The p110 δ isoform of PI 3-kinase negatively controls RhoA and PTEN

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Inactivation of PI 3-kinase (PI3K) signalling is critical for tumour suppression by PTEN. This is thought to be a unidirectional relationship in which PTEN degrades the lipids produced by PI3K, thus controlling cell proliferation, survival and migration. We now show that this relationship is in fact bidirectional, whereby PI3K reciprocally controls PTEN. We report that the p110 δ PI3K negatively regulates PTEN, through a pathway involving inhibition of RhoA. Inactivation of p110 δ in macrophages led to reduced Akt and Rac1 activation, but paradoxically to increased RhoA and PTEN activity. Partial inactivation of p190RhoGAP and a reduced binding of cytoplasmic RhoA to the cyclin-dependent kinase inhibitor p27 both contributed to the increased RhoA-GTP levels upon p110 δ inactivation. Pharmacological inhibition of ROCK, a downstream effector kinase of RhoA, restored all signalling and functional defects of p110 δ inactivation, including Akt phosphorylation, chemotaxis and proliferation. This work identifies the RhoA/ROCK pathway as a major target of p110 δ -mediated PI3K signalling, and establishes for the first time that PI3K controls itself, via a feedback loop involving PTEN.

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Introduction

Ligation of a wide variety of cell surface receptors leads to the generation of the PIP₃ lipid second messenger, which affects cell growth, cell cycle progression, survival, intracellular traffic, cytoskeletal changes and migration (Vanhaesebroeck *et al*, 2001; Hawkins *et al*, 2006). PIP₃ targets diverse downstream effector proteins through interaction with pleckstrin homology (PH) domains present in Ser/Thr kinases (such as Akt/PKB), Tyr kinases (such as Btk), adaptor proteins (such as Gab2) and regulators of small GTPases (such as guanine nucleotide exchange factors (GEFs) and GTPase-activating

proteins (GAPs)) (Vanhaesebroeck *et al*, 2001; Hawkins *et al*, 2006).

PIP₃ action in cells is counteracted by lipid phosphatases, among which PTEN is the most extensively investigated. Indeed, PTEN is mutationally inactivated in a wide variety of cancers, contributing to the constitutive activation of the PI3K pathway in these cells (Leslie and Downes, 2004; Parsons, 2004; Sansal and Sellers, 2004; Cully *et al*, 2006). Despite its importance, however, a clear picture on the biochemical regulation of PTEN, its responsiveness to extracellular stimuli and its control by PI3K itself has yet to emerge (Leslie and Downes, 2004; Gericke *et al*, 2006). Processes that have been implicated in PTEN regulation include oxidation, protein:protein interaction and phosphorylation on Tyr by unknown kinases (Koul *et al*, 2002; Sanchez *et al*, 2005) or phosphorylation on Ser/Thr, for example by CK2 and GSK3 (Vazquez *et al*, 2000; Torres and Pulido, 2001; Miller *et al*, 2002). All of these events may impact on PTEN stability and subcellular localisation. Recently, a role for RhoA and its effector kinase ROCK in positive regulation of PTEN activity has been reported (Li *et al*, 2005; Sanchez *et al*, 2005). Indeed, ROCK can increase the phosphorylation and activity of PTEN, through an unknown mechanism but most likely involving a physical interaction with PTEN (Li *et al*, 2005; Meili *et al*, 2005). The upstream signals that control this RhoA/ROCK/PTEN pathway are unknown. It is also unclear whether this pathway is operational in cells under non-overexpression conditions.

Downstream of tyrosine kinases and Ras, PIP₃ is produced by the class IA subset of PI3Ks. These are heterodimers made up of a 110 kDa catalytic subunit (p110 α , p110 β and p110 δ) in complex with one of five regulatory subunits (collectively called the 'p85s'). Whereas p110 α and p110 β are found in most tissues, the expression of p110 δ is most abundant in leukocytes.

We previously presented evidence that the class IA PI3K isoforms have nonredundant functions downstream of the receptors for colony-stimulating factor-1 (CSF-1) in macrophages (Vanhaesebroeck *et al*, 1999b) and epidermal growth factor (EGF) in breast cancer cell lines (Sawyer *et al*, 2003). Using microinjection of p110 δ -selective antibodies in the immortalised BAC1.2F5 macrophage cell line (Morgan *et al*, 1987), we showed that p110 δ controls actin reorganisation and chemotaxis downstream of the CSF-1 receptor (CSF-1R) (Vanhaesebroeck *et al*, 1999b). The intracellular signalling mechanism by which p110 δ achieves its biological functions in these cells has thus far not been delineated. Here we use genetic and pharmacological approaches to dissect the molecular mechanism of p110 δ signalling, using primary mouse macrophages as well as other cell types that express p110 δ , including breast cancer cell lines (Sawyer *et al*, 2003). We report the unexpected finding that p110 δ inactivates PTEN through a pathway involving RhoA and ROCK. This inhibition of PTEN is essential for p110 δ to exert its biological

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Figure 1 Impact of p110 δ inactivation on CSF-1-driven biological responses in BMMs. **(A)** Acute morphological changes in BMMs upon *random* stimulation with CSF-1. BMMs were starved of CSF-1 for 16–20 h, stimulated with 30 ng/ml of CSF-1 for the indicated time points, fixed and stained for F-actin, followed by confocal imaging/photography (scale bar: 20 μ m). **(B)** Cell polarisation and PIP₃ gradient formation in BMMs upon *gradient* stimulation with CSF-1. BMMs were exposed to a gradient of CSF-1, delivered via a microtip, for the indicated time periods, followed by fixation and indirect immunofluorescence using an anti-PIP₃ antibody (scale bar: 30 μ m). Cells with the pan-PI3K inhibitor LY294002 were used as control. **(C)** Chemotaxis in BMMs exposed to a gradient of CSF-1 in Dunn chambers, monitored for 16 h by time-lapse microscopy. The cell tracks from three separate experiments were merged into a single file for analysis. Circular histograms (upper panels) show the proportion of cells migrating into each of 20 segments of the angular trajectory plot (measured when each cell migrated past a horizon of 30 μ m from its starting point with the source of CSF-1 at the top). Arrows indicate significant mean directionality of the cell population. The shaded area marks the 95% confidence intervals of statistical significance. $n = x$ (y) below the histograms indicates the number of cells migrated past the particular horizon (x), out of the total number of cells tracked (y). Similar chemotaxis plots were obtained for horizon limits of 50, 80 and 100 μ m, although the cell numbers varied (not shown). Vector plots (lower panel) show the end point of the cells with the starting point of each cell at the intersection between X and Y axes and with the source of CSF-1 at the top of each plot. **(D)** Number of cells tested under (C) that migrated past the indicated horizons from their point of origin, relative to the total number of cells tracked. **(E)** Effect of genetic or pharmacological inactivation of p110 δ on DNA synthesis of BMMs. Data show a representative experiment performed with quadruplicate wells. LY = LY294002, IC = IC87114.

functions. This is the first documented pathway whereby PTEN activity is controlled by PI3K itself.

Results

Derivation of macrophages with inactive p110 δ

Bone-marrow-derived macrophages (BMMs) were derived from wild-type (WT) mice, or from mice in which both p110 δ alleles are replaced by a kinase-dead version of p110 δ (called p110 δ ^{D910A}), as a result of knock-in mouse gene targeting (Okkenhaug *et al*, 2002). Pharmacological intervention with p110 δ was achieved by the use of IC87114, a small molecule inhibitor with selectivity for p110 δ (Sadhu *et al*, 2003).

We first tested the effect of p110 δ inactivation on BMM biology. Culture of bone marrow precursor cells from WT and δ D910A/D910A mice in the presence of CSF-1 gave rise to similar numbers of mature BMMs (data not shown), expressing similar levels of the CSF-1R and the macrophage-specific marker F4/80 (Supplementary Figure S1). Mutant BMMs did not show compensatory expression of nontargeted class I PI3K isoforms (Supplementary Figure S1b) or other genes (as assessed by gene array studies; Clotilde Billottet and Bart Vanhaesebroeck, unpublished data). Under proliferating conditions in the presence of CSF-1, no changes in cell morphology, size and actin cytoskeleton were observed (data not shown). These data indicate that inactivation of p110 δ has no major effects on the differentiation of bone marrow precursor

Figure 2 p110 δ inhibits PTEN activity. (A) Effect of PTEN inhibition on Akt phosphorylation. BMMs were pretreated for 30 min with vehicle or 500 nM of the PTEN inhibitor VO-OHpic (Rosivatz *et al*, 2007), stimulated with CSF-1 (30 ng/ml) for the indicated time points followed by WB for P-Akt (S473) or GAPDH. Two independent experiments were performed with similar results. (B) Effect of p110 δ inactivation on Tyr phosphorylation of PTEN. BMMs were stimulated with CSF-1 (30 ng/ml) for the indicated time points, followed by IP of PTEN and WB for P-Tyr or total PTEN. Bottom panel: ratio of WB signal of Tyr-phosphorylated PTEN over that of total PTEN. Two independent experiments were performed with similar results. (C) Effect of p110 δ inactivation on PTEN lipid phosphatase activity. PTEN was immunoprecipitated from BMM lysates and its PIP₃ phosphatase activity determined using 1- α -phosphatidylinositol(3,4,5)-triphosphate as a substrate and the Biomol Green reagent. One representative experiment out of two independent experiments carried out in triplicate is shown (** $P < 0.01$). The blot above the graph shows WB of PTEN in the BMM lysates used for PTEN immunoprecipitation. (D) PTEN immunoprecipitates were incubated for 30 min with GST-SHP1 or with buffer control, followed by measurement of Tyr phosphorylation and lipid phosphatase activity of PTEN. (E) PTEN activity induced by IC87114 in breast cancer lines. MDA-MB-231 or T47D cancer cells were pretreated with 5 μ M of IC87114 or vehicle for 60 min, followed by PTEN IP and determination of its PIP₃ phosphatase activity by ELISA (Echelon). One representative experiment out of two independent experiments carried out in triplicate is shown (* $P < 0.05$). The blot above the graph shows a WB of PTEN in the lysates used for PTEN immunoprecipitation.

cells to mature macrophages. This conclusion is in line with previous reports that CSF-1-induced differentiation of myeloid precursors to macrophages does not depend upon PI3K activity, but rather on Erk activity (Gobert Gosse *et al*, 2005), which was unaltered in $\delta^{D910A/D910A}$ BMMs (see below).

CSF-1-induced BMM chemotaxis and DNA synthesis depend on p110 δ activity

Compared to WT cells, CSF-1-starved $\delta^{D910A/D910A}$ BMMs had a more rounded appearance (reduced ratio of length/breadth)

and a small but significantly reduced adhesive area (Supplementary Figure S2a).

Upon stimulation with a uniform and saturating concentration of CSF-1 (30 ng/ml), $\delta^{D910A/D910A}$ cells also showed reduced actin polymerisation, membrane ruffling and cell spreading (Figure 1A and Supplementary Figure S2a, b).

Upon gradient stimulation with CSF-1, administered by a micropipette (Figure 1B), WT cells rapidly polarised towards the source of CSF-1 and accumulated PIP₃ at the leading edge. At very early time points, a number of cells in

$\delta^{D910A/D910A}$ BMM cultures polarised but this was followed by quick retraction, so that at later time points polarised cells were no longer observed (Figure 1B). Similarly, IC87114 treatment of BMMs derived from WT mice, transgenic for an intracellular PIP₃/PI(3,4)P₂-binding GFP-Akt-PH probe (Nishio *et al*, 2007), blocked CSF-1-induced GFP recruitment to the leading edge (Supplementary Figure S2c).

Migration induced by a uniform concentration of CSF-1 was mildly affected upon p110 δ inactivation. Although WT and $\delta^{D910A/D910A}$ BMMs migrated with the same speed (Supplementary Figure S2d), the latter cells stayed closer to their starting point, as revealed by plots whereby the number of cells that migrated past a given distance from their point of origin is expressed, relative to the total number of cells tracked (Supplementary Figure S2d, bottom graph).

Chemotaxis of BMMs towards a gradient of CSF-1 in Dunn chemotaxis chambers was blocked by genetic or pharmacological inactivation of p110 δ (Figure 1C). Also under these conditions of directional migration, p110 δ inactivation led to a reduction in the number of cells that migrated past a given distance from their point of origin (Figure 1D).

In fully differentiated BMMs, inactivation of p110 δ led to reduced CSF-1-induced DNA synthesis, especially at higher concentrations of CSF-1 (Figure 1E). Treatment with IC87114 also decreased DNA synthesis of WT BMMs to the levels seen in $\delta^{D910A/D910A}$ cells, but did not affect DNA synthesis of the latter (Figure 1E). The pan-PI3K inhibitor LY294002 further reduced DNA synthesis of $\delta^{D910A/D910A}$ and WT cells (Figure 1E), suggesting that the remaining DNA synthesis in the absence of p110 δ is controlled by PI3K isoforms other than p110 δ . However, it is also possible that inhibition of non-PI3K signalling proteins (such as mTOR) by LY294002 (Knight *et al*, 2006) contributes to the antiproliferative effect of this compound.

Active p110 δ suppresses PTEN lipid phosphatase activity

As expected, CSF-1-induced Akt phosphorylation was reduced in $\delta^{D910A/D910A}$ cells (Figure 2A), indicative for reduced PIP₃ production in these cells upon CSF-1 stimulation. We aimed to overcome this effect of p110 δ inactivation by inhibiting PTEN, a major PIP₃ phosphatase. Pretreatment with VO-OHpic (3-hydroxypicolinate vanadium(IV) complex), a selective small molecule inhibitor of PTEN (Rosivatz *et al*, 2007), partially restored CSF-1-induced Akt phosphorylation in $\delta^{D910A/D910A}$ cells (Figure 2A).

The regulation of PTEN is complex and involves phosphorylation on tyrosine (Tyr) by unknown kinases (Koul *et al*, 2002; Sanchez *et al*, 2005) and on Ser/Thr, possibly by CK2, GSK3 and other kinases (Vazquez *et al*, 2000; Miller *et al*, 2002). Previous work has also implicated a RhoA/ROCK pathway in the control of PTEN whereby the Ser/Thr kinase ROCK activates PTEN, through an unknown mechanism (Li *et al*, 2005; Meili *et al*, 2005; Sanchez *et al*, 2005). Upstream signalling pathways controlling this RhoA/PTEN axis are also unknown.

Genetic or pharmacological inactivation of p110 δ led to increased (yet still low) Tyr phosphorylation of PTEN, both under basal and CSF-1-stimulated conditions (Figure 2B; Supplementary Figure S3a). Ser/Thr phosphorylation of PTEN

was not affected under those conditions (Supplementary Figure S3b).

Inactivation of p110 δ also led to increased PTEN activity under basal conditions (Figure 2C). CSF-1 stimulation did not affect PTEN activity in cells with inactive p110 δ , in contrast to WT cells in which CSF-1 stimulation reduced PTEN activity (Figure 2C). Treatment of PTEN, immunoprecipitated from cells with inactive p110 δ , with the tyrosine phosphatase SHP1 reduced PTEN activity to the levels seen in WT cells (Figure 2D). This indicates a correlation between increased Tyr phosphorylation and PTEN hyperactivity upon p110 δ inactivation.

PTEN activity was also constitutively increased in brain tissues from mice expressing inactive p110 δ (data not shown). Moreover, short-term treatment of p110 δ -positive breast cancer cell lines with IC87114 also led to increased PTEN activity (Figure 2E). These data indicate that p110 δ action inhibits PTEN activity.

p110 δ negatively regulates RhoA

To investigate how p110 δ could affect PTEN activity, we first investigated the effect of p110 δ inactivation on early signalling induced by CSF-1. Akt phosphorylation upon stimulation of $\delta^{D910A/D910A}$ BMMs with a saturating dose of CSF-1 (30 ng/ml) was reduced by approximately 50% (Figures 2A and 3A), whereas Erk phosphorylation was unaffected (Figure 3A and Supplementary Figure S4a). IC87114 mimicked this effect in WT cells (Figure 3A and Supplementary Figure S4a) but did not reduce Akt phosphorylation in $\delta^{D910A/D910A}$ BMMs (Figure 3A and Supplementary Figure S4a), in line with the p110 δ selectivity of this compound.

Compared to WT cells, CSF-1-induced GTP loading of Rac1 was decreased both in $\delta^{D910A/D910A}$ and IC87114-treated WT BMMs (Figure 3B and Supplementary Figure S4b). These observations are in line with the known positive role of PI3K in the regulation of Akt and Rac1 (Vanhaesebroeck *et al*, 2001; Welch *et al*, 2003).

Surprisingly, both basal as well as CSF-1-stimulated levels of RhoA-GTP were significantly increased in $\delta^{D910A/D910A}$ BMMs, compared to WT cells (Figure 3C and Supplementary Figure S4c). Pretreatment of WT cells with IC87114 also increased RhoA-GTP (Figure 3C and Supplementary Figure S4c). A similar increase in RhoA-GTP was also observed in CSF-1-stimulated BAC1.2F5 macrophages and EGF-treated MDA-MB-231 and T47D p110 δ -expressing breast cancer cell lines (Sawyer *et al*, 2003) in response to IC87114 (Figure 3C and Supplementary Figure S4d). Similar observations were made in brain cell lysates from $\delta^{D910A/D910A}$ mice (data not shown). These data imply that, under normal conditions, p110 δ activity suppresses RhoA function in cells.

RhoA and Rac1 have been shown to oppose each other in several biological settings, and low cellular levels of Rac-GTP most often correlate with high levels of RhoA-GTP, and *vice versa* (Sander *et al*, 1999; Raftopoulou and Hall, 2004). This appears not to be the case in BMMs given that basal RhoA-GTP levels were increased in p110 δ mutant BMMs, without an effect on basal Rac1-GTP levels (Figure 3B and C). This suggests that p110 δ -driven negative regulation of RhoA in these cells under basal conditions is independent of Rac1.

Inhibition of ROCK overcomes the biological effects of p110 δ inactivation

We next set out to investigate the importance of the increased RhoA activity in the biological responses controlled by p110 δ and regulation of PTEN. An important effector of RhoA-GTP is the Ser/Thr kinase ROCK (Riento and Ridley, 2003), which is sensitive to Y27632 (Uehata *et al*, 1997; Nobes and Hall, 1999; Ishizaki *et al*, 2000). Treatment of $\delta^{D910A/D910A}$ BMMs with Y27632 reverted p110 δ phenotypes to those of WT cells. This included the capacity of these cells to undergo CSF-1-induced cell polarisation and accumulation of PIP₃ at the leading edge (Figure 4A), chemotaxis (Figure 4B) and migration in a wound-healing assay (Supplementary Figure S5a). Y27632 also partially recovered the defects in Akt phosphorylation (Figure 4C) and DNA synthesis (Figure 4D), and blocked the increase in Tyr phosphorylation (Figure 4E) and lipid phosphatase activity of PTEN (Figure 4F). Y27632 also inhibited the increased phosphorylation of MLC, a downstream target of ROCK, observed in $\delta^{D910A/D910A}$ cells (Supplementary Figure S5b).

Alternative ways to interfere with Rho/ROCK pathways had similar effects as Y27632. For example, H1152, a ROCK inhibitor from a different chemical class to Y27632 (Sasaki *et al*, 2002), increased Akt phosphorylation in $\delta^{D910A/D910A}$ cells (Supplementary Figure S5c). Direct inhibition of RhoA by C3 transferase also annihilated the increased lipid phosphatase activity of PTEN in $\delta^{D910A/D910A}$ BMMs (Supplementary Figure S5d).

Similar data were observed in p110 δ -expressing breast cancer cells in which Y27632 restored IC87114-induced inhibition of EGF-stimulated Akt phosphorylation (Supplementary Figure S5e), demonstrating the generality of the link between p110 δ activity and RhoA inhibition.

These data are consistent with a model that inactivation of p110 δ leads to enhanced ROCK activity, as a consequence of increased RhoA-GTP levels, contributing to the observed phenotypes.

p110 δ inactivation leads to reduced p190RhoGAP activity

RhoA activity could be inhibited by p110 δ either through inhibition of a GEF or activation of GAP. Given that PI3K-dependent Rho-GEFs have not been reported, we first focused on the activity of p190RhoGAP, one of the key regulators of RhoA (Ren *et al*, 1999, 2000; Arthur and Burridge, 2001; Wakatsuki *et al*, 2003; Continolo *et al*, 2005). The *in vitro* GAP activity of p190RhoGAP immunoprecipitated from

$\delta^{D910A/D910A}$ BMMs under CSF-1-starved conditions towards RhoA was significantly reduced (Figure 5A). Given that the expression of p190RhoGAP was not altered in $\delta^{D910A/D910A}$ BMMs (Figure 5A, lower panel), this observation suggests an intrinsic change in p190RhoGAP in these cells. This was indeed the case, as p190RhoGAP showed reduced basal and CSF-1-stimulated Tyr phosphorylation in $\delta^{D910A/D910A}$ BMMs (Figure 5B).

The data above are indicative of defective Tyr kinase signalling downstream of the CSF1R upon p110 δ inactivation. p190RhoGAP is known to be phosphorylated and activated in an Src-dependent manner (Zrihan-Licht *et al*, 2000), via a complex mechanism that also involves PYK2, a FAK Tyr kinase homologue expressed in leucocytes (Ganju *et al*, 1997). Indeed, PYK2 has been reported to activate Src (Dikic *et al*, 1996) and to bind constitutively to p190RhoGAP, facilitating the activation of the latter by Src (Zrihan-Licht *et al*, 2000). Complex formation between PYK2 and p190RhoGAP was unaltered in $\delta^{D910A/D910A}$ BMMs (Supplementary Figure S6a), both under basal and CSF-1-stimulated conditions. However, CSF-1-induced Tyr phosphorylation of PYK2 (Figure 5C and Supplementary Figure S6b) was reduced in $\delta^{D910A/D910A}$ cells and IC87114-treated WT BMMs. Likewise, also Tyr phosphorylation of Src was decreased under these conditions (Figure 5D and Supplementary Figure S6c).

Taken together, these results are consistent with a model in which p110 δ activity leads to increased PYK2/Src Tyr phosphorylation and p190RhoGAP activity, leading to decreased RhoA-GTP levels. Conversely, inactivation of p110 δ leads to increased RhoA activity (schematically shown in Figure 8).

p110 δ inactivation leads to reduced association of cytosolic RhoA with p27

RhoA can also be regulated by p27, a member of the Cip/Kip family of cyclin-dependent kinase inhibitors (Besson *et al*, 2004a, b). Indeed, cytoplasmic p27 has been reported to bind and inhibit RhoA, by blocking its interaction with cytoplasmic RhoGEFs (Besson *et al*, 2004b). Akt is one of the regulators of p27, and reduction in Akt activity can lead to accumulation of p27 in the nucleus, contributing to inhibition of cell cycle progression (Zhou *et al*, 2001; Liang *et al*, 2002; Shin *et al*, 2002; Viglietto *et al*, 2002).

In CSF-1-starved BMMs, the ratios of cytosolic *versus* nuclear p27 were not significantly different between WT and $\delta^{D910A/D910A}$ cells (Figure 6A, black bars in lower bar

Figure 3 Effect of p110 δ inactivation on CSF-1-induced activation of protein kinases and small GTPases. (A) Effect of genetic or pharmacological inactivation of p110 δ on CSF-1-induced phosphorylation of Akt and Erk1/2 in BMMs. Top graphs: the indicated BMMs were stimulated with 30 ng/ml CSF-1 for different time points, followed by analysis of phosphorylation of Akt (on T308 and S473) or Erk1/2 (on T202/Y204) by WB of total cell lysates (80 μ g/lane). Graphs represent the mean $\pm$ s.e.m. of three separate experiments. Bottom graphs: WT BMMs were incubated with the indicated concentrations of IC87114 30 min before stimulation with CSF-1 (30 ng/ml, 5 min). Akt and Erk1/2 phosphorylation were assessed by WB of total cell lysates (80 μ g/lane). Graphs represent the mean $\pm$ s.e.m. of three separate experiments. (B) Effect of genetic or pharmacological inactivation of p110 δ on basal and CSF-1-induced Rac1 activation in BMMs. Equal volumes of cell lysates of the indicated BMMs were subjected to pull down assay with GTP-PBD, followed by detection of precipitated Rac1 by WB. Total cell lysates were resolved on the same SDS-PAGE gel and immunoblotted for Rac1. Graphs represent the mean $\pm$ s.e.m. of three separate experiments. (C) Effect of genetic or pharmacological inactivation of p110 δ on basal and stimulus-induced RhoA activation in macrophages and breast cancer lines. BMMs or BAC1.2.F5 cells were stimulated with CSF-1 (30 ng/ml), whereas MDA-MB-231 or T47D breast cancer cells were stimulated with EGF (50 ng/ml). When indicated, cells were pretreated with IC87114 (5 μ M, 1 h). Equal volumes of cell lysates of the indicated BMMs or breast cancer cell lines were subjected to pull down assay with GST-RBD, followed by WB detection of precipitated RhoA. Total cell lysates were resolved on the same SDS-PAGE gel and immunoblotted for RhoA. Graphs for BMMs represent the mean $\pm$ s.e.m. of three separate experiments. For breast cancer cell lines, two independent experiments were performed with similar results.

chart). However, upon stimulation with CSF-1, this ratio was increased in WT cells, but reduced in $\delta^{D910A/D910A}$ BMMs (Figure 6A). This suggests that in $\delta^{D910A/D910A}$ cells, less p27 is available in the cytosol to bind RhoA. In line with these observations, the amount of p27 co-immunoprecipitated with cytosolic RhoA, unlike in WT cells, did not increase in $\delta^{D910A/D910A}$ BMMs upon CSF-1 stimulation (Figure 6B).

Y27632 reversed the effect of p110 δ inhibition, and increased the ratio of cytosolic *versus* nuclear p27 in $\delta^{D910A/D910A}$ BMMs, without an effect on WT cells (Supplementary Figure 7).

Taken together, these data are compatible with a model whereby the reduction in p110 δ activity impairs Akt activation, resulting in less p27 being available in the cytoplasm of $\delta^{D910A/D910A}$ BMMs to bind RhoA, facilitating its activation (schematically shown in Figure 8).

Figure 4 Effect of the ROCK inhibitor Y27632 on CSF-1-induced biological responses of WT and $\delta^{D910A/D910A}$ BMMs. (A) $\delta^{D910A/D910A}$ BMMs were pretreated with 10 μ M Y27632 or vehicle for 30 min, exposed to a gradient of CSF-1 delivered through a microtip for the indicated time points, followed by fixation and indirect immunofluorescence using an anti-PIP₃ antibody (scale bar: 20 μ m). (B) $\delta^{D910A/D910A}$ BMMs were treated with 10 μ M of Y27632 and exposed to a gradient of CSF-1 in Dunn chambers. Cell migration was followed by time-lapse microscopy for 16 h. The circular histogram (left panel) shows the proportion of cells migrating into each of 20 segments of the angular trajectory plot measured when each cell migrated past a horizon of 70 μ m from its starting point with the source of CSF-1 at the top. Arrows indicate significant mean directionality of the cell population and the shaded areas mark the 95% confidence intervals of statistical significance. $n = x$ (y) below the histograms indicates the number of cells migrated past the particular horizon (x), out of the total number of cells tracked (y). Vector plot (right panel) shows the end point of the cells with the starting point of each cell at the intersection between X and Y axes and with the source of CSF-1 at the top of each plot. (C) BMMs were pretreated with 25 μ M of Y27632 or its vehicle for 15 min, stimulated with CSF-1 (30 ng/ml) for the indicated time points followed by WB for P-Akt (S473) or total Akt. Graph represents the mean $\pm$ s.e.m. of three separate experiments. (D) BMMs were incubated with the indicated concentrations of CSF-1 in the presence or absence of Y27632 (20 μ M), followed by measurement of [³H]thymidine incorporation 48 h later. Data show a representative experiment carried out in quadruplicate. (E) BMMs were stimulated with CSF-1 (30 ng/ml) for the indicated time points, followed by IP of PTEN and WB for P-Tyr or total PTEN. Bottom panel: ratio of WB signal of Tyr-phosphorylated PTEN over that of total PTEN. Two independent experiments were performed with similar results. (F) BMMs were pretreated with 25 μ M of Y27632 or vehicle for 15 min, followed by PTEN IP and determination of its PIP₃ phosphatase activity using L- α -phosphatidylinositol (3,4,5) triphosphate as a substrate and the Biomol Green reagent. One representative experiment out of two independent experiments carried out in triplicate is shown. The blot below the graph shows a WB of PTEN in the BMM lysates used for PTEN immunoprecipitation.

Figure 5 Effect of p110 δ inactivation on p190RhoGAP activity. (A) RhoA GAP activity in p190RhoGAP immunoprecipitates from unstimulated WT or p110 $\delta^{\Delta D910A/D910A}$ BMMs. The blot below the graph shows a WB of p190RhoGAP in the BMM lysates used for the GAP assay. Purified p50RhoGAP was used as positive control. (B) Top panel: p190RhoGAP immunoprecipitates were immunoblotted for pTyr or p190RhoGAP. Bottom panel: ratio of WB signal of Tyr-phosphorylated p190RhoGAP over that of total p190RhoGAP. (C) Ratio of the PYK2 WB signal in PTyr IP over that of total PYK2 in total lysates of the indicated cell types. Graph represents the mean $\pm$ s.e.m. of three separate experiments. (D) Ratio of the WB signal of Tyr (418)-phosphorylated Src over that of tubulin in total lysates of the indicated cell types.

Figure 6 Effect of p110 δ inactivation on p27 subcellular localisation and binding to cytosolic RhoA in BMMs. (A) Top panel: BMMs were stimulated with CSF-1 (30 ng/ml) for the indicated time points, followed by isolation of cytosolic and nuclear fractions and WB for p27, tubulin (marker for cytosol) or retinoblastoma protein (Rb; marker for nucleus). Bottom panel: ratio of WB signal of cytosolic vs nuclear p27. (B) Top panel: RhoA was immunoprecipitated from the cytosolic fraction of BMMs using a monoclonal antibody, followed by WB for p27 or RhoA. Bottom panel: ratio of WB signal of cytosolic p27 over that of cytosolic RhoA. Two independent experiments were performed with similar results.

Relative contribution of PYK2/Src- and p27-driven mechanisms in the regulation of RhoA activity

We next examined the relative contribution of the PYK2/Src and p27 pathways in controlling RhoA-GTP levels. This was

carried out in WT cells by pharmacological inhibition of Src or suppression of expression of PYK2 and/or p27 by siRNA, followed by assessment of RhoA levels, and comparing these with the RhoA-GTP levels in $\delta^{\Delta D910A/D910A}$ cells.

Figure 7 Relative contribution of PYK2/Src- and p27-driven mechanisms on RhoA activity. (A) BMMs, pretreated with the Src inhibitor PP2 (1 h at 1 μ M) were stimulated with CSF-1 for 10 min, followed by measurement of RhoA-GTP levels. (B) BMMs, transfected with siRNA either to PYK2 or p27, were stimulated with CSF-1 (30 ng/ml) for 10 min, followed by measurement of RhoA-GTP levels. (C) BMMs were transfected with siRNA to PYK2 and p27, or negative control siRNA, followed by determination of RhoA-GTP levels.

Under basal conditions, inhibition of Src by PP2 in WT cells increased RhoA-GTP levels almost to those seen in $\delta^{D910A/D910A}$ cells (Figure 7A, left panel and Supplementary Figure S8a). Inhibition of Src activity also made a significant, but less comprehensive contribution to the CSF-1-induced increase in RhoA-GTP (Figure 7A, right panel and Supplementary Figure S8a).

Under basal conditions, reduction of PYK2 expression in WT cells led to an increase in RhoA-GTP levels almost reaching those observed in $\delta^{D910A/D910A}$ BMMs (Figure 7B and Supplementary Figure S8b). Reduction of p27 expression had a more modest impact on RhoA-GTP (Figure 7B and Supplementary Figure S8b). Under conditions of CSF-1 stimulation, however, the contribution of p27 became more pronounced (Figure 7B and Supplementary Figure S8b). Silencing of both PYK2 and p27 in WT cells largely mimicked the effect of p110 δ inhibition on RhoA-GTP levels, both under basal and stimulated conditions (Figure 7C and Supplementary Figure S8c).

The data above suggest that the PYK2/Src-driven mechanism contributes almost solely to inhibition of RhoA activity under basal levels, whereas PYK2 and p27 effects are additive and both contribute to increased RhoA activity upon CSF-1 stimulation.

Discussion

Our data show that PI3K can control itself, through regulation of the PTEN tumour suppressor. This provides the first molecular link between PI3K and its key inactivator. We report that p110 δ signalling impinges on the previously documented RhoA/PTEN pathway (Li *et al*, 2005), which had thus far not been linked to upstream signalling, especially downstream of tyrosine kinases. We document that this RhoA/PTEN pathway is part of a larger signalling network in which p110 δ is in overall control of PTEN (Figure 8).

Our results also indicate that signalling by RhoA is a critical node in p110 δ signalling. Indeed, among the most unexpected findings reported here are the increased basal level of RhoA-GTP activity upon p110 δ inactivation, and the restoration of the biological defects induced by p110 δ inactivation by ROCK kinase inhibition. Increased levels of RhoA were found in diverse p110 δ -expressing cells such as macrophages, breast cancer cell lines and neurons. In these cells, the overall pattern of dynamic changes in RhoA-GTP levels following Tyr kinase receptor (CSF1-R, EGF-R) stimulation was not affected upon p110 δ inactivation, but the levels of RhoA-GTP remained consistently above those of cells with active p110 δ (Figure 3C). RhoA is important for cell body

contraction and tail retraction (Ridley, 2001; Sahai and Marshall, 2002) and tight control of this GTPase is critical for efficient cell migration and chemotaxis (Ridley, 2001). Elevated levels of active RhoA are incompatible with directional cell migration as they prevent membrane protrusion and cell polarity (Nobes and Hall, 1999; Arthur and Burridge, 2001; Ridley, 2001; Raftopoulou and Hall, 2004; Pixley *et al*, 2005; Wong *et al*, 2006).

That PI3K can be involved in keeping RhoA-GTP levels down has been suggested by the observation that treatment of neutrophils with the pan-PI3K inhibitor LY294002 enhances basal RhoA activity to levels reaching fMLP-stimulated RhoA activity (Xu *et al*, 2003). No explanation for this observation had been put forward at the time, but the notion that neutrophils have high levels of p110 δ (Vanhaesebroeck *et al*, 1997) makes it likely that this isoform of PI3K contributes to the effect of LY294002 on RhoA-GTP levels in these cells.

A number of pathways could potentially explain this p110 δ -dependent increase in levels of RhoA-GTP under basal conditions. Rac and Rho can suppress each others' activity, via several possible mechanisms (Sander *et al*, 1999; Ridley, 2001; Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004). In BMMs, we did not observe such inverse correlation. Indeed, under basal conditions of p110 δ inhibition, Rac1-GTP levels were unaltered while RhoA activity was increased (Figure 3B and C). We provide evidence for two mechanisms that contribute to the increased levels of RhoA-GTP upon p110 δ inactivation. The first one involves decreased binding of the p27 cyclin-dependent kinase inhibitor to cytosolic RhoA upon p110 δ inhibition (Figure 6B; summarised in Figure 8). Indeed, cytosolic p27 can bind RhoA, and prevent its activation by interfering with its binding to GEFs (Besson *et al*, 2004b). Thus, upon p110 δ inactivation, RhoA in the cytosol is not bound to p27 and has therefore more chance to interact with its RhoGEFs, facilitating its GTP loading. A second mechanism for increased RhoA-GTP upon p110 δ inactivation is reduced activation of Tyr kinases such as PYK2 and Src, leading to reduced p190RhoGAP activity (Figure 5C, and D; summarised in Figure 8). At present, we cannot exclude that other GAPs, such as ARAP3, a recently identified PIP₃/Rap-dependent RhoA-GAP (Krugmann *et al*, 2002; Donald *et al*, 2004), are also under the control of p110 δ .

Our data indicate that RhoA signalling downstream of p110 δ acts through another master regulator, namely PTEN. Indeed, PTEN lipid phosphatase activity was increased upon p110 δ inactivation (Figure 2C and E), correlating with increased Tyr phosphorylation of PTEN (Figure 2B and D). The effects of p110 δ inactivation on PTEN phosphorylation and activity were also reversed by the Y27632 ROCK inhibitor. Our results are in line with the evidence presented by Li *et al* (2005) that RhoA is upstream of PTEN, and mediates PTEN phosphorylation and activation through ROCK (Li *et al*, 2005; Meili *et al*, 2005). Dominant-negative RhoA mutants have no effect in PTEN-null cells (Sanchez *et al*, 2005), further demonstrating a link between these two proteins. At present, it is not clear how ROCK activates PTEN, and whether it directly phosphorylates PTEN and/or how it controls Tyr kinases. It is of interest to note that ROCK has also been implicated in the induction of Tyr phosphorylation of proteins (such as FAK) in other cell systems (Sinnott-Smith *et al*, 2001; Yagi *et al*, 2006). Whatever the mechanism of PTEN activa-

Figure 8 Schematic representation of the p110 δ →RhoA→PTEN signalling pathway described in the text. p110x refers to class I PI3K isoforms other than p110 δ . Indeed, the notion that inhibition of PTEN leads to increased PIP₃/P-Akt in cells with inactive p110 δ suggests that PTEN impinges on the PIP₃ produced by the remaining active class I PI3K isoforms in these cells (p110 α , p110 β or p110 γ). This implies that this pathway has the intrinsic capacity for indirect crosstalk between the class I PI3K isoforms.

tion by ROCK, the observation that treatment with inhibitors of ROCK or PTEN partially restored Akt phosphorylation in $\delta^{D910A/D910A}$ BMMs suggests that negative regulation of PTEN lipid phosphatase activity is a key function of p110 δ . The fact that inhibition of ROCK recovered all defective phenotypes (polarisation, chemotaxis, Akt phosphorylation, DNA synthesis) in $\delta^{D910A/D910A}$ BMMs while in parallel blocked the hyperactivation of PTEN in these cells is consistent with a feedback loop in which p110 δ keeps RhoA and PTEN in check to exert its biological effects (Figure 8).

It is unclear whether PI3K isoforms other than p110 δ can also negatively regulate RhoA. Preliminary data indicate that heterozygous inactivation of p110 α in BMMs does not affect GTP loading of RhoA (data not shown). At this point, though, it appears that the PIP₃ produced by non-p110 δ PI3K isoforms does not negatively affect PTEN. This is suggested by the following observations: in the absence of p110 δ activity, other PI3Ks still produce PIP₃ upon CSF-1 stimulation, given that Akt becomes activated (Figure 2A). The finding that pharmacological blockade of PTEN further increased Akt phosphorylation in $\delta^{D910A/D910A}$ cells indicates that this PIP₃ does not efficiently inhibit PTEN (Figure 2A). This is in line with the observation of unaltered PTEN activity upon CSF-1 stimulation of $\delta^{D910A/D910A}$ cells, despite the fact that one expects PIP₃ to be produced by non-p110 δ PI3Ks under those conditions (Figure 2C). These results suggest an isoform-selective role for p110 δ in the negative regulation of PTEN. How such isoform-selective signalling could be achieved is not clear at the moment, but may relate to isoform-selective protein kinase activity (Vanhaesebroeck *et al*, 1999a), compartmentalised signalling by class IA PI3Ks and other mechanisms (Vanhaesebroeck *et al*, 2001, 2005).

In summary, our work has uncovered a RhoA-mediated pathway by which p110 δ PI3K keeps PTEN lipid phosphatase activity in check through a mechanism involving RhoA and ROCK. This pathway is critical for p110 δ to exert its biological functions, including regulation of CSF-1-induced cell polarisation, chemotaxis and DNA synthesis. Our data also uncover a major role of p110 δ in regulating RhoA activity. It will be important in future work to assess the role of other class IA PI3K isoforms in the regulation of RhoA, especially in cell types which express low levels of p110 δ .

Materials and methods

Materials

The PTEN inhibitor 3-hydroxypicolinate vanadium(IV) complex (VO-OHPic) (Rosivatz *et al*, 2007) was provided by Dr Rudiger Woscholski, Imperial College, London. Polyclonal rabbit antibodies to PYK2 (sc-9019), CSF-1R (sc-692), p110 β (sc-602, used for western blots), p110 δ (sc-7176; used for western blot), goat polyclonal to PTEN (N19) and the monoclonal anti-RhoA antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against the PI3K subunits used for immunoprecipitation have been described previously (Vanhaesebroeck *et al*, 1999b). Upstate Biotechnology was the source of rabbit polyclonal anti-p85 (06-195), monoclonal anti-Rac1, the Rac1 activation assay kit (including GST-fusion p21-binding domain of PAK1 (GST-PBD) bound to glutathione-Agarose and lysis/wash buffer), monoclonal anti-p190RhoGAP and anti P-Tyr (4G10). GST-RBD and cell-permeable C3-transferase were from Cytoskeleton Inc. Phospho-specific (T308 and S473) Akt and anti-total Akt antibodies, anti-P-Src (Tyr418), polyclonal antibodies against P-PTEN (S380/T382/T383) and anti-total PTEN were from New England Biolabs. Other sources for reagents were as follows: monoclonal anti-PIP₃ IgG (Echelon Biosciences), rat anti-mouse F4/80-FITC (Serotec), PE anti-mouse CSF-1R (eBioscience), anti-rabbit P-MLC(S19) (Biosource), CSF-1 (Peprotech), rhodamine-phalloidin and Slow Fade Antifade kit (Molecular Probes), Y27632 and H1152 (Calbiochem-Novabiochem), ECL western blotting kit and protein A- or G-Sepharose (Amersham-Pharmacia). PYK2, p27 siRNA *smart* pools and negative control siRNA pool were from Dharmacon and Interferin transfection reagent from PolyPlus-Transfection. All other chemicals were obtained from standard commercial sources at the highest grade available.

Isolation and culture of BMMs

BMMs were derived from at least three 6- to 8-week-old mice per experiment and pooled. Cells were seeded on bacteriological plastic

plates at 10⁶ cells/ml in macrophage growth medium consisting of RPMI 1640 (Gibco-Invitrogen Ltd, Paisley, UK), 1 mM sodium pyruvate (Gibco), 1 $\times$ non-essential amino acids (Gibco), 0.029 mM 2-mercaptoethanol (Sigma), 10% heat-inactivated bovine Ultra low IgG FCS (Gibco) supplemented with 10% L-cell-conditioned medium as a source of CSF-1. After 3 days, nonadherent cells were collected and either cryogenically stored in FCS containing 10% DMSO or seeded at 6–8 $\times$ 10⁵ cells/ml on bacteriological plates and cultured for 4 days before use. Cells were detached using EDTA, centrifuged at 1000g, resuspended in macrophage growth medium and seeded for the experiments. All results were obtained from cells that had been cultured for no longer than 10 days after dissection. In all experiments described below (unless otherwise specified), the medium was changed to macrophage starvation medium (= macrophage growth medium without L-cell-conditioned medium) 16–20 h prior to the actual experiments. Unless otherwise indicated, the concentration of CSF-1 for cell stimulation was 30 ng/ml.

siRNA transfection

Cells were seeded for the experiments as described above and transfected the next day with siRNA to PYK2 and/or p27 (final concentration of each siRNAi was 50 nM) using Interferin transfection reagent, according to the manufacturers' instructions. Experiments were performed 4 days after transfection.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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