Activity of PLCε contributes to chemotaxis of fibroblasts towards PDGF

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Accepted 17 August 2012
Journal of Cell Science 125, 5758–5769
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doi: 10.1242/jcs.110007

Summary
Cell chemotaxis, such as migration of fibroblasts towards growth factors during development and wound healing, requires precise spatial coordination of signalling events. Phosphoinositides and signalling enzymes involved in their generation and hydrolysis have been implicated in regulation of chemotaxis; however, the role and importance of specific components remain poorly understood. Here, we demonstrate that phospholipase C epsilon (PLCε) contributes to fibroblast chemotaxis towards platelet-derived growth factor (PDGF-BB). Using PLCε null fibroblasts we show that cells deficient in PLCε have greatly reduced directionality towards PDGF-BB without detrimental effect on their ability to migrate. Furthermore, we show that in intact fibroblasts, signalling events, such as activation of Rac, are spatially compromised by the absence of PLCε that affects the ability of cells to enlarge their protrusions in the direction of the chemoattractant. By further application of live cell imaging and the use of FRET-based biosensors, we show that generation of Ins(1,4,5)P₃ and recruitment of PLCε are most pronounced in protrusions responding to the PDGF-BB gradient. Furthermore, the phospholipase C activity of PLCε is critical for its role in chemotaxis, consistent with the importance of Ins(1,4,5)P₃ generation and sustained calcium responses in this process. As PLCε has extensive signalling connectivity, using transgenic fibroblasts we ruled out its activation by direct binding to Ras or Rap GTPases, and suggest instead new unexpected links for PLCε in the context of chemotaxis.

Key words: Caenorhabditis elegans, Rab GTPase, Cortical granule exocytosis, Separase

Introduction
Phosphoinositide signalling plays an important role in various aspects of cell motility including cytoskeleton remodelling, regulation of cell adhesion and directional sensing (Kölsch et al., 2008). Directional movement is a property of most cell types during development and is subsequently critical for tissue remodelling and regeneration. Chemotaxis, or migration biased toward a gradient of soluble chemoattractant, has been studied extensively in two model cell types: neutrophils and the slime mould Dictyostelium discoideum. Another example of chemotactic sensing is that of fibroblasts in wound healing. Platelet-derived growth factor (PDGF), produced by platelets and macrophages, forms a gradient in the tissue and serves as a potent chemoattractant and mitogen, thus accelerating the rate of fibroblast invasion into the fibrin clot (Heldin and Westermark, 1999).

In the context of chemotaxis, several components of phosphoinositide signalling have been implicated in regulatory mechanisms. In particular, in D. discoideum and neutrophils the spatial segregation of PtdIns(3,4,5)P₃ by the actions of PI3K and PTEN have been well documented and the role of PI3K in chemokinesis and chemotaxis assessed using different in vitro and in vivo conditions (Afonso and Parent, 2011). Links with other important signalling components involved in cell movement and chemotaxis, small GTPases from Ras and Rho family, have also been explored and established in these model systems (Cain and Ridley, 2009). In fibroblasts, chemotaxis to PDGF is also characterised by polarised localisation of PtdIns(3,4,5)P₃. However, modelling predicts that localised PtdIns(3,4,5)P₃ accumulation primarily reflects receptor occupancy and is not regulated by feedback amplification or inhibition proposed for ameboid cells (Schneider and Haugh, 2005). Indeed, migration of fibroblasts is slower than that of amoeboid cells and although driven by membrane protrusion, it also involves substantial contribution of differential adhesion (Lauffenburger and Horwitz, 1996), suggesting distinct requirements for gradient sensing. Furthermore, it has been shown that robust PDGF sensing requires steeper gradients and a much narrower range of absolute chemoattractant concentration (Schneider and Haugh, 2005).

Another enzyme activity reported to be involved in chemotaxis in D. discoideum and several mammalian cell types is phosphoinositide-specific phospholipase C (PLC). In D. discoideum a single PLC activity, dDPLC, has been implicated in maintaining polarised distribution of PtdIns(4,5)P₂ and is suggested to affect localised regulation of PtdIns(3,4,5)P₃ production (Kortholt et al., 2007). In mammalian cells, however, there are six families of PLC enzymes (PLCβ, γ, δ, ε, ζ and η), consisting of 13 isozymes in humans, characterised by different regulatory mechanisms and expression pattern (Bunney et al., 2009). In particular, PLCδ2 is known to be involved in platelet aggregation and PDGF-BB mediated chemotaxis of human skin fibroblasts (Bunney et al., 2009). PLCδ2-null fibroblasts have greatly reduced directionality towards PDGF-BB without detrimental effect on their ability to migrate. Further analysis using FRET-based biosensors showed that generation of Ins(1,4,5)P₃ and recruitment of PLCδ2 were most pronounced in protrusions responding to the PDGF-BB gradient (Bunney et al., 2009). PLCδ2 has extensive signalling connectivity, using transgenic fibroblasts we ruled out its activation by direct binding to Ras or Rap GTPases, and suggest instead new unexpected links for PLCδ2 in the context of chemotaxis.
and Katan, 2011). PLCβ2 and PLCβ3 are abundant isoforms of PLC in hemopoietic cells and respond to agonists to G-protein coupled receptor, including potent chemoatractants fMLP and SDF-α. Studies of neutrophil chemotaxis demonstrated that cells lacking PLCβ2/PLCβ3 enzymes have slightly deficient chemotaxis towards fMLP gradient that is further affected by depletion of PI3K, thus functioning as components of complex and context-dependent regulatory networks (Tang et al., 2011). T cells deficient only in PLCβ2/β3, however, have substantially reduced chemotaxis when analysed in gradients of SDF-1α (Bach et al., 2007). Studies of several breast cancer cell lines demonstrated the involvement of PLC activity in chemotaxis towards EGF (Roussos et al., 2011; Wang et al., 2007) and the chemotaxis of fibroblasts towards PDGF has also been shown to require PLC (Wei et al., 2010). Neither of these studies demonstrated directly which of PLC isoforms are involved in the responses towards growth factor chemoattractant gradient although PLCγ1 enzyme was implicated in chemoattractant responses in several earlier studies (Kundra et al., 1994; Rönnstrand et al., 1999). Further complexity of PLC signalling is due to the involvement of several downstream events linked either to changes in PtdIns(4,5)P2 concentrations or generation of second messengers, diacylglycerol and Ins(1,4,5)P3. Studies in cancer cells emphasised reduction of local PtdIns(4,5)P2 and linked this to the activity state of coflin (Leyman et al., 2009; van Rheenen et al., 2007). In contrast, studies in fibroblasts focused on the role of Ins(1,4,5)P3 production and subsequent, more complex and dynamic changes in calcium levels in protrusions of cells responding to PDGF gradient (Wei et al., 2009). Thus, the role of specific PLC enzymes in chemotaxis, and particularly that towards growth factors, remains to be defined further and new insights are largely dependent on manipulation of expression of specific PLC enzymes and generation of cells from transgenic animals.

The studies reported here use fibroblasts derived from transgenic animals with null or mutated alleles of PLCγ1 encoding PLCγ enzyme characterised by extensive signalling connectivity, including several small GTPases involved in regulation of cells motility (Bunney and Katan, 2006; Smrcka et al., 2012). We demonstrate that PLCγ is required for chemotaxis of fibroblasts to PDGF-BB and contributes to localisation and persistence of signalling in protrusions responding to chemotactic gradient.

**Results**

**PLCγ contributes to responses of fibroblasts to PDGF-BB**

It has been previously established that PLCγ1, the only isoform in PLCγ family, is not uniformly expressed in different tissues and cell types and, compared to other PLCs, is generally at lower levels (Smrcka et al., 2012). Among cell types, appreciable levels of expression have been detected in fibroblast cell lines and murine fibroblasts (Ikuta et al., 2008; Kelley et al., 2006). Following generation of transgenic mouse strains with altered Plcγ1 alleles, we isolated mouse embryonic fibroblasts (MEFs) and established that fibroblasts from the Plcγ1−/− animals (KO) lack a PLCγ1 coding region and PLCγ protein expression (Fig. 1A–C).

Using a number of immortalised cell populations of WT and KO MEFs we established similar, significant differences in response to a potent fibroblast stimulus, PDGF-BB. Notably, PLC responses measured by inositol phosphate production were reduced to about 60% in KO MEFs (n=4, P<0.001, ANOVA) (supplementary material Fig. S1A). We further extended our analysis using a panel of different extracellular agonists, including growth factors for tyrosine kinase receptors (EGF, FGF and VEGF) and agonists for G-protein coupled receptors (LPA, thrombin, ephrinephrine, carbachol, bombesin and ATP) some of which have been previously linked to activation of PLCγ (Bunney and Katan, 2006; Smrcka et al., 2012). We also analysed several compounds that could act on potential intracellular mediators of PLCγ activation such as 8-pMeOPT (Dzhura et al., 2010). As shown in Fig. 1D, a PDGF-BB appears to be the most potent activator of PLCγ in MEFs also showing differences in PLC responses between the WT and PLCγ KO fibroblasts. Responses to some other agonists, for example thrombin, were also significantly reduced in KO MEFs as reported previously (Kelley et al., 2006).

The finding that PLC activity in PLCγ KO fibroblasts was only reduced and not abolished is consistent with the observations that PLCγ can be activated together with other PLC enzymes in response to the same stimulus. For example in HEK293T cells stimulated with EGF both PLCγ and PLCe enzymes are activated (Stope et al., 2004) while in fibroblasts stimulated by thrombin, activation includes PLCβ3 and PLCe (Kelley et al., 2006). This differs from overexpression systems where it could be clearly demonstrated that PLCγ, as the major expressing PLC, can respond to specific external signals, including PDGF (Kelley et al., 2004; Song et al., 2002). In the experiments shown here (Fig. 1D) the other activated PLC in response to PDGF most likely corresponds to PLCγ1, an isoform that has been clearly linked to PDGF stimulation (Hess et al., 1998; Liao et al., 2006; Wahl et al., 1989). Expression levels and activation of PLCγ1 in response to PDGF-BB remain unchanged in PLCγ KO fibroblasts (see further, Fig. 3A).

**Fibroblasts lacking PLCγ have impaired chemotaxis in PDGF-BB gradients**

As PLC activity has been implicated in chemotaxis towards growth factors (Kundra et al., 1994; Rönnstrand et al., 1999; Roussos et al., 2011; Wang et al., 2007; Wei et al., 2010), we analysed migration of the WT and PLCγ KO fibroblasts in gradients of PDGF-BB. Two of the methods that can be applied to generate gradients and monitor cell chemotaxis, Dunn chambers and delivery of PDGF using micropipette, were further adapted and characterised for this analysis (supplementary material Fig. S1C,D).

Experiments performed using Dunn chambers demonstrated a clear difference in the number of cells that migrated in the direction of chemotactic gradient (Fig. 2A; supplementary material Fig. S1B). While the majority of the WT cells were biased towards the gradient, KO cells migrated more randomly and had a considerably lower Forward Migration Index (n=4, P<0.01, ANOVA) (supplementary material Fig. S1B). Interestingly, Mean Speed of migration was slightly enhanced in PLCγ KO fibroblasts suggesting that PLCγ depletion has no detrimental effect on basal ability of cells to migrate but mainly affects chemotaxis (Fig. 2A).

We have also analysed the ability of migrating fibroblasts to turn towards PDGF-BB gradients by placing a PDGF-BB containing micropipette perpendicular to cell movement. For this cell-turning study, only fibroblasts with a clear leading protrusion were selected. Analysis and specific examples of the
WT and PLC-e KO fibroblasts in this assay are shown in Fig. 2B,C. Typically, WT cells formed a new protrusion towards the source, reversed their axes of polarisation and subsequently migrated in direction of higher PDGF-BB concentrations. In contrast, 7 out of 10 KO cells retained their initial direction or formed new protrusions randomly.

Signalling responses to PDGF-BB show differences that are highly localised
A number of intracellular signalling events are activated in fibroblasts following PDGF-BB stimulation. The key network nodes and links include PI3K mediated activation of PKB, activation of Ras and downstream kinases such as ERK and activation of PLCγ1 (Park et al., 2003). Several other small GTPases in addition to Ras, such as Rap, Rho and Rac, also participate in responses to PDGF-BB. Comparison of the WT and PLC-e KO fibroblasts following uniform stimulation by PDGF-BB for 5 min did not reveal any major differences in overall activation of the tested components; our analysis covered PDGFR, ERK, PKB, PLCγ1, K-Ras, Rap1 and Rac proteins (Fig. 3A). Analysis of several of these components at later times (not shown) did not reveal any differences between the WT and PLC-e KO MEFs.

Localisation or activation of signalling components in many physiological systems is not uniform. Migrating cells and cells responding to chemoattractant are well-documented examples of signalling segregation and compartmentalisation (Berzat and Hall, 2010). One of the components that can be robustly monitored regarding spatial distribution in real time is activation of Rac using FRET biosensors; localised activation of Rac1 has been shown in protrusions of migrating fibroblasts where it has an important role in formation of protrusions and cell movement (Machacek et al., 2009). Using a modified version of one of the previously described biosensors (FLAIR) (Hodgson et al., 2010) we analysed WT and PLC-e KO fibroblasts. We selected cells that show Rac activity in several protrusions and monitored the effect of PDGF-BB gradient formed using micropipette in the proximity of these cells. A summary of the change in FRET index between the membrane regions of the cell directed towards and away from the micropipette [based on analysis described in Wei et al. (Wei et al., 2009)] averaged over a number of WT and PLC-e KO fibroblasts, is shown in Fig. 3B and the differences illustrated by individual cells in Fig. 3C. In most WT cells, the protrusion size and the Rac activity increase in the protrusion nearest to the high concentrations of PDGF-BB. In contrast, in high proportion of
PLCε KO cells a protrusion closest to the PDGF-BB source is enlarged and has high Rac activity only transiently. Subsequently, the main Rac activity and protrusion extension occurs in other parts of the cell regardless of their position in PGDF gradient. Thus, despite the lack of overall differences in responses to uniform PDGF stimulation (Fig. 3A), signalling events in protrusions of fibroblasts lacking PLCε appear to be compromised when tested in the presence of a chemoattractant gradient.

**Phospholipase C activity of PLCε is required for normal chemotaxis**

PLCε is a dual function protein, incorporating PLC and GEF enzyme activities. In addition, it has a Ras-association domain (RA2) that mediates interaction with several GTPases from Ras family (H-, K-, N-Ras, Rap1 and Rap2) (Fig. 1A) (Bunney and Katan, 2006; Smrcka et al., 2012). Point mutations that result in specific inactivation of PLC activity and mutations that abolish Ras-binding and activation in transfected cells (Fig. 4A) have been based on previous structural studies (Bunney et al., 2006; Ellis et al., 1998). Mutations in the RA2 domain were also used to generate a transgenic mouse strain expressing RAm PLCε at physiological level (Fig. 4B).

To assess the importance of the RA2 domain in the context of chemotaxis and potential links with some of the Ras family members that bind this domain, we generated MEFs from Plce1 RAm/RAm mice and analysed their migration using Dunn chambers. These cells displayed clear directional movement towards a PDGF-BB gradient with forward migration index comparable to that of WT fibroblasts (Fig. 4C,D). Next, we used adenoviruses expressing PLCε variants deficient either in PLC activity or in Ras-binding (previously described in Citro et al., 2007; Oestreich et al., 2007) to rescue PDGF-BB-mediated chemotaxis in PLCε KO cells as well as the expression WT PLCε (Fig. 4C,D). This is also consistent with findings that MEFs lacking H-, K- and N-Ras (Drosten et al., 2010) had no significant difference in PLC responses to PDGF-BB stimulation despite a great reduction in the basal rate of cell movement (supplementary material Fig. S2A). Notably, in contrast to PLCε RAm, the variant lacking PLC activity (Ad-PLCm) was not able...
to rescue compromised chemotaxis of PLCε KO cells (Fig. 4C,D). Based on these observations, PLC activity of PLCε and the consequences of PtdIns(4,5)P2 hydrolysis (at least in part) underlay the role of this protein in chemotaxis. However, regulation of PLCε is not mediated by small GTPases that bind the RA2 domain.

Stimulation of PLCε PLC activity can be mediated by other small GTPases that do not require intact RA2 domain needed for action of Ras and Rap proteins. It has been shown that RhoA and RalA can stimulate PLCε lacking RA2 domain in co-expression or in vitro system (Gandarillas et al., 2009; Kelley et al., 2004; Seifert et al., 2004). The PLCε variant incorporating point-mutations within the RA2 domain used in our study is also fully activated by these small GTPases; we further established that other related family members, RhoB and RhoC and RalB, also stimulate PLCε (Fig. 5A). To elucidate which of these small GTPases could be involved in PLCε activation in the context of chemotaxis, we investigated whether these GTPases are activated in MEFs by PDGF-BB, tested MEFs deficient in specific GTPases and MEFs treated with C. botulinum C3 exoenzyme.
that impairs function of Rho GTPases by ADP-ribosylation and Ral by direct binding (Just et al., 2010) (Fig. 5B,C; supplementary material Fig. S3). Based on our data and previous reports, Rho GTPases are not readily activated in this system (Monypenny et al., 2009) while the use of MEFs deficient in RhoA was inconclusive due to concomitant upregulation of other GTPases (supplementary material Fig. S2B). In contrast, activation of RalA and RalB was observed in MEFs treated with PDGF-BB (Fig. 5B) and RalA or RalB deficient fibroblasts had both reduced PDGF-BB-induced PLC activation and compromised chemotaxis (Fig. 5C–E), suggesting that Ral proteins could mediate activation of PLCz in this context. This is further supported by the findings that depletion of Ral GTPases by siRNA affects preferentially PLC activation (supplementary material Fig. S3A,B) and directional migration (reduction of Forward Migration Index in WT MEFs by 38%, P<0.01, ANOVA) in WT MEFs. Similarly, the C. botulinum C3 exoenzyme, which reduces stimulation of PLC activity by PDGF-BB more potently in WT MEFs, also inhibits Ral activation by PDGF-BB (supplementary material Fig. S3C,D).

**Generation of Ins(1,4,5)P3 in cell protrusions can have a role in chemotaxis**

Following our observation that phospholipase C activity of PLCz is required for normal chemotaxis we measured the Forward Migration Index of fibroblasts treated with PKC inhibitor or calcium chelator BAPTA at concentrations that had little or no
effect on cell speed; as shown in Fig. 6A, under these conditions addition of BAPTA clearly affected directional motility.

We further analysed production of PLC-generated second messenger involved in calcium mobilisation, Ins(1,4,5)P$_3$, in fibroblasts exposed to PDGF-BB gradient; we used a modified version of previously described FRET biosensor, LIBRA (Tanimura et al., 2004); the data analysis is shown in Fig. 6B. Despite some impact of expression of LIBRA protein on cell viability (in particular when cells were stimulated with PDGF-BB) we were able to monitor oscillation in Ins(1,4,5)P$_3$ concentrations in cells exposed to PDGF-BB gradient, in some cells over a 40 minute period (Fig. 6C, top). In the WT fibroblasts the greatest change in FLIM-FRET was observed in protrusions closest to the source of PDGF-BB (Fig. 6C, bottom). Similarly as observed when using Rac biosensor FLAIR (Fig. 3B), PLCe KO fibroblasts appear to lack localised changes in Ins(1,4,5)P$_3$ production that are related to the direction of chemotactic gradient (Fig. 6C, bottom). In agreement with the proposed role of PLCe in protrusions of fibroblasts exposed to chemotactic gradient, we also observed

**Fig. 5. Analysis of PLCe regulation by different small GTPases.** (A) PLC activity of the WT and PLCe RAm variant was analysed in COS7 cells in the absence (black) or presence of either RalAV23 (grey) or RhoAV14 (white) (left panel). PLC activity of the WT PLCe was also tested in the presence of activated forms of RhoA, RhoB, RhoC, RalA and RalB (right panels). (B) Cell extracts from WT MEFs, untreated (−) and treated with PDGF-BB for 5 min (+), were analysed by western blotting using antibodies to RalA and RalB GTPases; total protein and protein present in the “pull-down” with the GST-RalBP1-RBD (GTP-bound forms) were analysed. (C) PLC activity was measured in immortalised cell populations of Rala flox/flox and Ralb flox/flox MEFs following infection with either Ad-GFP or Ad-Cre; untreated MEFs (basal) and MEFs treated with PDGF-BB (PDGF) were analysed. *P<0.05, t-test. (D) Expression of RalA and RalB proteins was analysed in Rala flox/flox and Ralb flox/flox MEFs following infection with either Ad-GFP or Ad-Cre. (E) Forward Migration Index was determined using Dunn chambers; immortalised populations of Rala flox/flox and Ralb flox/flox MEFs, following infection with either Ad-GFP (control) or Ad-Cre, were analysed. The data are from four independent experiments, *P<0.05, ANOVA. Random migration was not affected following infection with Ad-Cre.
Discussion

Despite intensive study and its biological importance the mechanism of chemotaxis remains unclear both more generally and in molecular detail. In particular, the role of cell signalling network either as a critical control mechanism (“compass model”) or a modulator of cell-generated cycle (“pseudopode-based model”), remains to be further characterised and the importance of specific signal transducer components defined within the context of specific cellular responses (Insall, 2010). Within the regulatory network some of the phosphoinositide signalling components and small GTPases have been linked to the interface between transmission of extracellular signals and pseudopode machinery. Here we identified one of the phosphoinositide-specific PLC enzymes, PLCɛ, as an important component required for regulation of chemotaxis of fibroblasts towards PDGF.

Physiological roles of PLCɛ have been addressed by generating transgenic animal models and cell lines derived from these animals (Bunney and Katan, 2006; Smrcka et al., 2012). Our strategy was to generate Plce1 null allele and a variant specifically deficient in some of signalling interactions (Figs 1, 4). We subsequently focused on fibroblasts that have not been previously studied with respect to cell migration or growth factor stimulation although being recognised as one of the cell types that express this isoform (Kelley et al., 2006; Ikuta et al., 2008). We found that PLCɛ KO fibroblasts have reduced PtdIns(4,5)P2 hydrolysis in response to PDGF and...
compromised chemotaxis towards this chemotactrant despite normal random motility; these cells lack the ability to form stable protrusions towards the PDGF-BB gradient (Figs 1–3). Previous work using PLCε fibroblasts from *Plec1* A/+ A/+ mice, expressing protein deficient in PLC activity, also revealed the importance of PLCε in fibroblast function, notably in the interplay between fibroblasts and cells from the immune system (Ikuta et al., 2008). It has been shown that PLCε is required for secretion of chemokines in response to phorbol ester and consequently for the ability of fibroblasts to mobilise cells of the immune system that constitute the tumour environment. From studies of PLCε KO mice reported so far (Smrcka et al., 2012) it is, however, not clear what other cell types could have impaired directional movement. Nevertheless, supporting evidence for the importance of PLCε in directional cell movement in vivo came from use of transgenic approaches in *C. elegans*, the first organism where PLCε was identified. Studies in *C. elegans* have revealed the defect in morphogenesis that involves a programmed series of migrations and fusions of epithelial sheets (Vázquez-Manrique et al., 2008). This process recapitulates many of the traits of wound healing after tissue damage in mammals. Specifically, the analysis of *C. elegans* using RNAi and mutant strains have shown that depletion of PLC-1/PLCε resulted in slower migration of the ventral epidermal cells. As a consequence, plc-1 loss of function resulted in ruptured embryos with a Gex (gut on exterior) phenotype and lumpy larvae (Vázquez-Manrique et al., 2008).

Among PLC enzymes PLCε is characterised by more complex regulatory interactions and greater degree of signalling connectivity. Notably, several small GTPases important for regulation of cell motility are involved in activation of PLCε (Bunney and Katan, 2006; Smrcka et al., 2012). This high degree of integration into signalling networks provides a scope for various regulatory mechanisms. In the context of chemotaxis to PDGF-BB, regulation of PLCε phospholipase activity is not mediated by direct binding of Ras or Rap proteins to RA2 domain (Fig. 4) and could be mediated by Rap GTPases (Fig. 5). A number of observations that support involvement of Rap proteins in regulation of cell motility, including Rap activation localised to membrane protrusions in response to EGF stimulation (Yoshizaki et al., 2007) and the control of the assembly and localisation of exocyst subunits and associated proteins at the leading edge of motile cells (Parrini et al., 2011), are consistent with the possibility that PLCε could also be regulated by Rap proteins.

Another distinct property of PLCε compared to other PLC enzymes is the capacity to participate in more sustained signal generation (Smrcka et al., 2012). Notably, it was shown that in fibroblasts stimulated by thrombin the immediate increase in Ins(1,4,5)P3 production is due to activation of PLCβ3 while the depletion of PLCε results in reduced Ins(1,4,5)P3 production between 3 and 60 min following stimulation (Kelley et al., 2006). Similar considerations were applied when stimulation of PLCγ2 and PLCε was observed by the same agonist (EGF) (Stope et al., 2004). The sustained signal generation of PLCε could be important in the context of chemotaxis where persistence of a signal could ensure continuous enlargement of a protrusion in the direction of chemotactrant gradient, the property that is lacking in PLCε KO fibroblasts (Fig. 3). In this respect, the role of PLCε in chemotaxis could differ from that suggested for PLCγ1, in most instances linked to transient activation.

In addition to the difference in responses observed in PLCε KO cells and inhibition of chemotaxis by calcium chelation (Fig. 1D; Fig. 6A), several other findings further support the possibility that PLCε contributes to signalling localised to growing protrusions by regulating Ins(1,4,5)P3 and calcium levels. These include observations that the function of PLCε in chemotaxis requires intact PLC activity (Fig. 4) and that protrusions are characterised by high levels of Ins(1,4,5)P3 generation (Fig. 6C). The recently proposed role for Ins(1,4,5)P3 in chemotaxis could provide one possible mechanism for the Ins(1,4,5)P3-mediated involvement of PLCε (Wei et al., 2009). The proposed model suggests that Ins(1,4,5)P3 receptor in the endoplasmic reticulum functions together with a stretch-activated cation channel (TRPM7) to generate local, dynamic sites of high calcium concentrations described as calcium flickers; the supporting data include the finding that administration of a membrane-permeable Ins(1,4,5)P3 analogue enhances both the calcium flicker activity and the turning process towards PDGF source. Importance of calcium flickers as a mechanism of gradient sensing was further suggested by observations that intracellular loading of EGTA to chelate intracellular calcium slows the turning. Interestingly, after complete abolition of the calcium flicker activity, cells can still migrate at even faster velocity; however, they are no longer able to make turns when exposed to a PDGF gradient (Wei et al., 2009; Wei et al., 2010).

In summary, we have found that PLCε contributes to regulation of fibroblast chemotaxis as an important component of signalling processes localised to cell protrusions that respond to PDGF-BB gradient. As a multifunctional protein with impact on several downstream events, PLCε has extensive signalling connectivity. In the context of chemotaxis it is likely that signalling links include Ral GTPases, as upstream regulators of PLC activity, and Ins(1,4,5)P3-mediated localised changes in calcium concentrations as downstream events affecting pseudopode machinery.

**Materials and Methods**

**Generation of PLCε transgenic animals and isolation of mouse embryonic fibroblasts**

Generation of PLCε transgenic mice was based on a standard homologous recombination strategy using stem cell manipulation. *Plec1*+/− targeting vector was constructed using DNA fragments flanking exon2 regional which were cloned into PGKNeo-F2L2DTA plasmid (Addgene plasmid 13445). *Plce1*KO targeting vector was constructed using DNA fragments of exon29/30 and using a modified version of PGKNeo-F2L2DTA plasmid where one of the loxp sites was replaced by a polylinker and the other loxp site was mutated by deletion of three nucleotides. The targeting vectors were electroporated into Bruce 4 ES cells and homologous recombination was checked by Southern blot. Positive clones were injected into albino C57BL/6J blastocysts and chimeric males were crossed with Cre or Flp recombinase transgenic mice, as appropriate, in order to remove the PGKNeo cassette. PLCε null allele (*Plce1−−*) was created by exon 2 deletion and frameshift termination in exon 3. RT-PCR, using primers in exon 1 and 3, was used to confirm genotype of *Plce1−−/−*, using the same plasmids, ES cells, blastocysts and Flp recombinase transgenic mice, was used to generate *Rala* and *Ralu* conditional alleles (Peschard et al., 2012).

WT, *Plec1*+/−, *Plce1KO/KO*, *RalaΔ/Δ* and *RaluΔ/Δ* MEFS were isolated from C57B1/6J mouse embryos (after at least four backcrosses to C57B/6J) at 13.5 days of gestation according to the method described in Michalska (Michalska, 2007).

**Cell culture, viruses and transfection**

Primary MEFS were cultured at 37°C in 3% O2 and 10% CO2. Primary MEFS were immortalised by infection with retrovirus pBABE-puro SV40 LT (Addgene) and selected in Puromycin at 2.5 µg/ml for 4 days. Immortalised MEFS were cultured in high glucose DMEM medium supplemented with 10% FBS and 2 mM L-glutamine at 37°C and 5% CO2.
immortalised MEFs with floxed alleles were infected overnight with either Adenovirus expressing only GFP (Ad-GFP) or Cre (Ad-Cre) Gene Transfer Vector Core) with 5 x 10^5 and 2.5 x 10^5 PFU at 50% confluence on a 175 cm^2 dish. 48 hours post-infection cells were trypsinised and, where required, 6 x 10^5 cells were first sorted by FACS before further cell culture. Prior to analysis of PLC activity or cell motility in Dunn chambers, the expression of proteins encoded by floxed alleles was analysed by western blotting.

Adenoviruses for transduction of PLC-ko MEFs with recombinant PLCs, described previously (Oestreich et al., 2007), were added in media containing 20% multiplicity of infection of each individual virus; prior to the infection 10^6 MEFs were let to adhere on a glass coverslip for 3 hours. Following protein expression analysis, cells were analysed in Dunn chambers.

Previously described FRET biosensors FLAIR (Hodgson et al., 2010) and LIBRA (Tanamura et al., 2004) were modified to incorporate the brighter and more photostable mTurquoise fluorophore (Goedhart et al., 2010) in place of CyPet or ECFP, respectively. To create a mTurquoise-Rac1 fusion construct a mTurquoise PCR fragment flanked with the restriction sites Nhel and BglII was subcloned into pCyPet-Rac1 digested with same restriction enzymes. The LIBRA probe consists of the N-terminal 20 amino acids from neuregulin (a membrane targeting signal) followed by the rat IP3, KD domain sandwiched between ECFP and EYFP. To produce mTurquoise-LIBRA a neuregulin-mTurquoise fusion fragment was created by overlap-extension of these two fragments and subcloned into the LIBRA vector, cut with Nhel and BspEI and lacking the neuregulin domain and ECFP. MEFs were transfection by electroporation using an Nucleofector II device (Lonza Group, Basel, Switzerland) with previously described plasmids encoding GFP-PLCs or GFP (Sorli et al., 2005). This was performed using Lonza Mouse/rat hepatocyte Nucleofector solution and seeded in glass-bottom 12 well glass bottom plates (MaTek Corporation, MA, USA) at a density of 10^4 cells per well. The cells were starved overnight in DMEM supplemented with 0.5% FBS prior to further analysis of cell localization.

Expression plasmids for the WT PLCc and its variants, plasmids encoding small GTPases as activators of PLC, were transfected into COS7 cells using Lipofectamine reagent (Invitrogen) were described previously (Bunney et al., 2006; Sorli et al., 2005).

**Analysis of protein expression, phosphorylation and activation status of small GTPases**

Protein expression and phosphorylation of different signalling proteins was analysed by western blotting using specific antibodies to PLCc (described in Bunney et al., 2006), PLCγ1 (Sigma), phospho-PLCγ1 (phospho-Tyr-783, Cell Signalling Technologies), PDGFR β (Pharmingen); ERK (phospho-Thr202/Tyr204), phospho-p42/p44 MAPK (Cell Signalling), PKB (Millipore), phospho-PKB (Cell Signalling), RhoA (Santa Cruz Biotechnology), RhoB and RhoC (Cell Signalling) and CDC42 (Santa Cruz Biotechnology). Antibody to α-actinin (Sigma) was used as a loading control.

Analysis of GTP-bound state of small GTPases was performed as described previously (Jones and Kathan, 2007). Briefly, cell lysates were incubated with GST-fusion binding proteins specific to GTP-bound forms, Raf-RBD for Raf, RapGDS-RBD for Rap, nPAK-RBD for Rac and BAP1-RBD GST for Ral. The protein present in the “pull-down” was subsequently analysed by western blotting using antibodies to K-Ras (Santa Cruz Biotechnology), Rap1 (BD Biosciences), Rac (Upstate), RalA (BD Transduction Laboratories) or RalB (Santa Cruz Biotechnology).

**Analysis of PLC activity**

Analysis of inositol phosphate formation in MEFs and COS7 cells was essentially as described previously (Sorli et al., 2005). Briefly, 24 hours after transfection of Cos7 cells and 24 hours after seeding MEFs, cells were labelled with 1.5 μM/ml myo-[3-3H]inositol (Perkin Elmer). For the treatment with c. butotalinum, C3 transferase, MEFs were transfected (by electroporation using an Nucleofector II) with 2 μg/ml of the C3 exoenzyme (Cytoskeleton) 24 hour before labelling. After a further 24 hours, the cells were incubated in 1.2 ml inositol-free DMEM, without serum, containing 20 mM LiCl with or without stimulation with indicated compounds for 1 hour. Concentrations were: 25 ng/ml PDGF-BB (Sigma); 2 μM LPA (Sigma); 1.3 U/ml Thrombin (Sigma); 50 nM Bombesin (Sigma); 100 ng/ml EGF (Sigma); 10 μM ATP (Sigma); 500 μM Carbachol (Sigma); 12 μM Epinephrine (Sigma); 100 μM Etiolothelin (Sigma); 50 ng/ml FGF (R&D Systems); 100 ng/ml VEGF (R&D Systems); 100 μM S-PmeOEP (BIOLOG Life Sciences Institute); 25 μM Isoprotoren (Sigma); 10 μg/ml TPA (Sigma). The cells were lysed and supernatants and pellets were separated for measurements of inositol phosphates and inositol lipids, respectively. PLC activity is expressed as the total inositol phosphates formed relative to the amount of [3H]inositol in the phospholipid pool. Mass measurements of Ins(1,4,5)P3 were performed using extracts from 2 x 10^5 cells, according to a protocol described in detail previously (Rodriguez et al., 2003). Data generation and analysis for transiently transfected COS7 cells was exactly as described in Sorli et al. (Sorli et al., 2005). Similarly, PLC activity measurements in MEFs, unless stated otherwise in figure legends, were from three independent experiments performed in duplicate; bars are the means of three data points, each defined by the average of two technical replicates. The error bars represent s.d.

**Dunn chamber analysis**

For the Dunn chamber assay 10^5 cells were seeded on a No. 2 glass coverslip and starved in DMEM supplemented with 0.5% FBS overnight. When applied 20 μM of BAPTA (Merck) or 390 nM of BIM (MERK) was added to the cells 15 min before and during the time of the chamber experiments. The coverslips were placed on the Dunn chamber (Zich et al., 1997) with a PDGF-BB concentration of 250 ng/ml in the outer annulus. Brightfield images of the cells were recorded in three positions around the bridge region under 4× magnification at 10 minutes intervals for 12 hours. For each image the cells were tracked using the ImageJ plugin Manual Tracking (Fabrice Cordelieres, Institut Curie, France.) Software was written in Matlab (Mathworks, MA, USA) to analyse the cell migration. Under low magnification the curvature of the bridge means that the direction of the gradient can vary substantially across the image. To address this issue the radial gradient was determined at three points on the bridge provided by the speed and direction of each cell was calculated relative to the local gradient using the cell track information. The Forward Migration (FMI) Index was calculated to quantification the directionality of the cells. The FMI is defined as the ratio of the sum of the displacement of a cell along the axes defined by the chemotactic gradient to the total path length of the cell. The mean angle of deviation from the chemotactic gradient was calculated to construct angular histograms of the direction of migration of the cell population relative to the chemotactic gradient. A hierarchical unbalanced analysis of variance (ANOVA) test was used to determine the significance of the differences between the FMI of different groups of Dunn chamber experiments (Zich et al., 1997). This test accounts for the potential variation between individual chamber experiments.

**Analysis using chemotactic gradients generated by perfusion**

Chemotaxis experiments were performed by continuously perfusing PDGF at a concentration of 0.5 ng/ml from a glass micropipette. Micropipettes were prepared from 0.7 μm borosilicate capillaries using a vertical needle puller (Narishige, Japan). This creates a steep PDGF concentration gradient in the region of the needle. The position of the micropipette was controlled using a three axis hydraulic micromanipulator (Narishige, Japan) and a nominal pressure of 420 hPa applied using KDS200 syringe pump (KD Scientific Inc., MA, USA). For FLAIR analysis, transfected cells were imaged on a Zeiss LSM 710 confocal scanning system mounted on an Axio Observer inverted microscope using a 40× oil immersion objective, NA 1.3. The microscope was equipped with an incubator maintained at 37°C. Fluorescence was excited at 405 nm while mTurquoise and YPet emission was simultaneously recorded between 465 and 500 nm and 525–620 nm, respectively. A FRET index was calculated as the ratio between the YPet and mTurquoise emission, as previously reported (Kraynov et al., 2000). YPet is inefficiently excited at 405 nm; cells transfected with YPet-PBD only showed no significant fluorescence above background levels in the acceptor channel when imaged under the same conditions. Control experiments were conducted to ensure there was no significant photobleaching over the time course at the power level used. Transfected cells on No. 1.5 glass coverslips were transferred to a custom-made open top slide holder. The cells were washed with PBS and the media replaced with Phenol-Red free Optimem (Gibco, NY, USA). Cells transfected with both mTurquoise-Rac1 and YPet-PBD were identified in the eyepiece; care was taken to select cells whose morphology was similar to that of untransfected cells. The micropipette was positioned in the “pull-down” was subsequently analysed by western blotting using antibodies to K-Ras (Santa Cruz Biotechnology), Rap1 (BD Biosciences), Rac (Upstate), RalA (BD Transduction Laboratories) or RalB (Santa Cruz Biotechnology).
Supplementary material available online at
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Fig. S1. Acute stimulation and chemotaxis of MEFs triggered by PDGF-BB. A) PLC responses in WT and PLCe KO MEFs. Inositol-phosphate analysis was performed on four different preparations of immortalized MEF populations (n=4); the data are from two independent experiments performed in duplicate, presented as means +/- SD.***, P < 0.001, ANOVA. B) Forward migration of WT and PLCe KO MEFs. Forward Migration Index was calculated for four different preparations of immortalized MEF populations (n=4) in five independent experiments. The comparison was performed using Dunn chambers. Data are shown as means +/- SD. ** P < 0.01, ANOVA. C) Analysis of gradient in the Dunn chamber. To quantify the gradient in the Dunn chamber Alexa-647 dye conjugated to 10,000MW dextran (similar molecular weight to PDGF-BB) was loaded in the outer annulus of the chamber and allowed to settle for 30 minutes. The fluorescence was monitored at 10 minute intervals over five hours. The fluorescence profile across the chamber (left, white line) is shown (right) at hour intervals. The gradient appears relatively constant across the chamber and as expected slowly reduces in magnitude over the time course as the PDGF-BB concentration in the two annuli begins to equilibrate. D) Analysis of gradient released by micropipette. Alexa-647-Dextran was loaded into a micropipette. The micropipette was positioned just above a coverslip and 420 hPa pressure applied. The fluorescence in the plane of the cell was monitored at 20 second intervals over 30 minutes. The fluorescence profile near the needle (left, white line) is shown. A stable gradient is established after one minute and appears stable over 30 minutes.
**Fig. S2.** A) PLC activity was determined in the WT (WT), PLCε KO (KO) and MEFs expressing PLCε RAm variant (KI) and in MEFs deficient in H- and N- Ras with further depletion of KRas flox/flox following infection with Ad-Cre (Rasless, RL). Immortalized cell populations were used. The PLC activity was measured in non-stimulated cells (basal) and cells stimulated by PDGF-BB (PDGF); **P < 0.01, t-test. Comparable PLC activity in WT and KI MEFs in this experiment is consistent with data from three independent preparations of immortalized cell populations (n=3, P<0.001, ANOVA). Expression of KRas analyzed by Western blotting (left panel). Forward Migration Index and Mean Speed of the WT (WT) and Rasless (RL) MEFs were calculated using Dunn chamber. *** P<0.001, ANOVA. B) PLC activity was measured in RhoA flox/flox population of MEFs following immortalization (RhoA) and subsequent expression of GFP alone (RhoA GFP) or Cre (RhoA Cre). *** P<0.001, t-test. Expression of indicated small GTPases was analyzed by Western blotting (left panel). Forward Migration Index of RhoA GFP and RhoA Cre MEFs was calculated using Dunn chamber (right panel).
Fig. S3. A) Basal and PDGF-BB stimulated PLC activity in immortalized populations of WT and PLCε KO MEF treated with either 200 nM of non-targeting si RNA (control, C) or siRNAs specifically targeting both, RalA and RalB (Ral). B) Expression of RalA and RalB in cells described in A was analyzed by Western blotting. C) Basal and PDGF-BB stimulated PLC activity in the immortalized populations of WT and PLCε KO MEFs; control cells and cells pretreated with *C. botulinum* C3 exoenzyme (+C3) were analyzed (left panel). D) Analysis of total and GTP-bound RalA in WT MEFs before and after treatment with C3 exoenzyme and/or PDGF-BB as indicated. The Western blotting was performed using total cell lysates and pull-downs obtained by GST-RalBP-1-RBD (left), followed by quantification (right). Differences indicated in the graphs correspond to *P*<0.05; **P*<0.01, t-test.