypes resulting from inhibition of PLCγ1 were difficult to dissect in these chemotaxis assays (Wells, 2000).

The possibility that PLCγ could also be an important signalling component in responses triggered directly by ECM receptors was initially suggested by observations that PLCγ1 can become phosphorylated upon integrin engagement, or recruited to integrin complexes in fibroblasts (Langholz et al., 1997; Zhang et al., 1999). Recently, several studies in highly specialized cell types (platelets and osteoclasts) supported the role of PLCγ isoforms in ‘outside-in’ signalling, downstream of ECM receptors (Inoue et al., 2003; Nakamura et al., 2001; Wonnerow et al., 2003). However, it is not known whether the participation of PLCγ enzymes in integrin signalling is a more general phenomenon, or which signalling components are involved or which physiological processes it could underlie in different cellular systems. In particular, the involvement in motility processes, such as those important for invasion and morphogenesis of cancer cells and endothelial cells during tumour progression, has not been addressed.

Here, we show that PLCγ has a key role in integrin-dependent cell motility required for invasion of diverse cancer cell types and morphogenesis of endothelial cells on basement membranes. Depletion of PLCγ and inhibition of PLC activity show that early events that follow attachment to the ECM are affected, resulting in lack of cell spreading and elongated cell morphology. By combining cellular studies and biochemical methods, we also suggest signalling components upstream (β1-containing integrins, adaptor protein GIT1 and Src kinases) and downstream (increase in intracellular calcium concentrations) of PLCγ1, providing a model of this novel signalling pathway. These data demonstrate that PLCγ1 is involved in processes related to cell motility more widely than previously suggested and that, in addition to chemotaxis triggered by growth factor receptors, PLCγ1 is stimulated through integrin activation in different cancer cell types and activated endothelial cells.

Materials and Methods

Materials

The following chemicals were obtained from Calbiochem (Merck): PP2, PP3 (structurally related control compound for PP2), U73122, U73343 (structurally related control compound for U73122), 2-APB, BAPTA-AM, bisindolylmaleimide I (GF109203X) and bisindolylmaleimide V. The PKC inhibitor R0-31-8220, Fluo3-AM, Fura2-AM, EGFT, FITC-conjugated anti-α-tubulin and mouse and rabbit HRPPO (horseradish peroxidase)-conjugated secondary antibodies were from Sigma. Texas Red-X Phallolidin and Cell tracker Green CMFDA (Total cytoplasm label) were from Molecular Probes. EGFT receptor inhibitor AG1478 and thapsigargin were from Biomol. siRNA oligonucleotides were obtained from Dharmacon and Oligofectamine Lipofectamine/Lipofectamine plus and OPTMEM were from Invitrogen. Anti-PLCγ1, anti-PKC(pan), anti-Src and anti-EGF receptor (blocking) were from Upstate, pPLCγ1(Y783), pPKC(δS660) and pSrc(Y416) were from Cell Signaling Technology. Anti-GAPDH was purchased from Research Diagnostics. Anti-PLCγ2 was supplied by Santa Cruz. Anti-integrin β1 (4B4 blocking) was supplied by Beckman Coulter and anti-integrin β1 (TS2/16 activating) from Pierce Endogen. Anti-GIT1, anti-phosphotyrosine, cell recovery solution, Matrigel, growth factor-reduced Matrigel, laminin, collagen I and collagen IV were obtained from BD Bioscience. Protein-G agarose was from Roche and fluorescent secondary antibodies from Jackson Immunologicals. Mouse PLCγ1−/− and PLCγ1+ (add back) fibroblast cells and rat PLCγ1-GFP were a kind gift from Graham Carpenter (Vanderbilt University. Nashville, USA) and the catalytically inactive PLCγ1 construct was a kind gift from Klaus Seedorf (Lilly, Hamburg, Germany).

Cell culture protocols

BE (colon carcinoma), DU145 (prostate carcinoma), A431 and Mouse PLCγ1−/− and PLCγ1+ fibroblast cells were maintained at 37°C in DMEM supplemented with 10% FBS. The cells were starved for 24 hours in DMEM containing 1% heat-inactivated FBS. Human umbilical vein endothelial cells (HUVECs; pooled donors, TCS CellWorks) were maintained at 37°C in large endothelial cell growth media plus supplements (TCS CellWorks). These cells were starved for 1 hour in MCDB-131 media supplemented with 0.2 mg/ml EGF, 0.1 µg/ml hydrocortisone and 0.1% (w/v) BSA. For certain experiments, cell cytoplasms were pre-labelled with a green fluorescent tracker dye (1 nM; Molecular Probes) for 1 hour prior to trypsinization and use in experimental procedures. For siRNA protocols, pre-annealed purified siRNA probes were from Dharmacon and were rehydrated prior to transfection using their standard protocol. The siRNA sequence targeting PLCγ1 is AAGAAGTCGCGACGCCGAG and the control non-targeting sequence is AAGCGCGCTTTGTAGGATTCG. siRNA probes (200 nM) were transfected using oligofectamine using a multiple transfection strategy that involved successive siRNA transfections on three consecutive days over a 3-day period. Treated cells were then either used for experiments 72 hours after the first transfection or were extracted for western blotting to check depletion levels. For siRNA rescue experiments, BE cells were transfected with either GFP or rat PLCγ1-GFP on day 3 of the multiple transfection protocol prior to the third and final siRNA transfection. Briefly, 1 µg of DNA was transfected into the cells for 4 hours using the lipofectamine plus transfection system. Subsequently, following extensive washing, the third and final siRNA transfection was performed in a similar fashion to the previous two siRNA transfections. The effects of the rescue were then analysed 24 hours later (72 hours after the first siRNA transfection). Transfection of other cell lines (i.e. mouse fibroblasts) was also performed in a similar fashion using the lipofectamine plus system with effects analysed 24 hours post-transfection.

Preparation and processing of cell lysates

Cells were scrapped into ice-cold lysis buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 50 mM KCl, 1 mM DTT, 1 mM Na3VO4, 5 mM MgCl2, 5 mM NaF, 10% [v/v] Glycerol, 1% [v/v] Triton X-100) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail II (Sigma) and then homogenized by passing the lysate through a Hamilton syringe (gauge 22) 10 times. After standing on ice for 10 minutes, lysates were clarified by centrifugation (15,800 g/10 minutes) and the supernatant used as the cell lysate. Lysates were either used for immunoprecipitation (see below), protein concentration determination (by Bradford methods) or directly for SDS-PAGE/blotting following the addition of SDS-sample buffer. In certain circumstances, cell lysates were obtained from Matrigel. Briefly, cells on Matrigel were washed and then incubated for 30 minutes with PBS containing containing phosphatase inhibitor cocktail 2 (Sigma). Following this, the cells were scraped into a suitable volume (2 ml per cm2) of ice-cold, cell recovery solution (BD Biosciences) and left on ice for 1 hour to digest the Matrigel. Following digestion, the cell pellet was recovered by centrifugation.
(160 μg/5 minutes) and the cells lysed and processed in accordance with the lysis protocol. For immunoprecipitation protocols, cell lysates were incubated first with washed protein-G agarose beads for 2 hours at 4°C as a pre-clearing step. Following this, the supernatant was incubated overnight at 4°C with the desired primary antibody. Washed protein-G agarose was added to this mixture and the incubation allowed to proceed for a further 2 hours at 4°C. The beads were then washed three times with lysis buffer and solubilized with SDS-sample buffer. Samples were separated by 10% SDS-PAGE and the proteins transferred to PVDF membrane. After blocking, membranes were incubated with suitable primary antibody (1 in 1000 dilution) overnight at 4°C. Following washing with 0.1% TBS-Tween, membranes were incubated with suitable HRPO-conjugated secondary antibody for 2 hours at room temperature. After further washing, proteins were visualized using the ECL detection method (Amersham Bioscience).

Immunofluorescence
Cells were fixed in 3.7% formaldehyde in PBS and permeabilized in 0.2% Triton X-100 in PBS. Following blocking in 2% BSA in PBS for 1 hour, fixed cells were incubated directly with either Texas-Red Phalloidin (1 in 1000) or FITC anti-α-tubulin (1 in 1000) for 2 hours. After extensive washing, cells were observed by immunofluorescent microscopy (Nikon Eclipse E600) and Bio-Rad confocal setup plus Laser Sharp software (Biorad MRC1024).

Analysis of cell morphology and motility on Matrigel
Cell culture dishes were coated with a thin layer of Matrigel (diluted 2 parts to 1 part DMEM). This was then allowed to set at 37°C for 90 minutes (approx. 200 μl of this mix was used per cm of dish area). Green Tracker dye-labelled cells were then plated in the Matrigel in the presence or absence of inhibitor compounds and the ability of the cells to elongate was analysed at various time points using time-lapse and fixed-time-point (phase contrast and fluorescent) microscopy (Nikon microscope camera, Open-Lab software). For time-lapse microscopy, cells were placed on a motorized stage (Prior Scientific) within an incubation chamber (5% CO2 37°C). Images were recorded every 10 minutes over a 20-hour period (Nikon Eclipse Microscope, Hamamatsu ORCA-ER camera, Simple PCI software). The images could then be converted into a movie and analysis carried out using Simple PCI software. For cell invasion studies, a similar method to that described before (Sahai and Marshall, 2003) was employed. Briefly, 100 μl of growth factor-reduced Matrigel was prepared in a 8 μM pore Transwell chamber (Costar). Tracker dye-labelled serum-starved cells (30,000) were seeded on the Transwell filter and into the matrix was assessed by Z-section confocal microscopy (see below for methodology) to confirm that invading cells not only crossed the Transwell filter but also invaded into the Matrigel. Where required, this methodology was adapted to enable 3D reconstruction of the invasion phenotype (Sahai and Marshall, 2003). Briefly, cells treated as above were seeded into growth factor-reduced Matrigel-coated 8 μM pore Transwell chambers and 10% FBS in DMEM was used as a chemoattractant in the lower well. At set times, cells were fixed with 3.7% formaldehyde, washed, and fluorescent cells were analysed by Z-section confocal microscopy (1 section every 4 μM through the Matrigel) using a Bio-Rad MRC1024 confocal microscope (Bio-Rad Laser Sharp software). Three-dimensional reconstruction of the cells was performed using velocity software (Improvision).

Results
Changes in cell morphology and cell migration on basement membrane extracellular matrices
It is well documented that morphogenesis of endothelial cells into capillary-like structures requires interactions between integrins and the ECM components present in basement membranes including collagen IV and laminin (Davis et al., 2002). Using time-lapse video microscopy, we analyse further the changes in cell morphology and different types of cell movements involved in this process (Fig. 1 and supplementary material Movie 1). It was observed that HUVECs first attached loosely to the matrix, followed by a transition to an increasingly elongated morphology and migration towards initial cell-cell contacts. Subsequently, small aggregates of aligned cells making short, cord-like structures were formed within 2 hours. The cells continued to move and, at this stage, the migration was along neighbouring cells by parallel sliding movements, or occasionally from one cord to another; this resulted in extension of the shorter structures into a complex interconnecting network of capillary tubes. These findings are consistent with the data reported previously using somewhat different experimental conditions (Connolly et al., 2002; Nehls et al., 1998), suggesting that the observed forms of guided migration are crucial in capillary assembly and remodelling.

Interestingly, two selected tumour cell lines, when placed on the same matrix, had sufficient plasticity to perform similar movements, including the initial cell grouping and guided

Calcium-release, PtdIns(4,5)P2-hydrolysis and in-vitro-phosphorylation assays
For calcium-release assays, BE cells were labelled with Fluo-3 dye (2 μM) for 1 hour prior to washing and trypsinization. Following centrifugation (5 minutes, 160 g) the cells were resuspended in serum-free DMEM at a concentration of 8×10^5 cells per ml. Calcium measurements were performed as described in (Rodriguez et al., 2001). Briefly, 2 ml of cell suspension was placed in a cuvette in the fluorometer (excitation 490 nm, emission 525 nm) and the baseline measured for a few minutes. Stimulants (1935 μg/ml T21/16 or 5 mM MnCl2) were added through a Hamilton syringe and the calcium release measured over a 40-minute period. Calcium release was then quantified using standard calcium calibrations procedures for Fluo-3 dye (Kao et al., 1989). For calcium signalling on Matrigel, BE cells were pre-labelled with Fura-2 dye (2 μM) for 1 hour prior to trypsinization. The labelled cells were then plated onto Matrigel and immediately visualized by fluorescent real-time video microscopy (dual-colour Nikon Elipse Microscope, Hamamatsu ORCA-ER camera, Simple PCI software) at excitation wavelengths of 380 nm (green: low/basal intracellular calcium) and 340 nm (red: high/released intracellular calcium) over a 30-minute period (emission 510 nm). For phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P2]-hydrolysis assay, previously described methodology (Mullinax et al., 1999) was used. The phosphorylation assay and purification of GST-tagged proteins was as previously described (Rodriguez et al., 2001). Briefly, 2 μg of purified specific antibody domain of PLCγ1 (pSA) protein (aa residues 484-936) was incubated for times indicated with Src-family kinases (0.5 μg) in a reaction buffer containing: 50 mM Tris (pH 8), 2 mM MnCl2, 2 mM MgCl2, 1 mM Na2VO4, 2 mM DTT and 50 μM ATP at 37°C. Reactions were stopped by the addition of 4× SDS sample buffer, boiled and analysed by western blotting with appropriate antibodies [phosphotyrosine and pPLCγ1(Y783)]. Where necessary, the degree of phosphorylation was quantified by densitometry (NIH image software).
migration along adjacent cells (Fig. 1; supplementary material Movies 2, 3, 5 and 6 and Fig. S1). However, as summarized in Fig. 1, their ability to elongate differed from this pronounced characteristic of endothelial cells (~380%), with colorectal BE cells elongating significantly (~160%) and the prostate cell line DU145 (right panel) only slightly (~40%). Consequently, the cancer cells were not capable of generating very extensive networks but instead made shorter formations supporting the cell movement.

In a more commonly used migration assay for cancer cells – invasion through Matrigel in response to a chemotactic gradient – both BE and DU145 cells showed a polarized morphology and migrated towards serum; BE cells invaded slightly further than DU145 over a 24-hour time period (Fig. 1B).

Depletion of PLCγ1 or pharmacological inhibition of PLC activity prevents early changes in morphology and subsequent movement of tumour and endothelial cells

To assess the role of PLCγ1 in processes that follow initial cell-ECM interactions, tumour cells and endothelial cells were treated either with siRNA to PLCγ1 or an inhibitor of PLC activity. The PLCγ1 downregulation was specific and most effective in BE cells (Fig. 2A). Since the direct action of the commercially available PI-PLC inhibitor U73122 on different PI-PLC families has not been demonstrated, we confirmed that U73122 inhibited PLCγ1 protein in a direct assay in vitro whereas the control compound U73343 had no effect (Fig. 2B).

The analysis of resulting cell phenotypes is shown in Fig. 2D and E and in supplementary material Movies 3 and 4. BE cells treated with PLCγ1 siRNA showed rounded morphology even after 4 hours following the attachment to the matrix and were observed to be poorly motile; in comparison at this time point, the control cells were elongated and moving. The same round phenotype and lack of motility was observed when using PI-PLC inhibitor U73122 in both BE and DU145 cells. A similar rounded morphology in PLCγ1 siRNA- or U73122-
Fig. 2. (A) PLCγ1-specific siRNA depletion of PLCγ1 protein levels. Cells were treated with siRNA probes for 72 hours and processed for western blotting. Protein levels of PLCγ1 were compared with control blots against GAPDH and PLCγ2 or PLCδ1. (B) PLCγ1 activity is inhibited by U73122 but not by U73343. Purified PLCγ1 activity was measured in the presence of the inhibitor (10 µM) using a PtdIns(4,5)P2 hydrolysis assay. Results are expressed as the average PtdIns(4,5)P2 hydrolysis from four experiments with standard deviations shown. (C) Left panel; cells pre-treated with siRNA [BE (∆), DU145 (●)] or U73122 [BE (○), DU145 (○)] were plated on plastic and the number of viable adherent cells counted at various time points and compared with matched controls (scrambled siRNA or U73343). Right panel; HUVECs cells were pre-treated with either siRNA (solid bar) or U73122 (hatched bar) and viable adherent cell counted after 16 hours and compared with controls (scramble siRNA or U73343). Results shown are an average of four experiments and are shown with standard deviations. (D) Cell elongation is inhibited by U73122 treatment or siRNA, and the inhibition can be rescued by expression of rat PLCγ1. Pre-treated [siRNA (PLCγ1 or scrambled), U73122 (2 µM) or U73343 (2 µM)] BE (top panels) or DU145 (bottom panels) cells were plated on Matrigel and the level of elongation analysed by phase contrast microscopy. BE cells were also transfected with GFP in the presence or absence of siRNA or with GFP-PLCγ1 in the in the presence of siRNA as indicated in the middle panel. Representative images are shown and average percentage cell elongation, which was analysed from 20 independent cell images is reported with standard deviations; BE: U73343 166.2±18.5%, U73122 4.3±14.4%, scrambled siRNA 151.1±25.9%, PLCγ1 siRNA 11.6±10.4%, scrambled siRNA+GFP 154.9±29.2%, PLCγ1 siRNA+GFP 13.4±11.8%. (E) HUVEC cell elongation and network formation is inhibited by siRNA or U73122. Pre-treated [siRNA (PLCγ1 or scrambled), U73122 (1 µM) or U73343 (1 µM)] HUVECs were plated on Matrigel and elongation analysed by fluorescent microscopy. (F) Cell invasion is inhibited by PLCγ1 siRNA or U73122. Serum-starved pre-treated (siRNA, U73122 or U73343) cells were plated onto Matrigel-coated Transwell chambers and invasion through the filter and into the matrix, towards serum (10%), was measured by fluorescent microscopy after 48 hours with the average percentage (number) of invading cells compared with control (untreated, 100%) sample from five experiments displayed with standard deviations: 1, control; 2, PLCγ1 siRNA; 3, scrambled siRNA; 4, U73122 (2 µM); 5, U73343 (2 µM).
treated HUVECs was also observed, with these cells unable to move, elongate further or form capillary-like structures. Using a chemoinvasion assay, we also demonstrated that the treated BE and DU145 cells were not able to transmigrate through this matrix (Fig. 2F).

In a set of control experiments, we also confirmed the data described in Fig. 2D and E using another siRNA probe of similar potency (data not shown). In addition, we analysed PLCγ1+/− fibroblasts (Ji et al., 1998) and found that the early morphological changes were delayed and partially inhibited (supplementary material Fig. S2) rather than diminished completely, as found following the treatment of BE and endothelial cells with PLCγ1 siRNA probes (Fig. 2). Since one likely reason for such discrepancy could be previously observed upregulation of PLCγ2 in PLCγ1−/− fibroblasts (Fleming et al., 1998), this finding further supports involvement of PLCγ1 in early ECM-induced cell morphological changes. Furthermore, transfection of rat PLCγ1 into BE cells and transfection of PLCγ1−/− fibroblasts with the wild-type and catalytically inactive PLCγ1 suggested that the elongated morphology could only fully be rescued by the wild-type PLCγ1 (Fig. 2D and supplementary material Fig. S2). The catalytically inactive PLCγ1 mutant could only partially rescue the elongated phenotype perhaps through the non-catalytic function of PLCγ1 in regulating agonist-induced calcium entry (Patterson et al., 2002). However, our data using siRNA, wild-type and catalytically PLCγ1 rescue and various inhibitors suggest that non-catalytic or scaffold roles of PLCγ1 do not make a major contribution in establishing this phenotype.

Further experiments established that the effects of PLCγ1 downregulation or inhibition on cell morphology were not significant when other substrates such as collagen I, collagen IV, laminin or plastic were used (supplementary material Fig. S3). Whereas some degree of cell elongation was observed on these substratum, it was far less pronounced than that seen on Matrigel and was not accompanied by the characteristic cell grouping observed on Matrigel. This suggests that multicompartment, three-dimensional ECM is required for the observed phenotype and that PLCγ1 is required specifically for the establishment of this Matrigel-dependent phenotype. It was also determined that the abilities of cells to adhere firmly to plastic and remain viable were not affected by the siRNA or U73122 treatments. The culture on this substrate, not accompanied by pronounced morphological differences, thus demonstrates that these treatments were not intrinsically harmful or toxic to the cells (Fig. 2C). Similarly, based on the time-lapse recordings, the initial attachment to and cell viability on the basement membrane matrices were not affected and, under the circumstances where U73122 was not replenished at suitable time points, cells recovered their elongated and motile phenotype over time (data not shown).

The data in Fig. 2 suggest that PLCγ1 could have a role at early stages following the initial attachment of cells to the matrix, where further cell-ECM linkages, integrin-triggered signalling and cytoskeletal reorganization lead to more spread and elongated morphology (Friedl and Brocker, 2000). Further studies of these early events were performed in BE cells, which are characterized by distinct morphological changes at these time points. As shown in Fig. 3, the early changes in actin organization included formation of protrusions that become more polarized, with the enrichment of actin in areas of cell-cell contacts; microtubules were excluded from the protrusions. The formation of these structures was greatly reduced in cells treated with PLCγ1 siRNA or U73122 (Fig. 3, bottom panels). These data are consistent with previously suggested involvement of PLCγ in cytoskeletal reorganization (Kassis et al., 2001; Wells, 2000). They were also further supported here by observations in the context of a different cellular response; using the same PLCγ1 siRNA probe, the formation of lamellipodia and filopodia in response to serum stimulation was dramatically reduced in DU145 prostate carcinoma cells (supplementary material Fig. S4).

Activation of PLCγ1 requires engagement of integrins and involvement of Src kinases

On the basis of previous studies of PLCγ isoforms (Inoue et al., 2003; Kassis et al., 2001; Nakamura et al., 2001; Wells, 2000; Wonerow et al., 2003), the requirement for PLCγ1 observed in our experiments could be due to its role in growth factor signalling leading to modulation of the cytoskeleton and integrins or to the involvement of PLCγ1 downstream of integrins. Although these pathways could have some of the components in common, the involvement of Src kinases in cell spreading and migration appears to be a distinct requirement.
for the integrin-triggered pathway yet is dispensable for motility responses to growth factors (Klinghoffer et al., 1999; Nakamura et al., 2001). Therefore, to distinguish between these possibilities, we used a combination of inhibitory and activating antibodies to integrins and inhibitors of Src kinases.

Since the phosphorylation of PLCγ1 on Y783 has been linked to activation of PLCγ1 (Rhee, 2001), the phosphorylation of this residue was analysed in BE cells after attachment to the ECM ligands (Fig. 4A). This phosphorylation was clearly observed in control cells extracted from Matrigel, but it was abolished in the presence of an inhibitory antibody to β1 integrin or the Src kinase inhibitor PP2. We also found that phosphorylation of Src kinases on residue Y416 (which in some, but not all, cell types is linked to the integrin engagement) (Cary et al., 2002), was prevented by the inhibitory antibody to β1 integrin. By contrast, treatment of BE cells on Matrigel with phosphoinositide 3-kinase (PI-3K) inhibitor, LY294002 compound, affected only phosphorylation of a PI-3K target, PKB-Akt kinase, without any effect on phosphorylation of Src and PLCγ1 (data not shown). The involvement of β1 integrins and Src kinases in signalling to PLCγ1 was further supported by experiments where a stimulatory rather than inhibitory antibody to β1 integrins or Mn2+ ions, known to trigger integrin ‘outside-in’ signalling, were used. An increase in phosphorylation of both Src and PLCγ1 was clearly detected following stimulation of serum-starved BE cells plated on plastic (Fig. 4B), where stimulation is performed in a more controlled and synchronized way than on Matrigel.

The link between PLCγ1 and Src kinases was examined further by analysis of protein complexes present in BE cells after attachment (Fig. 4C). This phosphorylation was clearly observed in control cells extracted from Matrigel, but it was abolished in the presence of an inhibitory antibody to β1 integrin or the Src kinase inhibitor PP2. We also found that expression of Src kinases on residue Y416 (which in some, but not all, cell types is linked to the integrin engagement) (Cary et al., 2002), was prevented by the inhibitory antibody to β1 integrin. By contrast, treatment of BE cells on Matrigel with phosphoinositide 3-kinase (PI-3K) inhibitor, LY294002 compound, affected only phosphorylation of a PI-3K target, PKB-Akt kinase, without any effect on phosphorylation of Src and PLCγ1 (data not shown). The involvement of β1 integrins and Src kinases in signalling to PLCγ1 was further supported by experiments where a stimulatory rather than inhibitory antibody to β1 integrins or Mn2+ ions, known to trigger integrin ‘outside-in’ signalling, were used. An increase in phosphorylation of both Src and PLCγ1 was clearly detected following stimulation of serum-starved BE cells plated on plastic (Fig. 4B), where stimulation is performed in a more controlled and synchronized way than on Matrigel.

The data in Fig. 4A,B suggest that PLCγ1 is placed downstream of Src kinases. In addition, it has been reported that PLCγ1 isoforms can be phosphorylated directly by several members of the Src family (Liao et al., 1993; Rodriguez et al., 2001). In agreement with these previous observations, we found that purified Src, Fyn and Lck can phosphorylate PLCγ1 in vitro. Furthermore, unlike Syk tyrosine kinase, they were capable of phosphorylating the tyrosine residue critical for activation, Y783 (Fig. 4D); this residue was also phosphorylated in BE cells attached to Matrigel (Fig. 4A). Although other tyrosine kinases could be responsible for phosphorylation of PLCγ1 in cells, it is therefore also possible that Src kinases could be directly involved. In BE cells, Src was found to be the most highly expressed member of the Src-family kinases, although Fyn was also detectable (data not shown).

**Fig. 4.** (A) Inhibition of integrin β1 or Src-family kinases diminishes Src(Y416)- and PLCγ1(Y783)-activating phosphorylations. BE cells were plated on Matrigel and either left untreated (control) or treated with either blocking β1 integrin (4B4) antibody (10 µg/ml), Src inhibitor PP2 (10 µM) or the control compound PP3 (10 µM) for 8 hours. Cells were recovered from the Matrigel and processed for western blotting using antibodies to phosphorylated or unphosphorylated forms of PLCγ1 and Src as indicated. An inhibitor of EGF receptor AG1478 (10 µM) did not prevent PLCγ1 phosphorylation in this type of experiment (not shown). (B) Stimulation of integrin β1 enhances Src (Y416) and PLCγ1 (Y783) phosphorylation. Serum-starved BE cells were stimulated with either Mn2+ (5 µM) or the activating integrin β1 antibody (TS2/16 10 µg/ml) for 30 minutes and then lysed and processed for western blotting using antibodies to phosphorylated or unphosphorylated forms of PLCγ1 and Src as indicated. (C) PLCγ1, Src and GIT1 co-immunoprecipitation is integrin β1 dependent. BE cells, either untreated (−) or treated (+) with the integrin β1 blocking antibody 4B4 (10 µg/ml), were plated on Matrigel for 8 hours, then recovered from Matrigel, lysed and immunoprecipitation (IP) was performed with either PLCγ1 or Src antibodies as indicated at the top. Subsequent western blotting (WB) of these samples was performed using different PLCγ1 or Src antibodies or the GIT1 antibody as indicated on the left. In the same experiment, EGF receptor could not be detected in the immunoprecipitates (not shown). (D) Src-family kinases Src, Fyn and Lck can directly phosphorylate the PLCγ1(Y783) site. Purified PLCγ1 was incubated with purified Src-family kinases, indicated at the top, in an in vitro phosphorylation assay for 30 minutes. PLCγ1(Y783) phosphorylation was analysed using specific antibodies whereas autophosphorylation of Src kinases was verified using general anti-phosphotyrosine antibodies by western blotting (top panels). Time course of Src phosphorylation of PLCγ1(Y783). Src phosphorylation of PLCγ1 was analysed using a time-course in vitro phosphorylation assay followed by western blotting. Amount of phosphorylation was quantified by densitometry (bottom panel).
Importantly, the resulting round cell morphology and invasion of BE and DU145 cells was also inhibited (Fig. 5C). The inhibition of integrin engagement and Src activity had a marked effect on morphology of BE cells and the ability of HUVECs to elongate and form extensive networks (Fig. 5A,B). Different results were obtained when using PKC inhibitors R0-31-8200 and GF109203X. These two inhibitory compounds affected cells at a later stage than observed with other inhibitors. Exposure to 2-ABP, an inositol (1,4,5) trisphosphate (Ins(1,4,5)P₃) receptor antagonist, the intracellular calcium-chelating agent BAPTA-AM or the sarcoplasmic-endoplasmic reticulum calcium ATPase inhibitor thapsigargin (which disrupts intracellular calcium gradients) resulted in rounded morphology, the phenotypes obtained using inhibitory reagents to these components were analysed (Fig. 5). The inhibition of integrin engagement and Src kinase activity had a marked effect on morphology of BE cells and the ability of HUVECs to elongate and form extensive networks (Fig. 5A,B). Chemoinvasion of BE and DU145 cells was also inhibited (Fig. 5C). Importantl, the resulting round cell morphology and subsequent reduction in cell movement demonstrated similarity between inhibition of β1 integrins, Src kinases and PLCγ1.

The possibility that stimulation of EGF receptor contributes to PLCγ1 signalling in responses to integrin engagement on Matrigel was also investigated. EGF receptor inhibitor AG1478 and a blocking anti-EGF receptor antibody did not have an effect on BE cell elongation on Matrigel (Fig. 5A). We also found that BE cells express low levels of EGF receptor and showed that, in A431 cells that express high levels of EGF receptor, inhibitor AG1478 was functional and prevented PKC phosphorylation (Fig. 6B). Furthermore, EGF receptor inhibition did not affect phosphorylation of PLCγ1 and Src on Matrigel, and the EGF receptor was found not to co-immunoprecipitate with Src or PLCγ1 (see legend to Fig. 4).

Stimulation of calcium mobilization through integrins and PLC is critical for early morphological changes and cell movement

Since the inhibition of PLC activity had a similar effect in HUVECs, BE and DU-145 cells compared with siRNA specific to PLCγ1, it is likely that the requirement for PLCγ1 is due to its catalytic function rather than to a possible role as a scaffold protein (Patterson et al., 2002; Ye et al., 2002). We therefore analysed the best-defined downstream mediators of PI-PLC activation, intracellular calcium mobilization and activation of PKC, following stimulation of β1 integrins (Fig. 6). Upon stimulation of the cell suspension with activating anti-β1 integrin antibodies or Mn²⁺ ions, sustained calcium accumulation was observed in BE cells that could be inhibited by the PI-PLC inhibitor U73122 and Src inhibitor PP2 (Fig. 6A). We could also show that BE cells loaded with Fura2 calcium dye and plated onto Matrigel show an increase in intracellular calcium concentration within 30 minutes of plating (Fig. 6A bottom panel). Using similar conditions as for calcium measurements, phosphorylation of PKC was also detected in BE cells. However, the level of phosphorylation was not substantially changed after stimulation (Fig. 6B, left). When BE cells present on the ECM were analysed, the phosphorylation of PKC was not affected by the anti-integrin antibody or the Src kinase inhibitor (Fig. 6B, right). Since PKC phosphorylation was analysed using pan-phospho antibody, changes in the phosphorylation level of a specific PKC isoform could not be excluded.

To assess involvement of PI-PLC-mediated calcium accumulation and possible requirements for PKC in early morphological changes and cell movement dependent on interactions with the ECM, we used several well-characterized inhibitors. Exposure to 2-ABP, an inositol (1,4,5) trisphosphate [Ins(1,4,5)P₃] receptor antagonist, the intracellular calcium-chelating agent BAPTA-AM or the sarcoplasmic-endoplasmic reticulum calcium ATPase inhibitor thapsigargin (which disrupts intracellular calcium gradients) resulted in rounded morphology of BE cells (Fig. 7A). Morphology of endothelial cells was similarly affected and their ability to form cellular networks was reduced (Fig. 7B). These compounds also inhibited chemoinvasion of BE and DU145 cells (Fig. 7C). Different results were obtained when using PKC inhibitors R0-31-8200 and GF109203X. These two inhibitory compounds affected cells at a later stage than observed with other inhibitory reagents.
inhibitors; both HUVECs and BE cells were elongated and grouped in smaller formations but were unable to form extensive networks (Fig. 7A,B). The invasion of cancer cells treated with these PKC inhibitors was also only partially affected (Fig. 7C). These data suggest the role of calcium mobilization downstream of PLCγ, however, further experimental evidence is needed to assess to what extent calcium contributes to the downstream responses. The other second messenger generated by PI-PLC action, DAG, could also be important but it is unlikely that the early morphological changes are mediated by PKC.

Discussion
The data shown here support the involvement of PLCγ1 in a signal transduction pathway leading to a more spread and elongated morphology. Other important components, summarized in Fig. 8, include integrins, Src-family kinases and intracellular calcium mobilization. The supporting data are shown in Figs 2, 4, 5 and 6. Several previous observations using different, often highly specialized, cell types have also suggested a link between the components of the pathway proposed here and cell spreading. Cells generated from mice deficient in one (src<sup>−/−</sup> osteoclasts) or several members (src<sup>−/−</sup>, fyn<sup>−/−</sup>, yes<sup>−/−</sup> fibroblasts) of the Src-family kinases demonstrated impaired cell spreading and migration in response to integrin engagement. The inhibition of Src kinase activity in platelets also resulted in reduced cell spreading and loss of PLCγ2 phosphorylation following the engagement of von Willebrand factor or αβ1 and αIIbβ3 integrins (Inoue et al., 2003; Mangin et al., 2003; Wonerow et al., 2003).

Furthermore, the importance of PLC isoforms as a critical target for Src kinases implied here (Fig. 4) was also suggested by other studies using an inhibitor of PI-PLC activity (U73122) or platelets deficient in the PLCγ2 isoform (Inoue et al., 2003; Mangin et al., 2003; Nakamura et al., 2001; Wonerow et al., 2003). The resulting cell phenotype was similar to that observed for deficiency in Src kinases in both cases, although it was more pronounced with U73122 than with disruption of PLCγ2 (Mangin et al., 2003; Wonerow et al., 2003). Consistent with the involvement of the PLCγ1 isoform in similar processes, initial observations have indicated that a fibroblast cell line derived from PLCγ1<sup>−/−</sup> mice could have a more rounded morphology (Ji et al., 1998). Using the same cell line, we confirmed this phenotype on basement membrane extracellular matrices (supplementary material Fig. S2). Overall, the data shown here for endothelial and two cancer cell lines, together with the previous observations, suggest that signalling from integrins to PLCγ could be an important and quite general pathway operating in a variety of cell types. Further comparison of different cell lines and ECM ligands described here also suggests that, depending on the type and strength of integrin-ECM interactions, the signalling through PLCγ could contribute to early morphological changes to different degrees.
Although several lines of experimental evidence support an overlap and synergy between integrin-mediated and growth factor pathways, there is also evidence that they could operate independently. In particular, the involvement of Src kinases in integrin signalling is not required for the effects of growth factors on similar processes; thus, the cell spreading or migration in response to soluble growth factors, as well as PLC\(\gamma\) phosphorylation, were not affected in fibroblasts and osteoclasts from mice deficient in Src kinases (Klinghoffer et al., 1999; Nakamura et al., 2001). It has also been shown that PDGF receptor lacking the tyrosine residue phosphorylated by Src retained and had even more enhanced chemotaxis towards PDGF, mediated by enhanced phosphorylation and activation of PLC\(\gamma\) (Ronnstrand et al., 1999). The link between Src kinase and PLC\(\gamma\) shown here (Fig. 4) indicates that the main function of PLC\(\gamma\) in our experimental system is in integrin rather than growth factor-triggered responses.

On the basis of broader studies in different cellular responses, PLC\(\gamma\) isoforms can be linked to downstream processes though generation of second messengers or reduction in PtdIns(4,5)\(P_2\) concentrations, both of which require PLC catalytic activity, as well as through protein-protein interactions that are independent of this PLC activity (Katan, 1998; Putney, 2002; Rebecchi and Pentyala, 2000; Rhee, 2001). Although the role of PLC\(\gamma\) as a scaffold protein was originally linked to cell proliferation (Smith et al., 1994), other evidence from cellular systems where PLC\(\gamma\) functions as an essential component of growth factor-induced chemotaxis suggests that, at least in this type of cell movement, the catalytic activity was required (Chen et al., 1994; Ronnstrand et al., 1999). Our experiments demonstrate that the inhibition of PLC activity leads to changes similar to those seen with depletion of PLC\(\gamma\) and that only catalytically active PLC can fully rescue elongated morphology (Fig. 2 and supplementary material Fig. S2). On the basis of these observations, it is likely that integrin-regulated cell motility mediated by PLC\(\gamma\) also requires its catalytic activity. Some of the previous studies have documented that an increase in cellular calcium resulting from PLC\(\gamma\) activation coincided with the cytoskeletal

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**Fig. 7.** Inhibition of Ins(1,4,5)\(P_3\) and calcium but not PKC inhibits early changes in cell morphology. (A) BE cells plated on Matrigel and treated for 8 hours with either 2-APB (40 \(\mu\)M) an Ins(1,4,5)\(P_3\) receptor antagonist, BAPTA-AM (20 \(\mu\)M), an intracellular calcium-chelating agent, thapsigargin (THAPS; 100 nM) an inhibitor of the sarcoplasmic endoplasmic reticulum calcium ATPase or the PKC inhibitor compounds R0-31-8220 (3 \(\mu\)M) or GF109203X (3 \(\mu\)M) and its structurally related control bisindolylmaleimide V (BiV) (3 \(\mu\)M). Cell elongation was analysed by microscopy with representative images shown and average percentage cell elongation, analysed from 20 independent cell images reported with standard deviations; control 161.2±17.8%, APB-2 7.7±6.4%, BAPTA-AM 1.8±6.5%, thapsigargin 3.2±4.3%, RO-31-8220 45.0±17.9%, GFX109203X 41.0±21.3%, BiV 154±16.7%. (B) HUVECs were plated on Matrigel and treated as for part A. Cell elongation and networking was analysed by fluorescent microscopy. (C) Serum-starved cells treated as for part A were plated onto Matrigel-coated Transwell chambers and invasion through the filter and into the matrix, towards serum (10%) was analysed by microscopy after 48 hours with the average percentage (number) of invading cells compared with control (untreated, 100%) sample from five experiments displayed with standard deviations. 1, control; 2, 2-APB; 3, BAPTA-AM; 4, R0-31-8220; 5, GFX109203X; 6, BiV.

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**Fig. 8.** Model of PLC\(\gamma\) involvement in the \(\beta_1\) integrin-Src signalling cascade.
rearrangements and formation of lamellipodia and filopodia; both calcium responses and formation of such protrusions were reduced in PLCγ2-deficient platelets (Mangin et al., 2003; Wonerow et al., 2003). Although a similar conclusion can be drawn from this work (Figs 3, 6 and 7), a direct link between an increase in intracellular calcium concentrations and changes in actin cytoskeleton remain speculative. More generally, despite considerable circumstantial evidence for the involvement of PLCγ in reorganization of cytoskeletal components (Kassis et al., 2001; Wells, 2000), there are only a few observations that suggest an underlying mechanism or a link with the known regulators of actin cytoskeleton such as small GTPases from the Rho family (Fleming et al., 1998; Connolly, J., Simpson, N., Hewlett, L. and Hall, A. (2002). Rac regulates endothelial morphogenesis and capillary assembly. Mol. Biol. Cell 13, 2474-2485.

We thank Hugh Patterson for his assistance regarding microscopy work, and Wynne Aherne and Yvette Newbatt for assistance with the PsdIns(4,5)P2 hydrolysis assay. We also thank Graham Carpenter for his gift of the Mouse Fibroblast cell lines and the Rat PLCγ1-GFP construct, and Klaus Seedorff for his gift of the catalytically inactive PLCγ1 construct. Work is funded by Cancer Research UK.

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