A new link between the c-Abl tyrosine kinase and phosphoinositide signalling through PLC-γ1

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The c-Abl tyrosine (Tyr) kinase is activated after platelet-derived-growth factor receptor (PDGFR) stimulation in a manner that is partially dependent on Src kinase activity. However, the activity of Src kinases alone is not sufficient for activation of c-Abl by PDGFR. Here we show that functional phospholipase C-γ1 (PLC-γ1) is required for c-Abl activation by PDGFR. Decreasing cellular levels of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) by PLC-γ1-mediated hydrolysis or dephosphorylation by an inositol polyphosphate 5-phosphatase (Inp54) results in increased Abl kinase activity. c-Abl functions downstream of PLC-γ1, as expression of kinase-inactive c-Abl blocks PLC-γ1-induced chemotaxis towards PDGF-BB. PLC-γ1 and c-Abl form a complex in cells that is enhanced by PDGF stimulation. After activation, c-Abl phosphorylates PLC-γ1 and negatively modulates its function *in vivo*. These findings uncover a newly discovered functional interdependence between non-receptor Tyr kinase and lipid signalling pathways.

The activation mechanism of the c-Abl Tyr kinase has remained elusive, despite its identification more than 20 years ago. c-Abl is located in multiple cellular compartments and its activity is modulated by various stimuli[1–3]. The membrane pool of c-Abl is activated downstream of the PDGFR in a manner partially dependent on the Src family kinases[4] and c-Abl is important in membrane ruffling downstream of PDGF[4]. Here, we uncover a novel component of the signal transduction pathway that results in c-Abl activation downstream of the PDGFR.

Two PDGFRs (α and β) bind to four PDGF growth factors (A–D). A major downstream effector of the activated PDGFRs is PLCγ, and PLC-γ1 is the major isoform expressed in fibroblasts[5]. Activated PDGFRs phosphorylate PLC-γ1, resulting in its subsequent activation[5]. Once activated, PLC-γ1 hydrolyses PtdIns(4,5)P_2 to produce the second messengers inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). PLC-γ1 contains a pleckstrin homology (PH) domain, split PH and catalytic lipase domains (X and Y), which are separated by two SH2 (Src-homology domain-2) domains (amino- and carboxy-terminal) and an SH3 domain[5]. PLC-γ1 is directly phosphorylated by the PDGFR at three sites (Tyr 771, Tyr 783 and Tyr 1254)[6]. Phosphorylation of Tyr 783 is essential for activation of PLC-γ1 by PDGF[6]. PtdIns(3,4,5)P_3, the product of phosphatidylinositol-3 kinase (PI-(3)K activation, is required for full activation of PLC-γ1 in some systems[5]. Phosphorylation of Tyr 771 may negatively regulate PLC-γ1 (ref. 6) and protein kinase C (PKC) may downregulate PLC-γ1 through serine phosphorylation *in vitro*[7]. However, the mechanisms that result in inactivation of PLC-γ1 *in vivo* after PDGF stimulation remain to be defined.

PLC-γ is important in directed cell migration[8] and wound-healing[9], as well as in neurite outgrowth[10], membrane ruffling[11], macropinocytosis[12] and possibly mitogenesis[5]. The substrate and products of PLC-γ1 are critically important in cytoskeletal reorganization, cell adhesion and migration[13,14]. Similarly, the c-Abl Tyr kinase and the related Arg tyrosine kinase (Abl2) are linked to cytoskeletal reorganization[4,15–17], cell migration (wound healing, chemotaxis)[18,19] and neurite outgrowth[20,21]. Similar to PLC-γ1, c-Abl localizes to membrane ruffles and translocates to the plasma membrane after growth factor stimulation[19,22]. Here, we uncover a bidirectional link between PLC-γ1 and the c-Abl Tyr kinase.

Results

PLC-γ1 activity is required for activation of c-Abl by PDGF. To determine the mechanism of c-Abl activation by PDGF, we used Ph cells (a murine 3T3 line carrying a deletion of the endogenous PDGFR-α) to express various forms of a chimeric receptor containing the extracellular domain of the PDGFR-α and the intracellular domain of the PDGFR-β. These chimeric receptors are activated by PDGF-AA, but stimulate PDGFR-β signalling events[23]. Ph cells expressing wild-type, kinase-dead and receptors with various Tyr to Phe mutations (which disrupt the binding of specific SH2-containing proteins in the intracellular domain) were generated[23]. F72/74 lacks the Src-binding sites, whereas the F5 mutant retains the Src-binding sites but lacks the binding sites for SHP-2, PI(3)K, Ras-GTPase-activating proteins (Ras-GAP) and PLC-γ1 (Fig. 1a). After serum withdrawal, cells were stimulated with PDGF-AA and endogenous c-Abl activity was measured by an *in vitro* kinase assay.

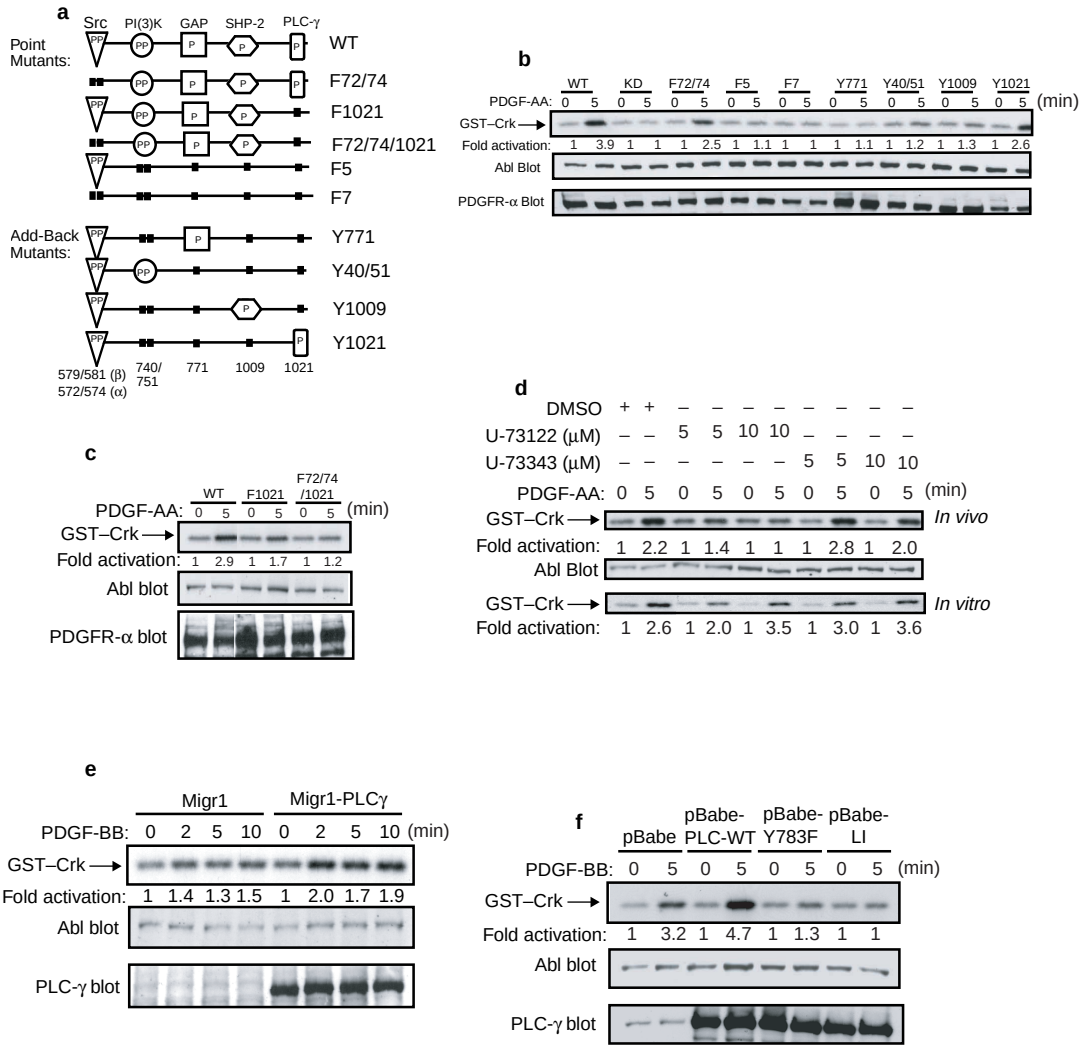

Figure 1 **c-Abl activation by PDGF is dependent on PLC-γ1 activity.** **a**, Schematic diagram of wild-type and mutant chimeric α/β PDGFR intracellular domains. P denotes phosphotyrosine and filled squares denote Tyr that have been mutated to Phe (F). Top: point mutations. Bottom: add-back mutants contain Phe in place of Tyr at the five sites indicated (F5), and have single add-back phosphotyrosines. **b**, c-Abl kinase activity after PDGF stimulation in Ph cells expressing chimeric α/β PDGFRs. c-Abl protein was immunoprecipitated with Pex4 antibody, and immunoprecipitates were assayed for c-Abl kinase activity using GST–Crk as a substrate. KD denotes a kinase-dead receptor. The average fold activation from three experiments was 3.5 ± 0.4, 1.9 ± 0.6, and 2.5 ± 0.1 fold for cells expressing wild-type, F72/74 or Y1021 receptors, respectively. **c**, c-Abl kinase activity in Ph cells expressing PDGFRs lacking the PLC-γ1-binding site (F1021) alone or lacking both the PLC-γ1 and Src-binding sites (F72/74/1021). The average activation of c-Abl from three independent experiments was 3.1 ± 0.5, 1.7 ± 0.1 and 1.4 ± 0.3 fold in wild-type, F1021 or F72/74/1021-expressing cells, respectively. **d**, Top: Ph cells expressing a wild-type chimeric receptor were serum-starved, treated for 10 min with U-73122, its inactive analogue U-73343, or vehicle (DMSO) at the indicated concentrations, and stimulated with PDGF. The average fold activation of c-Abl in three independent experiments was 2.4 ± 0.2 or 1.3 ± 0.1 fold after pretreatment with DMSO or U-73122, respectively. U-73122, U-73343, or vehicle were incubated in the c-Abl *in vitro* kinase assay at the indicated concentrations (bottom). **e**, c-Abl activity in PLC-γ1-null mouse embryo fibroblasts infected with vector (Migr1), or with PLC-γ1 (Migr1–PLC-γ1). c-Abl was immunoprecipitated with AB-3 antibody. Average c-Abl activation in three experiments was 1.3 ± 0.1 and 1.4 ± 0.2 fold in cells expressing Migr1 5 and 10 min after PDGF stimulation respectively, whereas in cells expressing Migr1–PLC-γ1, c-Abl was activated 2.2 ± 0.4 and 2.0 ± 0.1 fold at the same times. **f**, NIH3T3 cells were infected with retroviruses containing either vector (pBabe), PLC-γ1 wild-type, or PLC-γ1 dominant-negative mutants, PLC-γ1^{Y783F} and pBabe–PLC-LI (lipase-inactive). Cell lysates were then analysed for c-Abl kinase activity using K12 antibody. The average activation in the three experiments was 2.6 ± 0.9, 4.2 ± 0.8, 1.3 ± 0.0, and 1.0 ± 0.0 fold in cells expressing vector, wild-type PLC, PLC-γ1^{Y783F} or PLC–LI respectively.

c-Abl was activated in cells expressing the wild-type PDGFR, but not in cells expressing a kinase-dead version (Fig. 1b). As reported previously, mutation of the Src-binding sites (F72/74) reduced activation of c-Abl by PDGF (Fig. 1b)[4]. Unexpectedly, activation of c-Abl by PDGF was completely abolished in cells expressing the F5 receptor which retains the Src-binding sites (Fig. 1b). These findings indicate that Src kinase activity is necessary, but not sufficient, for full activation of c-Abl by PDGF and that at least one of the molecules unable to bind to this receptor (SHP-2, PI(3)K, Ras-GAP or PLC-γ1) is required for activation of c-Abl.

To identify the molecules involved in c-Abl activation, we used a series of receptor mutants that add back each Tyr mutated on the F5 receptor (Fig. 1a). Addition of Tyr 771, Y740/Y751 and Y1009 had little effect on activation of c-Abl by PDGF (Fig. 1b). In contrast, addition of Tyr 1021 (which binds PLC-γ1) partially restored activation of c-Abl by PDGF (Fig. 1b). To confirm that Tyr 1021 is

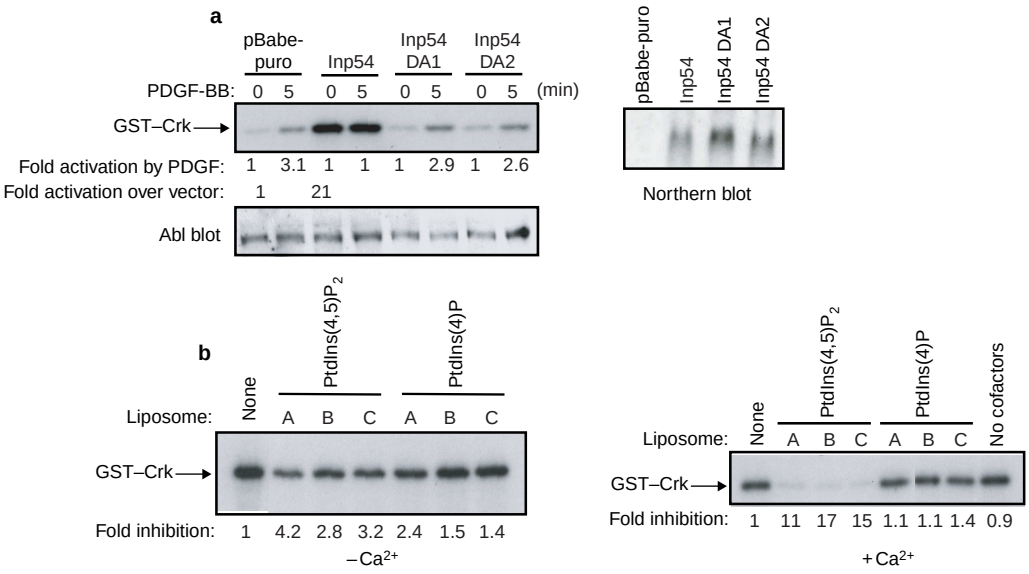

Figure 2 **PtdIns(4,5)P_2 inhibits c-Abl kinase activity. a**, NIH3T3 cells were infected with retroviruses containing vector (pBabe–puro), Inp54, or phosphatase-inactive mutants (D280A) (DA1, DA2), and c-Abl kinase activity was examined *in vitro* after immunoprecipitation with K12 antibody (top). The average activation of c-Abl after expression of the Inp54 was 22 ± 0.2 fold. Expression of the active and inactive phosphatases was determined by northern blotting (right) using the entire phosphatase cDNA as a probe. **b**, An equal amount of lysate from 293T cells over-expressing c-Abl was immunoprecipitated with the K12 antibody and the immuno-precipitates were incubated with liposomes containing PIPs, and subjected to *in vitro* kinase assays. Three independent sets of liposomes were utilized (A–C) using different times of sonication. Kinase reactions were carried out in the absence (left) or presence (right) of calcium chloride.

required for c-Abl activation, Tyr 1021was mutated to Phe in the context of the wild-type and F72/74 receptors. Cells expressing the F1021 receptor displayed reduced activation of c-Abl by PDGF and cells expressing the F72/74/1021 receptor had even lower levels of c-Abl activation. This suggests that activation of c-Abl by PDGF requires both the Src and PLC-γ1 binding sites (Fig. 1c). The dependence of PLC-γ1 on PI(3)K activation[5] may explain why the PDGFR Tyr 1021 add-back only partially restores c-Abl kinase activity (Fig. 1b), as the PI(3)K binding sites were absent in this mutant.

To test whether PLC-γ1 functions upstream of c-Abl in a PDGF signalling pathway, Ph cells containing a wild-type chimeric receptor were pretreated with a pharmacological inhibitor of PLC-γ (U73122), its inactive analogue (U73343) or vehicle dimethyl sulphoxide (DMSO). Activation of c-Abl by PDGF was reduced in a dose-dependent manner after pretreatment with U-73122, but not with vehicle or the inactive analogue (Fig. 1d, top). Activity of the c-Abl kinase was not directly affected by the addition of U73122 *in vitro* (Fig. 1d, bottom), suggesting that inhibition of PLC-γ1 in the cell blocked the activation of c-Abl by PDGF.

To test further whether PLC-γ1 is required for activation of c-Abl by PDGF, we compared *PLC-γ1*$^{-/-}$ fibroblasts[24] to these cells reconstituted with wild-type PLC-γ1. Activation of c-Abl in response to PDGF was reduced in *PLC-γ1* null fibroblasts when compared with cells reconstituted with PLC-γ1 (Fig. 1e). Blocking endogenous PLC-γ1 activity with the PLC-γ1 dominant-negative mutants PLC-Y783F (which is not activated by PDGF; ref. 6) and a lipase-inactive (LI) mutant (which binds to the PDGFR and becomes Tyr phosphorylated but lacks lipase activity[25]) inhibited PDGF induced c-Abl activation (Fig. 1f). In contrast, expression of wild-type PLC-γ1 increased the c-Abl activation by PDGF (Fig. 1f). These data show that PLC-γ1 activity is required for endogenous activation of c-Abl by PDGF and that its overexpression can potentiate c-Abl activation.

Mechanism of c-Abl activation. Activation of c-Abl downstream of PLC-γ1 could occur through one of two mechanisms. PLC-γ1 activation produces $InsP_3$ and calcium, which could alter the activity of c-Abl. Alternatively, depletion of the PLC-γ1 substrate, PtdIns(4,5)P_2, may affect c-Abl activity. Neither calcium nor inositol phosphates altered the activity of c-Abl (Fig. 2b; data not shown). We next tested whether depleting cellular PtdIns(4,5)P_2 affects c-Abl kinase activity *in vivo* by expressing a yeast Inp54 highly specific for PtdIns(4,5)P_2 (ref. 26). This phosphatase functions in mammalian cells, and a constitutively membrane-bound form produces lowered cellular levels of PtdIns(4,5)P_2 (ref. 27). We expressed the native active phosphatase, rather than the form targeted to the membrane, as the membrane-targeted form induces cell rounding whereas the native form does not alter cellular morphology. NIH3T3 cells expressing the active phosphatase had markedly elevated basal c-Abl kinase activity (over 20-fold) when compared with cells expressing the vector or a phosphatase-inactive (D280A) mutant (Fig. 2a). Activity of the c-Abl kinase was stimulated by PDGF-BB in cells infected with vector or the inactive phosphatase, but in cells expressing the active phosphatase, the basal c-Abl kinase activity was significantly elevated and could not be further increased by the addition of PDGF (Fig. 2a). These results suggest that PtdIns(4,5)P_2, or a protein that binds to PtdIns(4,5)P_2, might function to inhibit c-Abl kinase activity *in vivo*. The ability of both PLC-γ1 and Inp54 expression to activate c-Abl suggests that PtdIns(4,5)P_2, rather than the products of either enzymatic reaction, affects c-Abl kinase activity.

To determine if PtdIns(4,5)P_2 directly binds to c-Abl, various fragments of c-Abl fused to glutathione *S*-transferase (GST) were used in a binding assay with immobilized phosphatidyl inositol lipids (PIPs). The c-Abl SH2–SH1 domain bound to immobilized PtdIns(4,5)P_2 in the absence or presence of calcium (data not shown). In contrast, the c-Abl SH2 and SH3 domains alone did not bind to PtdIns(4,5)P_2 (data not shown). As PtdIns(4,5)P_2 was able to bind to the Abl kinase domain, we examined whether PtdIns(4,5)P_2 directly inhibited c-Abl kinase activity *in vitro*. In the absence of calcium, PtdIns(4,5)P_2 liposomes partially inhibited c-Abl kinase activity *in vitro*, whereas PtdIns(4)P had a lesser effect

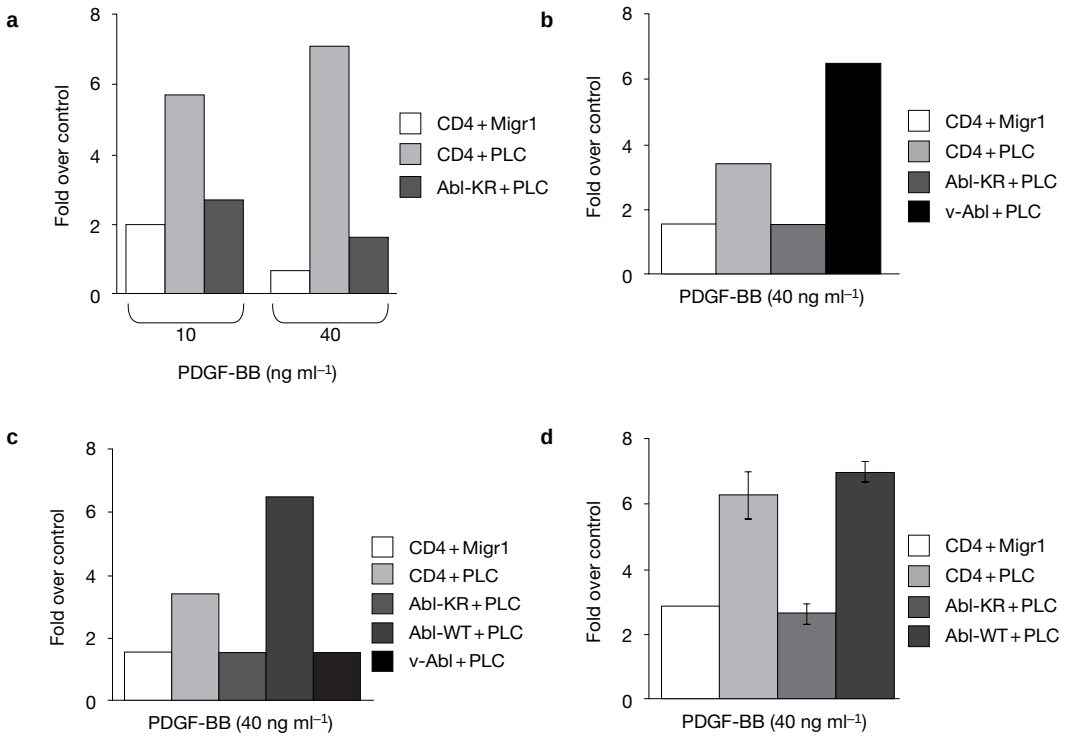

Figure 3 **c-Abl and PLC-γ1 function together to regulate chemotaxis.** PAE cells were transfected with the GFP bicistronic vector, Migr1 or Migr1–PLC together with the Mig-CD4 vector, or Mig–CD4 expressing either wild-type c-Abl, kinase-inactive c-Abl or v-Abl. Chemotaxis towards PDGF-BB (10 or 40 ng ml^{-1}) was measured in Transwell assays. The number of transfected cells (GFP-positive) that migrated in wells containing PDGF-BB were compared with the number of GFP-positive cells that migrated in the absence of PDGF-BB. To ascertain that an equivalent percentage of cells in each experimental condition expressed PLC-γ1, cells were plated on coverslips and counterstained with DAPI to determine GFP transfection efficiency. PLC-γ1 transfection efficiency was equivalent in the absence or presence of c-Abl constructs. Expression of Abl constructs was confirmed by western blotting (data not shown). Four independent experiments **(a–d)** are shown. The experiment in **d** was performed in duplicate.

(Fig. 2b, left). Calcium alters the specificity of lipids for kinases such as PKC[28]. Therefore, we examined the effect of adding calcium to the c-Abl kinase assay in the presence and absence of phospholipids. Addition of calcium alone had no effect on the kinase activity of c-Abl (Fig. 2b, right). In the presence of 400–800 μM calcium, PtdIns(4,5)P_2 markedly inhibited the activity of c-Abl kinase (Fig. 2b, right) and the inhibitory effect was highly specific for PtdIns(4,5)P_2, as no effect was observed for PtdIns(4)P under the same conditions. Under conditions where lower levels of calcium (2 μM) were used, PtdIns(4,5)P_2 did not inhibit the activity of the c-Abl kinase *in vitro* (data not shown). These data suggest that PtdIns(4,5)P_2 may indirectly inhibit c-Abl and PtdIns(4,5)P_2 may recruit a molecule that inhibits activity of the c-Abl kinase *in vivo*. Alternatively, higher concentrations of calcium may be necessary to observe inhibition of the c-Abl kinase activity *in vitro*, and therefore PtdIns(4,5)P_2 cannot be ruled out as the inhibitor of c-Abl.

c-Abl functions downstream of PLC-γ1 in PDGF-induced chemotaxis. To determine whether activation of c-Abl downstream of PLC-γ1 after PDGF stimulation affects known PLC-γ1-mediated cellular responses, we analysed whether c-Abl is important in PLC-γ-dependent chemotaxis towards PDGF-BB. Overexpression of PLC-γ1 increased chemotaxis of porcine aortic endothelial (PAE) cells expressing a PDGFR-β, as previously reported[29] (Fig. 3a–d). Expression of a kinase-inactive form of c-Abl blocked the migration induced by PLC-γ1 (Fig. 3a–d). Expression of wild-type c-Abl had no effect on chemotaxis, whereas expression of a constitutively active form of Abl (v-Abl) potentiated PLC-γ1-mediated chemotaxis in response to PDGF (Fig. 3b, c). Thus, c-Abl functions downstream of PLC-γ1 in the regulation of cellular migration toward PDGF.

c-Abl and PLC-γ1 interact. As PLC-γ1 is required for activation of c-Abl by PDGF, we examined whether the two proteins interact. Both the amino- and carboxy terminal SH2 domains from PLC-γ1 strongly interacted with overexpressed c-Abl (Fig. 4a, left). Overexpression of wild-type c-Abl to high levels (20–50-fold) in 293T cells results in enhanced Tyr phosphorylation of c-Abl and activation of its kinase activity[30]. Mutation of the Arg residue within the Phe-Leu-Val-Asn-Glu-Ser (FLVRES) motif in the PLC-γ1 C-SH2 domain eliminated binding to c-Abl, suggesting that the interaction involves phosphotyrosine binding (Fig. 4a, left). c-Abl contains SH3, SH2 and kinase (SH1) domains and the C terminus contains filament-(F) and globular-(G) actin-binding domains. The c-Abl SH2–SH3 GST-fusion fragment strongly interacted with AU1-tagged PLC-γ1, whereas the SH2 domain alone showed a weaker interaction (Fig. 4a, right) that was evident after longer exposure (data not shown). The SH3 domain alone did not interact and the Abl C terminus interacted weakly with overexpressed PLC-γ1 (Fig. 4a, right). Thus, the isolated SH2 domains of PLC-γ1 interact with c-Abl and the combined c-Abl SH2–SH3 domains interact with PLC-γ1 after overexpression in 293T cells.

We next examined whether a c-Abl–PLC-γ1 complex could be detected in intact cells. A c-Abl and PLC-γ1 complex was formed before and after PDGF stimulation (Fig. 4b, right panel). PDGF stimulation increased the amount of c-Abl in the complex when low levels of a cytoplasmic form of c-Abl was introduced into NIH3T3 cells (Fig. 4b, left). To further characterize the inducibility of the interaction, we examined the binding of various c-Abl fragments to endogenous PLC-γ1 in NIH3T3 cells before and after PDGF stimulation. Both the SH2 and SH2–SH3 domains of c-Abl

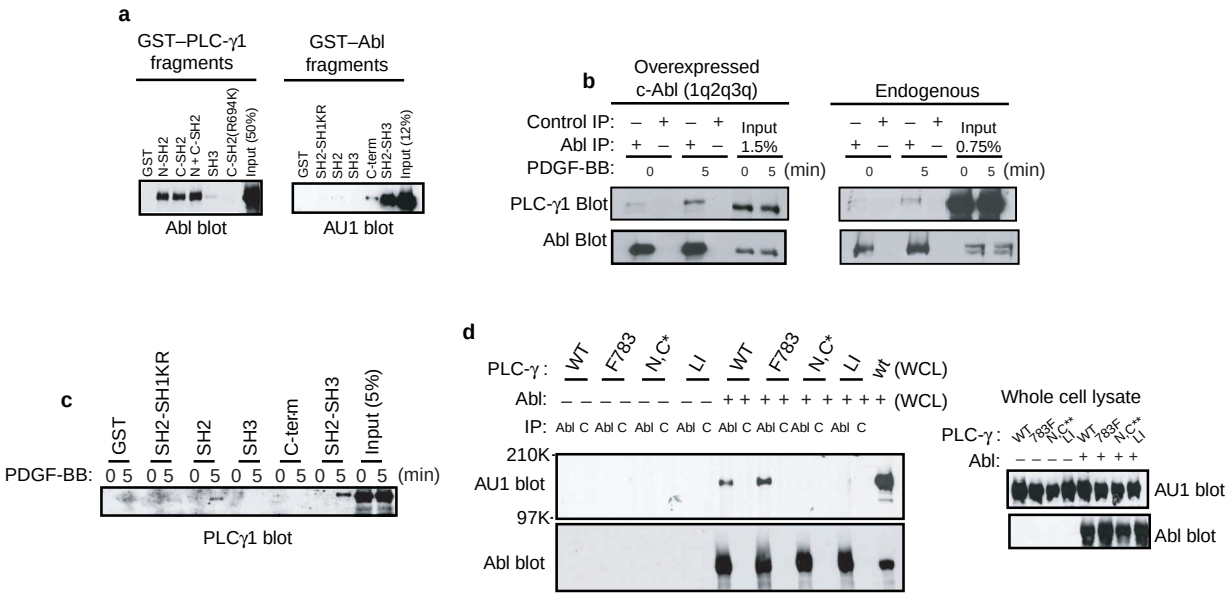

Figure 4 **PLC-γ1 and c-Abl form a complex. a**, c-Abl was overexpressed in 293T cells, and 1 μg of GST fusion proteins containing various domains of PLC-γ1 or GST alone were used in a pulldown assay (left). Coprecipitating c-Abl protein was detected by blotting with 8E9 antibody (left). AU1-tagged PLC-γ1 was overexpressed in 293T cells, and GST fusions of c-Abl or GST alone were used to pull down PLC-γ1 (right). Equal loading of all GST proteins was confirmed by Ponceau staining (data not shown). **b**, c-Abl lacking nuclear localization signals (1q2q3q) was expressed in NIH3T3 cells by retroviral infection. Cellular lysates were incubated with anti-c-Abl or a polyclonal control antibody, and the immunoprecipitates were analysed by blotting with anti-PLC-γ1 (top). The blot was stripped and reprobed for c-Abl (bottom). Endogenous c-Abl was immunoprecipitated from NIH3T3 cells and the immunoprecipitates were blotted as above (right). **c**, GST fusion proteins containing c-Abl fragments were used to pull down endogenous PLC-γ1 from starved (0) or PDGF-stimulated (5 min) NIH3T3 cells. **d**, AU1-tagged wild-type and mutant forms of PLC-γ1 were expressed alone or together with c-Abl in 293T cells. Lysates were incubated with anti-Abl K12 antibody (Abl) or a control polyclonal antibody (C) and the immunoprecipitates were blotted with anti-AU1 antibody (upper panel). The blot was stripped and reprobed with anti-Abl antibody (bottom). Whole cell lysates were blotted for c-Abl and PLC-γ1 expression (right). N,C* is a double SH2 domain PLC-γ1 mutant; LI is a lipase-inactive mutant.

interacted with endogenous PLC-γ1, but only after stimulation with PDGF (Fig. 4c). The preformed complex identified before stimulation (Fig. 4b) may represent pools of PLC-γ1 and c-Abl that do not localize to the plasma membrane, and therefore may not be affected by growth factor stimulation. The c-Abl SH3 and C-terminal domains did not bind to PLC-γ1 in starved or PDGF-stimulated cells (Fig. 4c). These results suggest that the interaction of c-Abl with the endogenous PLC-γ1 protein is primarily mediated by the combined c-Abl SH3–SH2 domains.

Mutant or wild-type forms of PLC-γ1 were overexpressed with c-Abl in 293T cells to identify the PLC-γ1 domains that mediate binding to c-Abl. PLC-γ1 co-immunoprecipitated with c-Abl when the two proteins were overexpressed (Fig. 4d, top). Mutation of the Arg in the FLVRES motifs (N,C*, where * indicates mutation) in both of the PLC-γ1 SH2 domains disrupted binding to c-Abl (Fig. 4d). Mutation of the N-SH2 domain alone had little effect on the co-immunoprecipitation of PLC-γ1 with c-Abl, whereas mutation of the C-SH2 (C*) domain greatly reduced the interaction (data not shown). Therefore, the C-SH2 domain provides a greater contribution to the binding. A lipase-inactive mutant of PLC-γ1 did not interact with c-Abl (Fig. 4d). The lipase-inactive PLC-γ1 mutant co-immunoprecipitates with the activated PDGFR to the same levels as wild-type PLC-γ1, demonstrating that the SH2 domains of the mutant are functional (data not shown). In addition, this lipase-inactive mutant retains the ability to induce DNA synthesis[25], suggesting that the protein is folded properly. These findings suggest that PLC-γ1 lipase activity may be required for the interaction with c-Abl.

c-Abl phosphorylates PLC-γ1. As there is an interaction between c-Abl and PLC-γ1, we examined whether PLC-γ1 is a substrate for c-Abl *in vivo*. In the presence of overexpressed, activated c-Abl, PLC-γ1 became heavily Tyr phosphorylated (Fig. 5a, left). Tyr phosphorylation of PLC-γ1 was abolished when both SH2 domains of PLC-γ1 were mutated (Fig. 5a). Mutation of the N-SH2 domain alone resulted in only a slight reduction in the level of phosphotyrosine, whereas mutation of the C-SH2 domain or the lipase domain markedly reduced phosphotyrosine levels (Fig. 5a). These results are consistent with previous findings (Fig. 4d, data not shown) and demonstrate that mutants of PLC-γ1 that cannot bind to c-Abl have reduced Abl-induced Tyr phosphorylation.

The PLC-γ1 Tyr phosphorylation sites induced by c-Abl *in vivo* were mapped by mass spectrometry. Three PLC-γ1 peptides were identified that contained phosphorylated Tyr (Fig. 5b). These include a peptide containing Tyr 771 and Tyr 775, which was phosphorylated at one site, and peptides containing Tyr 572 and Tyr 1003. The c-Abl kinase phosphorylated PLC-γ1 fragments fused to glutathione *S*-transferase (GST) *in vitro* (Fig. 6a). The sites phosphorylated by c-Abl on the N+C-SH2–SH3 and the C-SH2 fragments of PLC-γ1 were mapped by mass spectrometry. Tyr 771 was the most abundant (63%) *in vitro* Tyr phosphorylated site, followed by Tyr 783 (44%) and Tyr 703 (27%) (Fig. 6b). In contrast to the *in vitro* results, Tyr 783 phosphorylation by c-Abl was not detected *in vivo* (Fig. 5b, top). A highly sensitive targeted ion tandem mass spectra (TIMM) analysis[31] demonstrated that the phosphorylated peptide containing Tyr 783 was not detected in cells co-expressing c-Abl, although the unphosphorylated peptide was easily isolated (data not shown). To test which sites identified by mass spectrometry were true c-Abl *in vivo* phosphorylation sites, we co-expressed PLC-γ1 mutants containing Tyr to Phe mutations with c-Abl and their Tyr phosphorylation status was assessed after immunoprecipitation. Mutation of Tyr 1003 or Tyr 771 to Phe reduced the phosphorylation of PLC-γ1 by c-Abl *in vivo* (Fig. 5b, bottom). Mutation

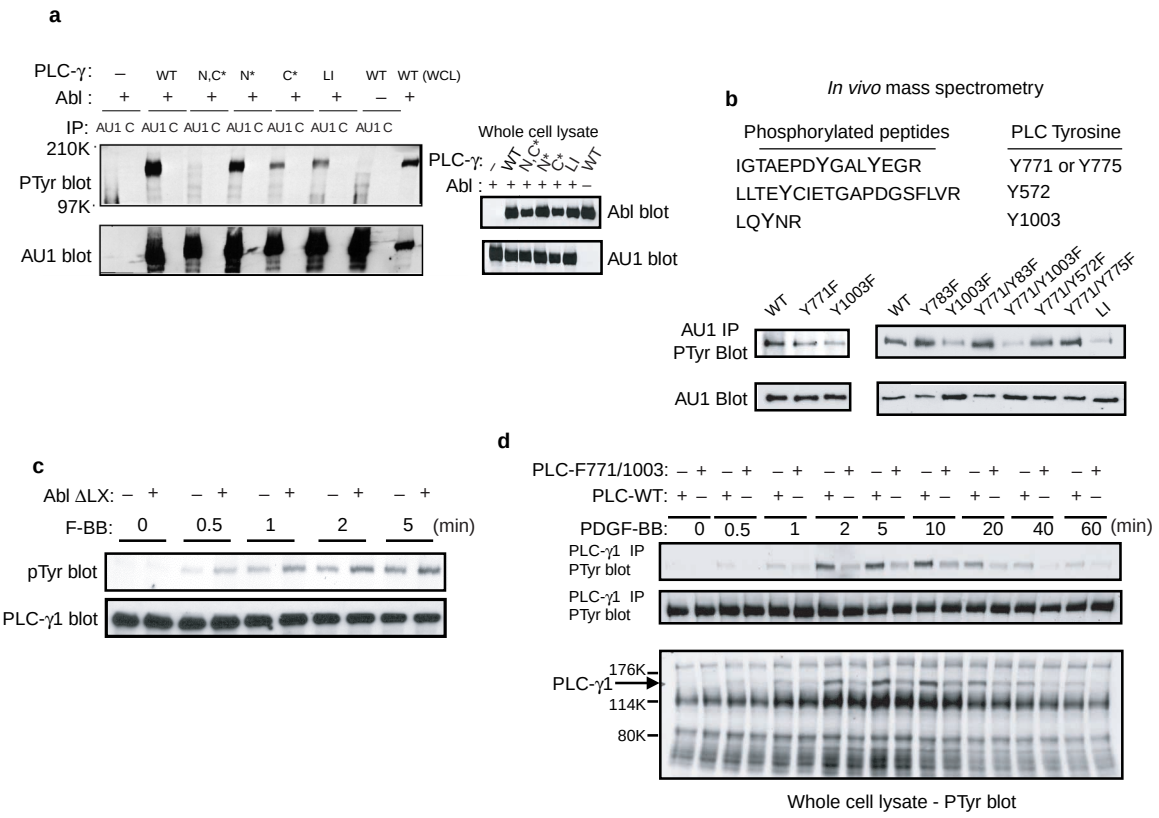

Figure 5 **c-Abl induces Tyr phosphorylation of PLC-γ1 *in vivo*. a**, c-Abl was co-expressed with wild-type or mutant PLC-γ1 in 293T cells. Exogenous PLC-γ1 was immunoprecipitated with α-AU1 antibody and the immunoprecipitates were probed with α-phosphotyrosine antibodies (4G10/PY99; top). The blot was stripped and reprobed for PLC-γ1 (AU1; bottom). **b**, PLC-γ1 was coexpressed with c-Abl in 293T cells and Tyr phosphorylation sites in the immunoprecipitated PLC-γ1 were identified by mass spectrometry (top). **b**, PLC-γ1 mutants were coexpressed with c-Abl in 293T cells, and PLC-γ1 phosphorylation was assessed as in **a** (bottom). The blot was stripped and reprobed with AU1 (bottom). **c,** NIH3T3 cells were infected with vector (–) or c-Abl-ΔLX (+). Endogenous PLC-γ1 was immunoprecipitated from starved and PDGF-stimulated cells, and the immunoprecipitates were blotted with anti-phosphotyrosine antibody (upper panel). The blot was stripped and reprobed for PLC-γ1 (lower panel). **d,** Wild-type or mutant PLC-γ1 was expressed in PLC-γ1-null cells by retroviral infection. Cells were starved and stimulated with PDGF, PLC-γ1 was immunoprecipitated with anti-PLC-γ1 antibody and the immunoprecipitates were probed with anti-phosphotyrosine antibodies (top). The blot was stripped and probed for PLC-γ1 (middle). Overall Tyr phosphorylation was assessed by blotting whole cell lysates with antiphosphotyrosine antibodies (bottom).

of both Tyr 771 and Tyr 1003 significantly reduced the c-Abl-induced Tyr phosphorylation of PLC-γ1 to levels similar to that observed for the lipase-inactive mutant (Fig. 5b). These findings demonstrate that c-Abl phosphorylates PLC-γ1 *in vivo* predominantly at Tyr 771 and Tyr 1003.

To determine whether c-Abl phosphorylates PLC-γ1 after PDGF stimulation, we examined whether increasing kinase activity of c-Abl enhanced PDGF-induced PLC-γ1 Tyr phosphorylation. NIH3T3 cells expressing a weakly-activated form of c-Abl containing a deletion of the C terminus[32] (c-Abl-ΔLX) had increased phosphorylation of endogenous PLC-γ1 in response to PDGF (Fig. 5c). To determine whether PLC-γ1 Tyr 771 and Tyr 1003 are phosphorylated in response to PDGF stimulation, we reconstituted PLC-γ1 null cells with wild type or Y771F/Y1003F form of PLC-γ1, where both Tyr are mutated to Phe. Phosphorylation of the immunoprecipitated PLC-γ1 771/1003F protein was significantly reduced in response to PDGF when compared with wild-type PLC-γ1 (Fig. 5d). These results demonstrate that Tyr 771 and Tyr 1003 are targets of phosphorylation downstream of PDGF stimulation.

Functional consequence of PLC-γ1 phosphorylation by c-Abl. To determine whether phosphorylation of PLC-γ1 by c-Abl affected activity of PLC-γ1, we co-expressed c-Abl and PLC-γ1 in 293T cells and assessed PLC-γ1 activity by analysis of inositol phosphates using high pressure liquid chromatography (HPLC). Overexpression of PLC-γ1 (but not a lipase-inactive PLC-γ1 protein) induced a two to threefold increase in activity that was reflected by increased levels of $Ins(1,4,5)P_3$ (Fig. 7a). Overexpression of wild-type or kinase-inactive (KR) c-Abl alone did not change $Ins(1,4,5)P_3$ levels (Fig. 7a). Co-expression of PLC-γ1 with wild-type c-Abl resulted in decreased PLC-γ1 activity, as measured by a consistent decrease in $Ins(1,4,5)P_3$ (Fig. 7a). This effect was not observed when a kinase-inactive form of c-Abl was co-expressed with PLC-γ1. Therefore, phosphorylation of PLC-γ1 by c-Abl may serve as a feedback mechanism to turn off PLC-γ1 activity.

To determine whether c-Abl downregulates PLC-γ1 in response to PDGF, we compared $InsP_3$ production after PDGF stimulation of quiescent mouse embryonic fibroblasts (MEFs) lacking Arg and c-Abl with that observed in the null MEFs reconstituted with c-Abl and Arg. Lithium chloride is used to trap downstream metabolites of $Ins(1,4,5)P_3$, which is short-lived after PDGF stimulation. In response to PDGF stimulation, $Ins(1,4,5)P_3$ is rapidly converted to $Ins(1,3,4,5)P_4$ by phosphorylation and this product in turn is converted to $Ins(1,3,4)P_3$. Lithium chloride treatment inhibits the 1-phosphatase that dephosphorylates $Ins(1,3,4)P_3$, producing a large accumulation of $Ins(1,3,4)P_3$ and thereby allowing indirect measurement of $Ins(1,4,5)P_3$ production by measuring $Ins(1,3,4)P_3$. To examine the downregulation of PLC-γ1 after PDGF stimulation, lithium chloride was added at various times after PDGF stimulation.

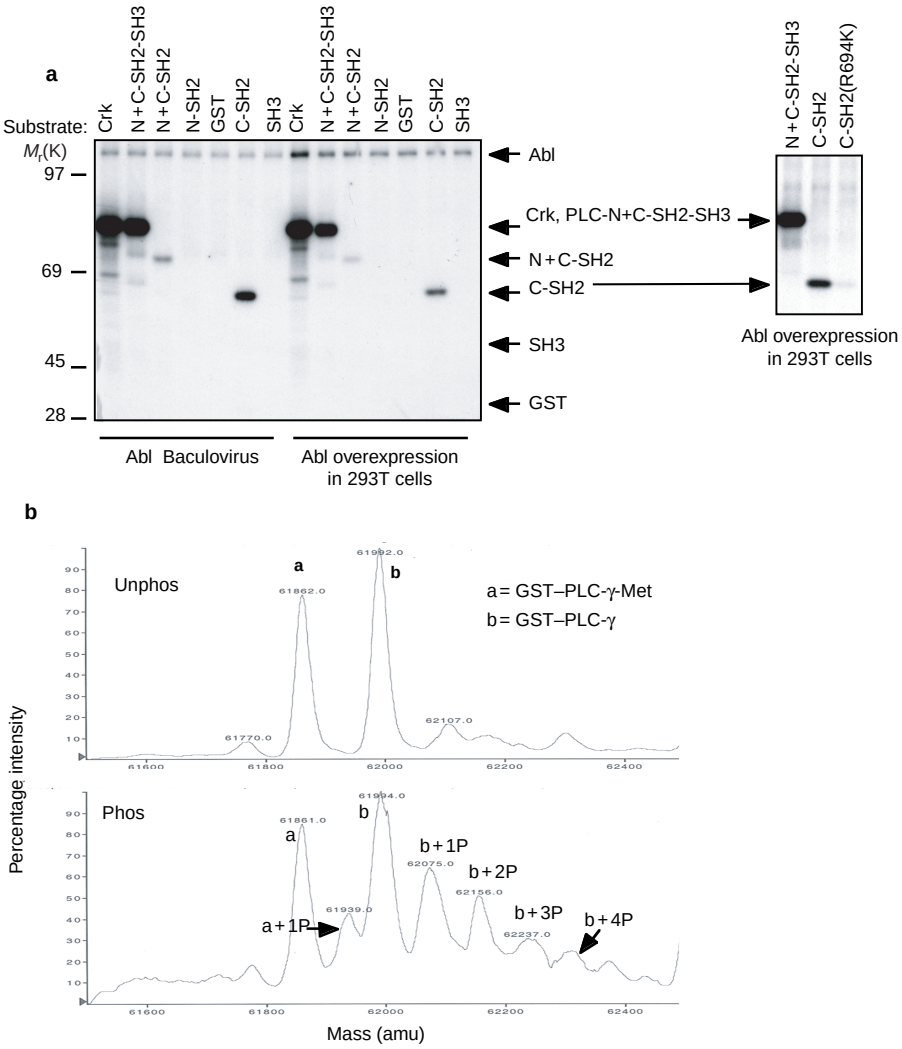

Figure 6 **c-Abl phosphorylates PLC-γ1 *in vitro* a**, c-Abl expressed in baculovirus-infected SF9 insect cells (left) or overexpressed in 293T cells (right) was immunoprecipitated with K12 antibody, and incubated in a kinase assay with various GST fusion proteins containing fragments of PLC-γ1. GST–PLC-γ-N+C-SH2–SH3 contains both SH2 domains and the SH3 domain. The amount of GST fragments used was equivalent as determined by Coomassie blue staining (data not shown). The positions of the GST fusion proteins are indicated with arrows. **b**, c-Abl expressed in 293T cells was immunoprecipitated with K12 antibody and incubated with GST–PLC-γ-N+C-SH2–SH3 in a kinase assay. Kinase reactions were analysed by liquid chromatography/mass spectrometry. Tyr phosphorylated peaks are noted by the characteristic increase of 80 daltons in mass per mole of phosphate added. –MET refers to species lacking the initial methionine residue. Phosphorylated Tyr residues were identified by trypsin digestion and tandem mass spectrometry of phosphopeptides

Arg/Abl null MEFs exhibited significantly greater levels of Ins(1,3,4)P_3 produced 5 and 10 min after PDGF stimulation, compared with the same MEFs reconstituted with normal cellular levels of c-Abl and Arg (Fig. 7b). Thus, loss of the c-Abl and Arg tyrosine kinases impairs the normal downregulation of PLC-γ1 observed in PDGF-stimulated cells. These data reveal that the Abl family kinases, in addition to functioning downstream of PLC-γ1, are also important in downregulation of PLC-γ1 activity *in vivo* in response to growth factor stimulation.

Discussion

c-Abl is activated by a variety of stimuli. However, little is known regarding the mechanisms of c-Abl activation by these various upstream signals. Here we demostrate that activation of c-Abl by PDGF stimulation is dependent on the earlier activation of PLC-γ1, thus uncovering a novel link between the c-Abl non-receptor Tyr kinase and phosphoinositide turnover catalysed by PLC-γ1. The mechanism of c-Abl activation downstream of PLC-γ1 involves the release of PtdIns(4,5)P_2-mediated inhibition of the c-Abl kinase activity (Fig. 8). Activation of the c-Abl kinase by PDGF is potentiated by phosphorylation by the Src family kinases[4,33] (Fig. 8).

Inhibition of c-Abl kinase activity by PtdIns(4,5)P_2 provides a unique mechanism of c-Abl regulation. The kinase activity of c-Abl is tightly regulated in the cell by intra- and inter-molecular interactions. Purification of c-Abl increases its kinase activity, as does overexpression (20–50 fold)[30,34,35]. These findings suggest the presence of a soluble inhibitor that may be titrated out by overexpression or removed by purification[30,34,35]. Several potential direct inhibitors of c-Abl have been described, including PAG, RB and AAP-1 (refs 1, 36) and more recently, F-actin[21]. However, the importance of these proteins in regulating c-Abl in physiologically

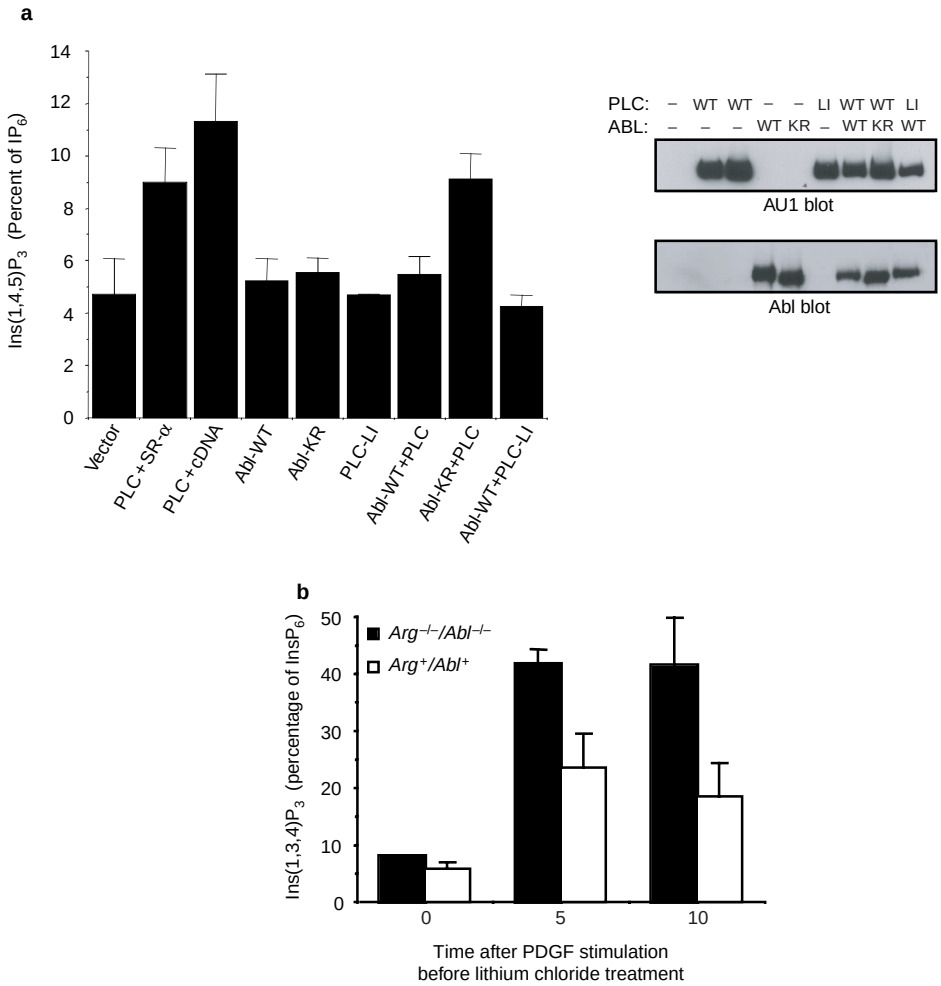

Figure 7 **c-Abl phosphorylation of PLC-γ1 causes downregulation of PLC activity a**, 293T cells were cotransfected with PLC-γ1 and c-Abl constructs, and labeled with ^{3}H-myoinositol. Labeled inositol phosphates were analysed by HPLC. To correct for differences in cell number, the amount of Ins(1,4,5)P_3 was normalized to $InsP_6$, which does not change after PLC-γ1 expression. The graph is representative of three independent experiments, each performed either in triplicate (above) or duplicate. A separate set of cells was transfected and probed for PLC-γ1 or c-Abl expression (right). c-Abl-WT and c-Abl-KR were cloned into different vectors, pcDNA3 (WT) and pSRα (KR), respectively, so that the expression of c-Abl-KR was equal to that of c-Abl–WT. The two different empty vectors were transfected with PLC as controls. WT = wild-type, LI = lipase-inactive, KR = kinase-inactive. **b**, Arg/Abl double null MEFs (solid bars) or the same MEFs reconstituted with c-Abl and Arg (Arg+/Abl+) by retroviral infection (open bars) were stimulated with PDGF-BB for the indicated times or left unstimulated, followed by treatment of all plates with lithium chloride (10 min). Labeled inositol phosphates were analysed as above, and Ins(1,3,4)P_3 levels were normalized to $InsP_6$.

relevant processes remain unclear. Intramolecular interactions may also contribute to the negative regulation of c-Abl. Constitutive activation of the c-Abl kinase may result through disruption of intramolecular SH3-proline sequence interactions, by deletion or point mutation of the c-Abl SH3 domain, or mutation of proline residues in the linker region between the SH2 and catalytic domains[37,38]. It has also been proposed that c-Abl is auto-inhibited by its first 81 amino acids[39]. After activation by PDGF, c-Abl is downregulated by dephosphorylation through the action of the PEST-type protein tyrosine phosphatases[40]. Activated c-Abl is also downregulated by ubiquitin-dependent degradation[41].

A model for c-Abl activation has been proposed, on the basis of enzymatic analysis of c-Abl[35]. In this model, c-Abl exists in an unphosphorylated inhibited conformation and purification of c-Abl or a signalling event that results in removal of inhibitory interactions causes c-Abl activation. Activation can also be induced by phosphorylation of Tyr 412 in the SH1 domain and Tyr 245 in the linker region, which disrupts the SH3–linker interaction, and results in the full activation of c-Abl[35]. Our results are in agreement with this model. We suggest that PtdIns(4,5)P_2 (and possibly other molecules bound to PtdIns(4,5)P_2) are involved in maintaining c-Abl in an inactive or low activity state before PDGF stimulation and that activation of c-Abl by PDGF occurs only after PtdIns(4,5)P_2 is destroyed either by hydrolysis (PLC-γ1-mediated) or dephosphorylation (inositol polyphosphate 5-phoshatase-dependent; Fig. 8). c-Abl is further activated through phosphorylation by the Src family kinases on Tyr 412 and a second site, Tyr 245[4,33]. These events may occur concurrently in PDGF-stimulated cells.

PLC-γ1 and c-Abl form a complex *in vivo* and after kinase activation, c-Abl phosphorylates PLC-γ1, causing inhibition of PLC-γ1 activity (Fig. 8). Unlike the PDGFR, c-Abl does not phosphorylate positive regulatory residues on PLC-γ1 *in vivo*. Downregulation of PLC-γ1 by c-Abl *in vivo* may involve phosphorylation of Tyr 771, which negatively regulates PLC-γ1 *in vitro*[6], as c-Abl phosphorylates Tyr 771 both *in vitro* and *in vivo*. Additionally, c-Abl expression elicits phosphorylation of Tyr 1003 in the PLC-γ1 Y-catalytic

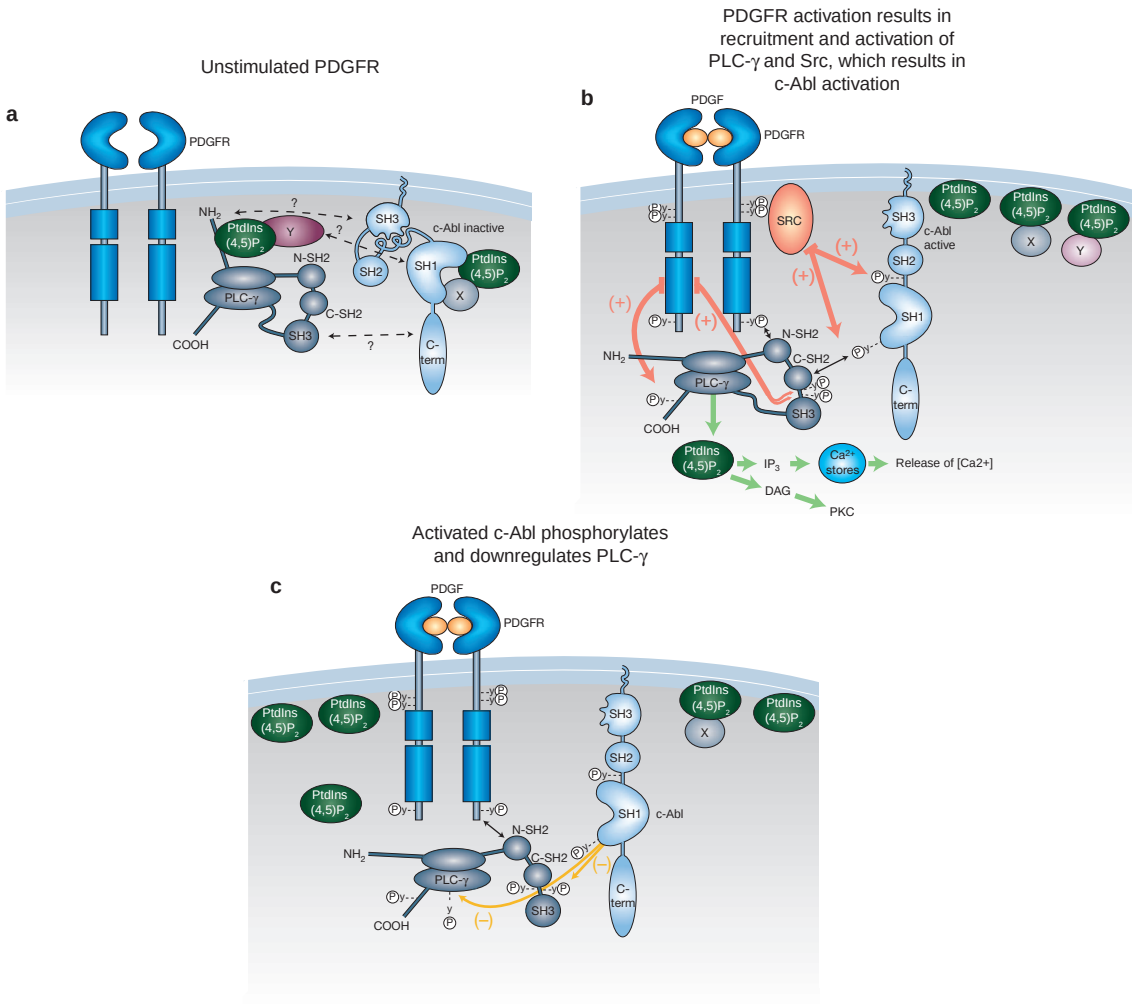

Figure 8 **Model of the interaction between c-Abl and PLC-γ1 signalling pathways. a**, Before PDGF stimulation, c-Abl activity is partly suppressed through a mechanism involving PtdIns(4,5)P_2. This model is also consistent with the existence of c-Abl inhibitory intramolecular interactions that may be stabilized by the binding of PtdIns(4,5)P_2-binding proteins (X) to c-Abl. Before PDGF stimulation, a c-Abl–PLC-γ1 complex is detected that may be dependent on direct or indirect (Y) binding between the two proteins. **b,** PDGF stimulation enhances the interaction between c-Abl and PLC-γ1, which is mediated by SH2-phosphotyrosine binding, and may be strengthened by SH3–proline sequence interactions between the two proteins. Activated PLC-γ1 hydrolyses PtdIns(4,5)P_2, releasing c-Abl kinase inhibition. Full activation of c-Abl by PDGF also requires phosphorylation of c-Abl by Src family kinases. **c,** The activated c-Abl in turn phosphorylates and downregulates PLC-γ1. After c-Abl-induced PLC-γ1 downregulation, PtdIns(4,5)P_2 levels increase, thereby inhibiting the c-Abl kinase and returning its activity to basal levels. Black arrows denote protein–protein interactions and dotted black arrows represent possible interactions. Red arrows correspond to positive regulatory phosphorylation events, whereas yellow arrows are negative regulatory phosphorylation events.

domain. Tyr 1003 has not previously been identified as a target of other Tyr kinases and therefore Tyr 1003 represents a novel phosphorylation site that may contribute to downregulation of PLC-γ1 by c-Abl.

PLC-γ1 has been implicated in the control of cell migration, axon guidance and neurite outgrowth[8–10]. The Abl kinases are important in the positive regulation of axon guidance and neurite outgrowth[20,42], and in negative regulation of migration of fibroblast and epithelial cells (R.P. and A.M.P., unpublished results)[18,19]. These findings may be explained by the potential dual roles of c-Abl in modulating actin polymerization at the leading edge, while also signalling at focal adhesions[2,19]. Here, we show that both c-Abl and PLC-γ1 positively regulate chemotaxis toward PDGF-BB in PAE cells expressing a PDGFR-β. Therefore, the PLC-γ1-dependent activation of c-Abl is physiologically relevant, as PLC-γ1 requires c-Abl kinase activity to enhance chemotaxis towards PDGF.

Our findings suggest that Abl family kinases may be important in signalling downstream of PtdIns(4,5)P_2 in multiple signalling pathways. PtdIns(4,5)P_2 has been implicated in the regulation of a variety of cellular processes, in addition to modulating cytoskeletal dynamics. Among these processes are endocytosis, exocytosis, vesicle targeting and synaptic plasticity[43]. Additionally, c-Abl regulates PLC-γ1 and thereby affects the turnover of phospholipids. Together, these findings reveal the existence of bidirectional regulation of Abl Tyr kinases and lipid signalling pathways. □

Methods

Materials

U73122 and its inactive analogue U-73343 were purchased from BioMol Research Labs (Plymouth Meeting, PA). Antibodies directed against c-Abl (K12, Santa Cruz Biotechnologies, Santa Cruz, CA; AB-3, Oncogene, San Diego, CA; 8E9, Pharmingen, San Diego, CA), the AU1 tag (Covance/Babco, Richmond, CA), antiphosphotyrosine (4G10, UBI; PY99, Santa Cruz) and GST (Santa Cruz,) were purchased commercially. Antibodies directed against the extracellular domain of PDGFR-α (80.8), the intracellular domain of PDGFR-β (30A; ref. 23), PLC-γ1 (ref. 44) and c-Abl (Pex4; ref.4) were previously described.

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL) and ^{3}H-myoinositol was purchased from American Radiolabeled Chemicals Inc (St Louis, MO). PDGF-AA and PDGF-BB were obtained from Upstate Biotechnologies (Lake Placid, NY).

DNA constructs

Chimeric PDGF α/β receptors containing F1021 and F72/74/1021 mutations were created by swapping a fragment containing F1021 from a PDGFR-β mutant[45] into wild-type chimeric receptors[23]. pcDNA3-c-Abl (murine) was obtained from R. V. Etten. Migr1–c-Abl, pSRα–c-Abl–KR and PLC-γ1 mutants (Y783F, LI (H335F), N*-SH2(R586K),C*-SH2 (R694K), N*C*-SH2 (R586/694K)) were described previously[4,44]. Constructs of c-Abl and v-Abl were cloned into the bicistronic vector Mig-CD4 (obtained from W. Pear). Migr1–PLC-γ1 was created by blunting the PLC-γ1 *Xba*I fragment and cloning into the blunted EcoR1 site of Migr1. PLC-γ1 mutants were created using Quikchange mutagenesis (Stratagene, Cedar Creek, TX). pBabe-puro Inp54 (ref. 26) was created by removing the phosphatase from a green fluorescent protein (GFP)–myristoylated version[27], without the GFP tag or Lyn myristoylation sequence. A phosphatase-dead 5-ptase construct was created by mutating pBabepuro-Inp54 D280 to Ala by mutagenesis (QuikChange, Stratagene, Cedar Creek, TX). All PLC-γ1 and Inp54 mutants were sequenced.

Cell lines

Ph cells, which lack endogenous PDGFR-α, and Ph cells expressing wild-type and mutant chimeric PDGFRs were previously described[23]. *PLC-γ1*-null fibroblasts were a kind gift of G. Carpenter and were maintained as described[24]. *PLC-γ1*-null fibroblasts were reconstituted with PLC-γ1 by retroviral infection with Migr1–PLC-γ1, followed by FACS to sort for GFP-positive cells. *Arg/Abl* double null MEFs (a kind gift of A. Koleske, Yale University, New Haven, CT) were reconstituted with vector (Migr1) or c-Abl as previously described[4]. The bicistronic PK1–Arg construct was introduced into MEFs by liposome-mediated transfection followed by puromycin selection. PAE cells were infected with the PDGFR-β in a LXSH retroviral vector, followed by hygromycin selection to produce a stable cell line.

GST fusion proteins

GST–PLCγ-N-SH2 and GST–PLCγ–N+C-SH2 were obtained from T. Pawson. GST–PLCγ–C–SH2 and GST–PLCγ–N+C–SH2–SH3 were previously described[23,46]. GST–PLCγ-SH3 was constructed by PCR amplification and cloning into pGEX–KG. GST–PLCγ–C–SH2 (R694) was obtained by mutating the GST–PLC-γ–C-SH2 construct[46] using transformer mutagenesis (Clonetech, Palo Alto, CA). GST–Crk and GST fusion proteins containing fragments of c-Abl were previously described[4] .

Immunoprecipitation, western blotting, GST pulldown and kinase assays

Cells were lysed in a Triton lysis buffer (used for kinase assays, GST pulldowns, co-immunoprecipitations) or RIPA buffer (used for immunoprecipitation-phosphotyrosine assays). Kinase assays were performed as previously described[4]. PIPs in chloroform:methanol were dried down, resuspended in 10 mM Tris at pH 7.5 and sonicated to create liposomes. Liposomes were added directly to the kinase assay, in the absence or presence of calcuim chloride. Significant inhibition of c-Abl kinase activity by PtdIns(4,5)P_2 (6–15-fold) with no effect of PtdIns(4)P was observed for all batches of lipids at concentrations of 40–60 μM lipid and 400–800 μM calcuim chloride.

Identification of phosphorylation sites by mass spectrometry

GST–PLCγ–C-SH2 and GST–PLCγ–N+C-SH2–SH3 were phosphorylated in a cold kinase reaction utilizing immunoprecipitated c-Abl from 293T cells transfected with murine c-Abl. The extent of phosphorylation was determined by direct analysis of the kinase reaction by liquid chromatography/mass spectrometry (LC/MS) using an HP1090 liquid chromatography system and a Sciex (Toronto, Ontario, Canada) API-III mass spectrometer. Protein mass spectra were deconvoluted from mass/charge to molecular weight using the BioSpec Reconstruct (Sciex, Toronto, Ontario, Canada) routine within the BioMultiview (Sciex, Toronto, Ontario, Canada) data processing software for the API-III. Specific sites of phosphorylation were identified by liquid chromatography/tandem mass spectrometric analysis (LC/MS/MS) of proteolytic digests of the kinase reaction mixtures. To determine site-specific phosphorylation stoichiometry, reconstructed ion chromatograms were plotted from LC/MS analyses of proteolytic digests of the kinase reaction mixtures for both phosphorylated and nonphosphorylated forms of each phosphopeptide. Peaks corresponding to phosphorylated and nonphosphorylated forms of each peptide were integrated and the percentage of phosphorylation for each site was calculated on the basis of peak areas.

Identification of *in vivo* phosphorylation sites on PLC-γ1 was performed by co-expressing c-Abl and PLC-γ1 in 293T cells and immunoprecipitating PLC-γ1. Phosphorylation sites on PLC-γ1 were identified by the Harvard Microchemistry Facility (Cambridge, MA) using microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry on a Finnigan (San Jose, CA) LCQ DECA quadrupole ion trap mass spectrometer. TIMM was conducted on the peptide containing Tyr 783 as previously described[31].

Analysis of inositol phosphates by HPLC

After transfection of 293T cells, the media was replaced with inositol-free DMEM containing 10% dialysed foetal bovine serum (FBS) (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA) and ^{3}H-myoinositol (10 μCi ml^{-1}). After 60–70 h, cells were lysed in 1M hydrochloric acid and the soluble supernatant was loaded onto the HPLC. Arg/Abl double-null MEFs and double-null MEFs reconstituted with Arg and c-Abl were labelled with ^{3}H-myoinositol for 48 h in 15% serum. These cells were serum-starved for 48 h in medium containing label and 0.2% serum and then stimulated with PDGF-BB or left unstimulated. Lithium chloride (10 mM) was added at the indicated times for 10 min before cell lysis and lipid analysis.

Transwell migration assays

PAE cells expressing a PDGFR-β were transfected with the indicated DNAs using Fugene (Roche, Indianapolis, IN). Two days later, cells were serum starved overnight in DMEM alone. Transwell chemotaxis assays were performed by coating the bottom surface of the transwell membrane (Costar, Corning, NY) with collagen (Collaborative Research; BD Biosciences, Bedford, MA) (100 μg ml^{-1}) for 2 h at 37 °C. Cells were trypsinized, washed in migration media (DMEM/1% BSA), resuspended to 1 × 10^6 cells per ml and filtered through a 70-μm nylon strainer to remove clumped cells. Transwells were washed with DMEM and migration media was placed in the bottom well with or without PDGF. 200 μl of cell suspension in migration media was placed in the upper wells and cells were allowed to migrate for 4 h. After migration, cells on the upper surface were removed and cells on the bottom surface were fixed in 4% paraformaldehyde for 10 min and stained with DAPI (0.0002%) for 5 s. Cells were mounted in antifade solution and transfected cells (GFP-positive) were counted (8–20× fields each).

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COMPETING FINANCIAL INTERESTS
The authors declare that they have no competing financial interests.

The cover of the April issue of *Nature Cell Biology* should have included a credit to Wei Yu, Mirjam Zegers and Keith Mostov, who kindly provided the original images.

In Dernick *et al.* (*Nature Cell Biol.* **5,** 358–362 (2003)) the formula on page 362 in the methods section was incorrect, and should read:

$$G_p = \frac{\omega C_v}{\sqrt{(\frac{\omega C_v}{\mathrm{Im}}) - 1}}$$

rather than:

$$G_p = \omega C_v / \sqrt{\omega C_v / \mathrm{Im} - 1}$$

This has now been corrected online.

In Plattner *et al.* (*Nature Cell Biol.* **5,** 309–319 (2003)), there was an error in Fig. 3c. The correct version of the figure is shown below, and has also been corrected online.