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Sabine Suire Sabine, Charlotte Lécureuil, Karen Anderson E, George Damoulakis George, Izabella Niewczas Izabella, et al.. GPCR activation of Ras and PI3Kc in neutrophils depends on PLCb2/b3 and the RasGEF RasGRP4. *EMBO Journal*, 2012, 31 (14), pp.3118-3129. 10.1038/emboj.2012.167 . hal-00776864

HAL Id: hal-00776864

<https://hal.science/hal-00776864>

Submitted on 29 May 2020

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GPCR activation of Ras and PI3K γ in neutrophils depends on PLC β 2/ β 3 and the RasGEF RasGRP4

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The molecular mechanisms by which receptors regulate the Ras Binding Domains of the PIP₃-generating, class I PI3Ks remain poorly understood, despite their importance in a range of biological settings, including tumorigenesis, activation of neutrophils by pro-inflammatory mediators, chemotaxis of Dictyostelium and cell growth in Drosophila. We provide evidence that G protein-coupled receptors (GPCRs) can stimulate PLC β 2/ β 3 and diacylglycerol-dependent activation of the RasGEF, RasGRP4 in neutrophils. The genetic loss of RasGRP4 phenocopies knock-in of a Ras-insensitive version of PI3K γ in its effects on PI3K γ -dependent PIP₃ accumulation, PKB activation, chemokinesis and reactive oxygen species (ROS) formation. These results establish a new mechanism by which GPCRs can stimulate Ras, and the broadly important principle that PLCs can control activation of class I PI3Ks.

The EMBO Journal (2012) 31, 3118–3129. doi:10.1038/emboj.2012.167; Published online 22 June 2012

Subject Categories: signal transduction; immunology

Keywords: neutrophil; PI3K; PLC β ; Ras; RasGRP4

Introduction

G protein-coupled receptors (GPCRs) control a variety of neutrophil responses, including chemotaxis and reactive oxygen species (ROS) formation, which are important in health and disease (Simon and Green, 2005; Ley *et al.*, 2007). The intracellular signals coordinating these responses are complex, operate over time scales spanning

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Received: 13 January 2012; accepted: 15 May 2012; published online: 22 June 2012

seconds to hours, are GPCR and context dependent and far from understood. There are, however, a relatively limited number of primary intracellular signals that encode the spatiotemporal characteristics of GPCR and G-protein activation that are of known physiological importance. These include class I PI3Ks (phosphoinositide 3 kinases, particularly PI3K γ), PLC β s (phospholipase C) and small GTPases such as Rac1 and 2, cdc42, RhoA and Rap1 and 2. In this work, we have focused on mechanisms controlling activation of PI3K γ .

PI3K γ is a key effector in a number of myeloid-derived cells (Hirsch *et al.*, 2000; Li *et al.*, 2000; Sasaki *et al.*, 2000) that can be activated directly by G $\beta\gamma$ subunits (Stoyanov *et al.*, 1995; Stephens *et al.*, 1997). PI3K γ synthesizes the signalling lipid PIP₃ and hence can drive activation of PIP₃-binding proteins, such as PKB and several specific regulators of small GTPases. It comprising a regulatory (p84 or p101) and a catalytic (p110 γ) subunits (Stoyanov *et al.*, 1995; Stephens *et al.*, 1997; Suire *et al.*, 2005; Voigt *et al.*, 2006). Full activation by G $\beta\gamma$ s depends on p101 (Suire *et al.*, 2006). The p110 γ subunit contains a Ras Binding Domain (RBD) capable of binding and conveying activation by Ras-family GTPases (Rodriguez-Viciana *et al.*, 1994; Pacold *et al.*, 2000; Suire *et al.*, 2002). The RBD is clearly essential for activation of PI3K γ by GPCRs (Suire *et al.*, 2006). It is unclear, however, whether Ras proteins regulate the RBD of PI3K γ *in vivo* in the context of studies indicating that other small GTPases can control class I PI3Ks (Shin *et al.*, 2005).

Current thinking about small GTPases, such as Ras, suggests that they are molecular switches that exist in either a GTP-bound, signalling-competent state or a GDP-bound, basal state. GTP-, but not GDP-, bound GTPases can activate specific proteins with domains, for example, CRIB or RBD, evolved to bind them. Guanine nucleotide Exchange Factors (GEFs) act to stimulate GTP loading. GTPase Activating Proteins (GAPs) act to return the GTPases to their GDP-bound state. Hence, Ras can in principle be activated by activation or inhibition of relevant GEFs and/or GAPs.

Ras-family GTPases, in neutrophils K- and N-Ras, are activated rapidly and substantially following engagement of GPCRs (Zheng *et al.*, 1997). This signal is important for activation of the protein kinase, Raf (Marshall, 1996), and hence the canonical p42/p44-MAPK pathway and also has the potential to activate PI3K γ (see above and below) and hence a range of PIP₃-regulated responses.

Despite the central role of Ras proteins in neutrophils the mechanism by which they are activated is entirely unknown. Further, there is no clear picture of how Ras can be activated by GPCRs in other cell types that could be assumed to apply in neutrophils (Downward, 2003).

PLC β 2 and PLC β 3 are expressed in neutrophils and can be activated by a range of GPCRs, *via* both G $\beta\gamma$ and G α_q subunits (Smrcka *et al.*, 1991; Taylor *et al.*, 1991; Camps *et al.*, 1992), to hydrolyse PI(4,5)P₂ and drive accumulation

of its products, diacylglycerol (DAG) and Ins(1,4,5)P₃. DAG can activate DAG/phorbol ester-binding, C1 domain-containing effectors, such as the conventional or novel PKCs, and Ins(1,4,5)P₃ activates intracellular Ca²⁺ mobilization, both of which are important regulators of ROS formation and cell migration (Li *et al*, 2000; Shi *et al*, 2007). Although PI3K γ and PLC β 2/ β 3 use a common substrate lipid it is thought they represent independent, master regulators of ‘parallel’, GPCR-sensitive, signalling cassettes in neutrophils (Tang *et al*, 2011).

In summary, it is clear that the RBD of PI3K γ is crucial for GPCR-stimulated accumulation of PIP₃ (Suire *et al* 2006), despite this and the demonstrated importance of the RBDs of other class I PI3Ks in many other broadly important biological settings such as chemotaxis in *Dictyostelium* (Sasaki *et al*, 2004), control of cell size in *Drosophila* (Orme *et al*, 2006) and tumorigenesis (Gupta *et al*, 2007), the signalling networks controlling these domains and whether Ras-, or another family of, GTPases, regulate the RBDs *in vivo* are unclear. Furthermore, the molecular mechanisms by which GPCRs activate Ras, in both neutrophils and more broadly, are poorly defined.

We have addressed these issues by attempting to define how GPCRs control Ras and the RBD of PI3K γ in neutrophils.

Results

A targeted shRNAi screen to identify a RasGEF in a human neutrophil-like cell line required for fMLP-stimulated activation of Ras

We addressed the nature of the GPCR-sensitive mechanism driving activation of Ras in neutrophils. On the basis of the rapid response, the very low basal levels of Ras-GTP and precedent, we hypothesized that a relevant RasGEF would be receptor sensitive. We screened human neutrophils for expression of all of the potential RasGEFs in the human genome (cdc25 domain-containing proteins that were either known to use Ras-family proteins as substrates or were of unknown specificity). Nine candidates could be detected by reverse transcriptase PCR (RT-PCR) in mRNA from either differentiated PLB-985 cells (human neutrophil-like) or human peripheral blood-derived neutrophils (RasGEF1c, RasGEF2, RasGRF1, Bcar3, Sos1, Sos2, RasGRP3, RasGRP4 and Sh2d3c). We positively selected PLB-985 cells stably expressing a single shRNAi directed against candidate RasGEFs or a control target (typically three different, stable, shRNAi-expressing cell lines for each target). fMLP-stimulated activation of Ras was then assessed by a Raf-RBD ‘pull-down assay’ in differentiated cells. This revealed that shRNAi suppression of RasGRP4 (about 85% reduction in protein levels, see Supplementary Figure 1) but not any of the other candidate RasGEFs reduced activation of Ras by fMLP (Figure 1). Further experiments showed that suppression of RasGRP4 also reduced fMLP-stimulated PIP₃ accumulation, indicating that the GTP-Ras which we were assaying was relevant to activation of PI3K γ (see Supplementary Figure 1).

RasGRP4 message has a restricted distribution within the myeloid compartment and although expressed strongly in mast cell lineages, it is also detectable in monocytes and granulocytes (Reuther *et al*, 2002; Yang *et al*, 2002). RasGRP4 has RasGEF activity (Reuther *et al*, 2002; Yang *et al*, 2002), can regulate the MAPK pathway and transcriptional targets in

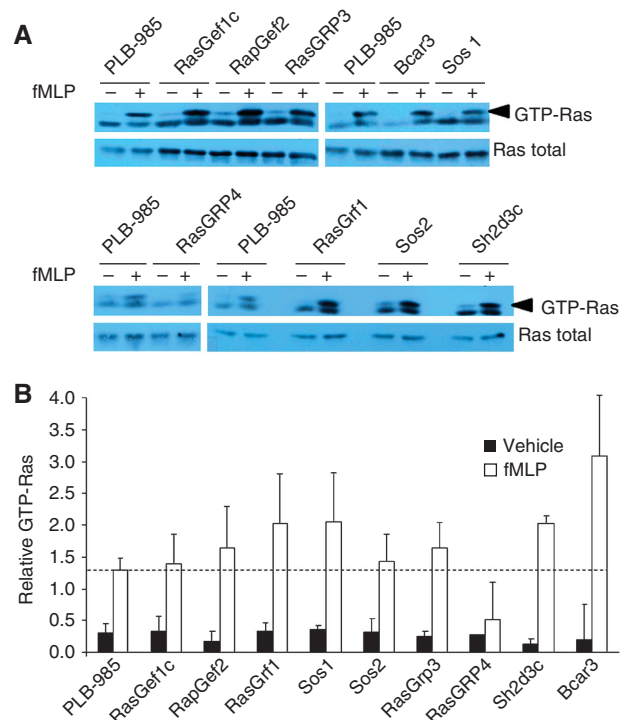


Figure 1 An shRNAi screen for suppressors of fMLP-stimulated Ras activation in PLB-985 cells. Nine potential RasGEFs were detected in human neutrophils or differentiated PLB-985 cells. A panel of PLB-985 cell lines was established expressing, shRNAi directed against these RasGEFs, control targets or vector alone (two, or mostly three, independent cell lines each expressing a different shRNAi for each target were derived by lentiviral transduction and use of bis-cistronically expressed eGFP as a selectable marker). Cell lines were differentiated, stimulated with fMLP (100 nM, 30 s) and Ras activation was assayed by pull-down assay with Raf-RBD. (A) Representative immunoblots showing fMLP (+), or its vehicle (-), stimulated Ras activation in parental PLB-985 cells or in lines expressing shRNAi against the indicated RasGEFs. Immunoblots of the total Ras in the lysates from which the pull downs were prepared are also shown. (B) Quantification of data, like that shown in (A), from at least three experiments for each shRNAi-expressing cell line presented as the relative amount of GTP-Ras corrected for total Ras, in the form of mean values \pm s.e. Figure source data can be found with the Supplementary data.

mast cells (Katsoulotos *et al*, 2008). Aberrant expression of RasGRP4 may be associated with disease (Watanabe-Okochi *et al*, 2009). Recent reports have suggested that Fc ϵ RI receptor-dependent, and PMA-induced, responses in mouse mast cells and inflammatory reactions in, some but not all, mouse models of inflammation are reduced in the genetic absence of RasGRP4 (Adachi *et al*, 2012; Zhu *et al*, 2012). However, the position of RasGRP4 in the fabric of intracellular signalling and its physiological roles in neutrophils are unclear.

Disruption of the mouse RasGRP4 gene

To validate the results of the screen and address the physiological role of RasGRP4 and Ras activation in mouse neutrophils, we disrupted the mouse *RasGRP4* gene using standard homologous targeting strategies (see Supplementary Methods and Supplementary Figure 2). Two independent, correctly targeted ES clones were used to derive mouse strains

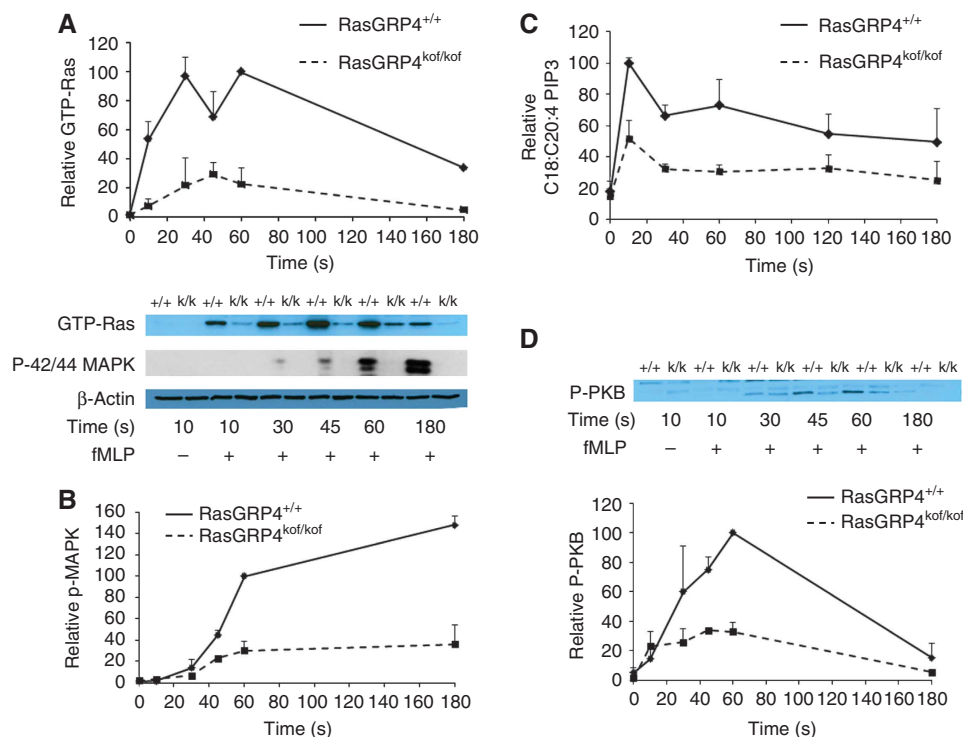


Figure 2 fMLP-stimulated activation of Ras, p42/p44 MAPKs, PKB and PIP₃ accumulation is reduced in RasGRP4^{kof/kof} mouse neutrophils. Neutrophils were prepared from either RasGRP4^{kof/kof} or RasGRP4^{+/+} mice and their responses to fMLP (10 μM) in a range of assays was compared. (A) The lower part shows representative immunoblots measuring fMLP-, or vehicle- (plotted at 0 s in the graphs), stimulated Ras activation (by RafRBD pull down) and p42/p44 MAPK activation, with anti-phospho-T202/Y204 antibodies, and the amount of β-actin, as a loading control, in aliquots of the lysates from which the pull downs were performed. The upper part presents quantification of data from three independent experiments, like those shown in the lower part, showing activation of Ras. Data are presented as means ± s.e., n = 3 and are expressed as % of the samples stimulated for 1 min (100%), normalized to their respective loading control. (B) Quantification of p42/p44 MAPK phosphorylation from three separate experiments, like those shown in the lower part of (A) is shown. Data are presented as means ± s.e., n = 3 and are expressed as % of the samples stimulated for 1 min (100%), normalized to their respective loading controls. (C) Quantification of stearoyl/arachidonoyl-PIP₃, as a ratio with the recovered PIP₃-ISD, in fMLP- (10 μM) or vehicle (plotted at 0 s) -stimulated neutrophils, from three independent experiments is shown. Data are presented as means ± s.e., n = 6 (D) The upper part shows a representative immunoblot measuring fMLP-, or vehicle- (plotted at 0 s in the graph), stimulated activation of PKB, with anti-phospho-S473-PKB antibodies, aliquots of lysates from the experiment shown in (A). The lower part shows quantification of phosphorylation of S473-PKB from three independent experiments for RasGRP4^{+/+} and two independent experiments for RasGRP4^{kof/kof}, like that shown in the upper part, as means ± s.e., n = 3 for RasGRP4^{+/+} or as means ± s.d., n = 2 for RasGRP4^{kof/kof} and are expressed as a % of the samples stimulated for 1 min (100%), normalized *via* their respective loading controls. Figure source data can be found with the Supplementary data.

carrying the targeted RasGRP4 allele in their germline. RasGRP4^{+/+} and RasGRP4^{kof/kof} mice were derived from each strain and were used in the experiments described below. We observed no significant differences between the two independent RasGRP4^{kof/kof} strains. RasGRP4^{kof/kof} mice were; born at expected Mendelian ratios, of normal weight and appearance, fertile and had normal blood cell counts (see Supplementary Figure 2). These results suggested that haematopoiesis was unperturbed by loss of RasGRP4 expression. Isolated, bone marrow-derived neutrophils from RasGRP4^{kof/kof} mice appeared to be fully responsive in a number of assays (details below), suggesting that they were fully differentiated and broadly functional. We crossed RasGRP4^{kof/kof} mice with FlpE- and Cre-expressing strains sequentially to derive a strain, RasGRP4^{-/-}, containing minimal heterologous DNA and lacking the cDNA for exons 5 and 6, encoding the catalytic domain, of RasGRP4 (see Supplementary Figure 3 and Supplementary Materials and methods). RasGRP4^{-/-} mice were viable and fertile and their neutrophils responded indistinguishably to those from RasGRP4^{kof/kof} mice in specific experiments (see below).

RasGRP4 is required for fMLP-stimulated activation of Ras and PI3K γ pathways

fMLP failed to stimulate significant activation of Ras in neutrophils from RasGRP4^{kof/kof} mice (Figure 2A). The simplest explanation of this result is that RasGRP4 has a unique role as an fMLP-sensitive RasGEF in both mouse and human neutrophils.

Next, we addressed whether the reduction in Ras activation in RasGRP4^{kof/kof} neutrophils also reduced activation of predicted Ras effector pathways. We found fMLP-stimulated phosphorylation of p42/p44 MAPKs (T202/Y204), PIP₃ accumulation and phosphorylation of PKB (S473) were severely reduced in RasGRP4^{kof/kof} neutrophils (Figure 2). This indicates that the reduction in fMLP-stimulated Ras activation in neutrophils lacking RasGRP4 is sufficient to suppress stimulation of the p42/p44-MAPK and PI3K γ /PKB pathways.

Role of RasGRP4 in regulation of Rac and Rap GTPases in neutrophils

Rac GTPases are central regulators of neutrophil responsiveness and can be rapidly activated by GPCRs *via* the RacGEFs

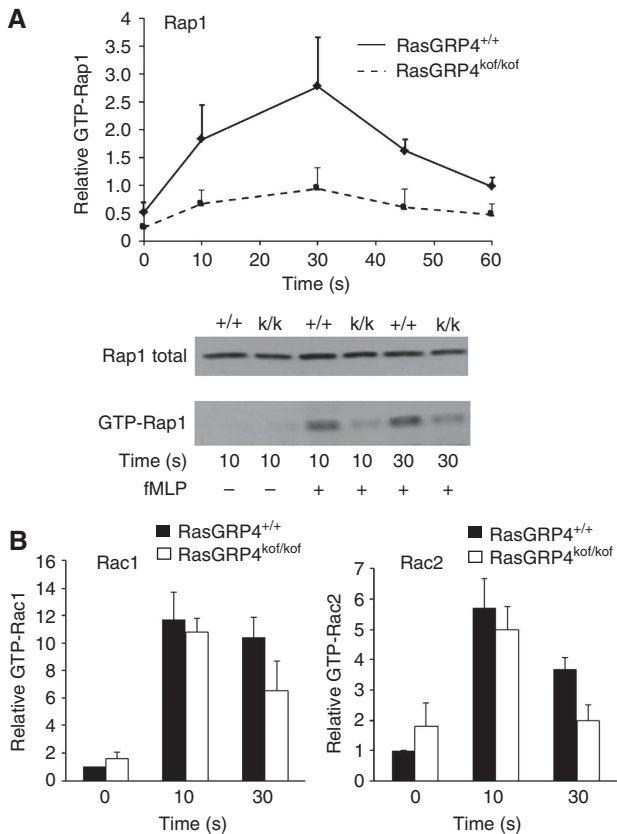


Figure 3 fMLP-stimulated activation of Rap1 and Rac1 and 2 in RasGRP4^{kof/kof} mouse neutrophils. Bone marrow-derived neutrophils were prepared from either RasGRP4^{kof/kof} or RasGRP4^{+/+} mice and assayed for their ability to support fMLP-stimulated activation of Rap1 and Rac1 and 2. (A) The lower part shows a representative immunoblot of fMLP (+, 10 μ M), or vehicle (-), induced activation of Rap1, measured by pull down with RalGDS bait, in RasGRP4^{+/+} (+/+) or RasGRP4^{kof/kof} (k/k) neutrophils. The upper part shows quantification of data, like that shown in the lower part, from five independent experiments, presented as the relative amount of Rap1-GTP/total Rap1, normalized between experiments by correcting the overall mean ratio for each experiment to the same value, in the form of means \pm s.e. ($n = 4$ or 5). The reduction in Rap1 activation was judged significant by comparing the ratio of the area under the curve for RasGRP4^{kof/kof}/RasGRP4^{+/+} by a one sample of a t -test, $P = 0.0357$. (B) fMLP-induced activation of Rac1 and Rac2 was quantified by pull down with PAK-CRIB bait and immunoblotting with anti-Rac1 or Rac2-specific antibodies. Results were normalized *via* the amount of GTP-Rac in unstimulated RasGRP4^{+/+} neutrophils in each experiment. The data are presented as means \pm s.e., $n = 4$ (the reduction in activation, in RasGRP4^{kof/kof} compared with RasGRP4^{+/+} neutrophils, of Rac2 was significant at $P = 0.03$ while the one of Rac1 was not significant at $P = 0.11$). Figure source data can be found with the Supplementary data.

DOCK2 (Kunisaki *et al*, 2006) and PReX-1 (Welch *et al*, 2005). PIP₃ is thought to regulate the intracellular distribution of both RacGEFs but the activity of only PReX-1. We measured activation of Rac1 and 2 by fMLP using a PAK-CRIB pull-down assay and found that in RasGRP4^{kof/kof} neutrophils fMLP-induced activation of Rac1 and 2 was not significantly changed after brief stimulation, but slightly reduced after longer times of stimulation (Figure 3B). The timescale over which the absence of RasGRP4 has an effect on Rac activation is in-keeping with the relatively slow appearance of a phenotype in the activation of another PIP₃ effector, PKB

(Figure 2D) and the multifactorial regulation of Rac activity in these cells. This suggests that rapid activation of Rac by fMLP is largely unchanged by the absence of RasGRP4 and hence that this phenotype is not a result of a broad reduction in G-protein signalling and that the weak reduction in Rac activation at later times probably results from reduced PIP₃-dependent activation of RacGEFs.

In the context of the similarities between Ras and Rap GTPases and the observation that RasGRP2 is a physiological RapGEF (Crittenden *et al*, 2004), we assayed fMLP-stimulated activation of Rap1 using RalGDS-RBD and found it to be reduced in RasGRP4^{kof/kof} compared with RasGRP4^{+/+} neutrophils (Figure 3A). This result was unexpected in the context of work that indicated RasGRP4 is a RasGEF but not a RapGEF when transfected into cells (Reuther *et al*, 2002) and genetic evidence that suggested RasGRP2 is required for GPCR-stimulated Rap1 activation in mouse neutrophils (Bergmeier *et al*, 2007). The simplest explanation of this result is that RasGRP4 can be both a Ras and Rap1GEF although it remains possible that the reduction in GTP-bound Rap1 is an indirect result of inhibition of Ras activation.

The role of RasGRP4 in regulation of ROS formation and neutrophil migration

We sought to establish the consequences of loss of RasGRP4 on the functional responses of neutrophils. We measured ROS formation in response to the GPCR ligands C5a, LTB₄, fMLP and the phorbol ester, PMA, that bypasses cell surface receptors and activates a subset of C1 domain-containing proteins, such as PKCs and RasGRPs, directly. The amount of ROS produced in response to all three GPCR ligands was reduced substantially in neutrophils from both RasGRP4^{kof/kof} and RasGRP4^{-/-}, compared with RasGRP4^{+/+} mice, across all times of stimulation and doses of agonist (Figure 4A and B). In contrast, PMA-stimulated ROS formation was increased slightly in RasGRP4^{kof/kof} and RasGRP4^{-/-} compared with RasGRP4^{+/+} mouse neutrophils (Figure 4A and B).

These results are entirely consistent with past work showing that either loss of PI3K γ or knock-in of a Ras-insensitive mutant PI3K γ substantially reduced fMLP-stimulated ROS formation (Li *et al*, 2000; Suire *et al*, 2006) and hence that RasGRP4 and Ras regulate ROS formation *via* the RBD of PI3K γ . These results also suggest that PMA-stimulated ROS formation is not dependent on activation of Ras and its downstream targets and is presumably mediated entirely by conventional and/or novel PKC species. In addition, the results suggest that the reduction in GPCR-mediated ROS formation in both the RasGRP4^{kof/kof} and RasGRP4^{-/-} neutrophils is extremely likely a result of loss of RasGRP4 specifically.

We also assayed chemotaxis of isolated mouse neutrophils in gradients of fMLP in an EZTaxiscan chamber. These experiments revealed that RasGRP4^{kof/kof} neutrophils moved in the fMLP gradients with the same migratory index, mean velocity when moving and mean total accumulated distance on both glass and fibrinogen-coated glass as RasGRP4^{+/+} neutrophils (Figure 5B). However, the proportion of RasGRP4^{kof/kof} neutrophils that moved in response to fMLP on both glass and fibrinogen-coated glass was lower than for RasGRP4^{+/+} neutrophils (Figure 5C), which is

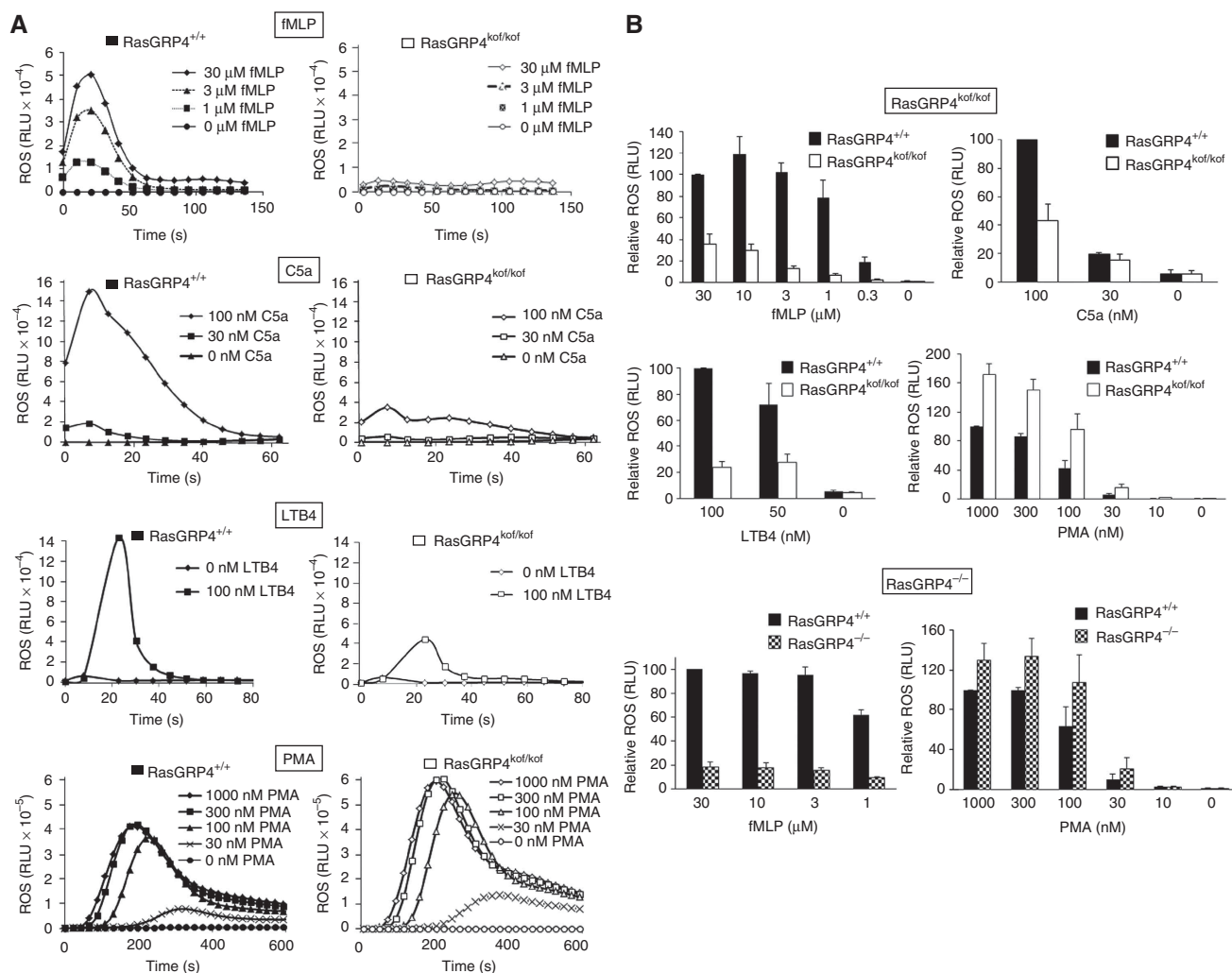


Figure 4 ROS responses displayed by neutrophils from RasGRP4^{kof/kof} and RasGRP4^{-/-} mice. (A) Neutrophils were prepared from either RasGRP4^{kof/kof} (right side) or RasGRP4^{+/+} (left side) mice and stimulated with different concentrations of fMLP, C5a, LTB4 or PMA (from top to bottom panels) and the rate of ROS formation was measured. The data presented are from a single experiment typical of three or four experiments. (B) In the upper four panels, data from experiments shown in (A) were integrated over time to generate an estimate of the total ROS generated in response to each stimulus (RasGRP4^{+/+} in solid black, RasGRP4^{kof/kof} in white). The two lower panels show integrated ROS in response to either fMLP (left) or PMA (right) for neutrophils derived from RasGRP4^{+/+} (solid black) or RasGRP4^{-/-} (chequered) mice. The data are means \pm s.e., from four independent experiments (fMLP and PMA for RasGRP4^{kof/kof}) or three experiments (C5a, LTB4 and RasGRP4^{-/-} data).

remarkably similar to the phenotype of PI3K γ -deficient cells (Ferguson *et al*, 2007). Surprisingly, however, when we tested the ability of neutrophils to migrate into an aseptically inflamed peritoneum, there was no difference between RasGRP4^{kof/kof} and RasGRP4^{+/+} mice, despite the fact that a number of mouse models with loss of PI3K γ function show reduced responses in these assays (Suire *et al*, 2006; Figure 5A). We consider this is in part a result of the fact that PI3K γ has roles in endothelial cells, which do not express RasGRP4, that support extravasation of neutrophils (Puri *et al*, 2005) and in part due to an undefined, additional, inhibitory, PI3K γ -independent role for RasGRP4 in migration of neutrophils into the peritoneum (see below, possibly involving PLC β 2/ β 3).

The molecular mechanism regulating activation of RasGRP4

We sought to define the molecular mechanism by which fMLP activates RasGRP4. Other members of the RasGRP4

family are either argued to be activated by increased free cytosolic Ca²⁺ acting *via* the tandem EF-hand domain in the case of RasGRP2 (Stefanini *et al*, 2009) (the C1 domain of RasGRP2 does not appear to bind DAG (Johnson *et al*, 2007) or *via* coincident PKC-mediated phosphorylation of T133/T184 and binding of DAG to the C1 domain in the case of RasGRP1/3 (Zheng *et al*, 2005). RasGRP4 possesses a similar overall topology to its other family members but is very unlikely to be regulated by Ca²⁺ as its tandem EF hand-like domain lacks key residues known to be required for binding of Ca²⁺. Furthermore, RasGRP4 lacks the PKC sites equivalent to those required for activation of RasGRP1/3 (Stone, 2011). Interestingly, the mouse genome-sequencing project has predicted the existence of two splice variants of RasGRP4, differing on the basis of a 5aa insert into a loop forming part of the lipid-binding pocket of the C1 domain (Johnson *et al*, 2007) (the longer variant is not found in the human genome). The sequence of the shorter variant and human RasGRP4 fits well with a subfamily of C1 domains

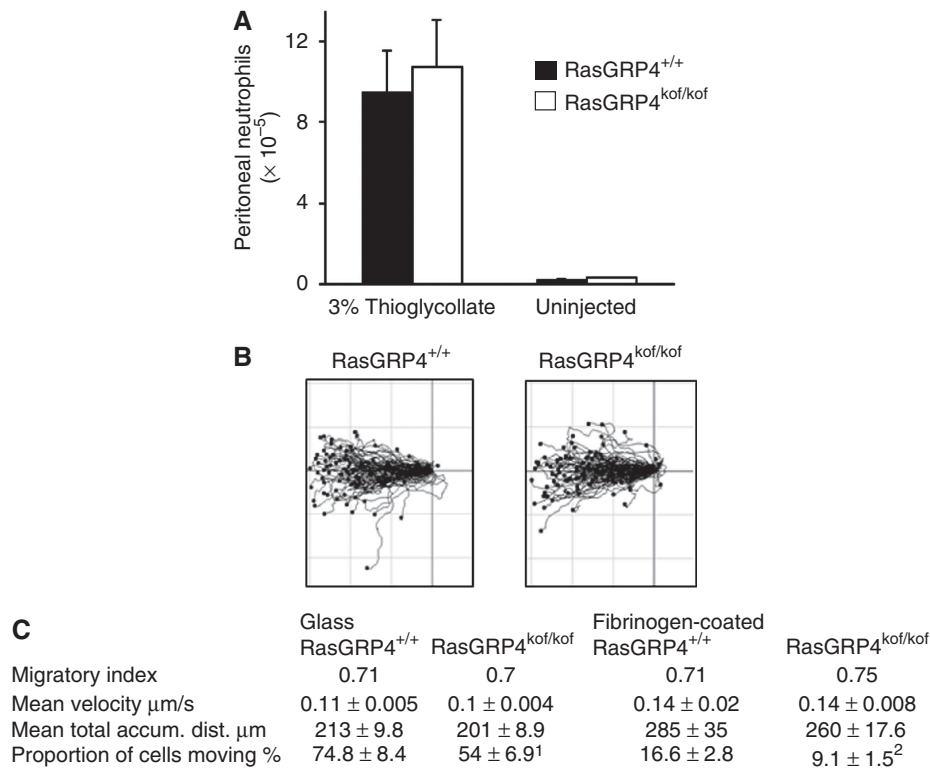


Figure 5 Neutrophil migration *in vitro* and *in vivo*. (A) RasGRP4^{kof/kof} and RasGRP4^{+/+} mice were injected intraperitoneally with thioglycollate. After 3.5 h, the neutrophils that had migrated into the peritoneum were quantified. The data are presented as means \pm s.e. from 4 independent experiments including a total of 14 RasGRP4^{+/+} and 15 RasGRP4^{kof/kof} mice. (B) Neutrophils were isolated from RasGRP4^{kof/kof} or RasGRP4^{+/+} mice and assayed for their ability to respond to, and migrate within, fMLP gradients on either untreated glass or fibrinogen-coated glass. Data are presented as centre-zeroed individual tracks (untreated glass only) for cells that moved a total accumulated distance of $>25 \mu\text{m}$. (C) Data were extracted from individual cell tracks like those in (B) using the ImageJ-plugin Chemotaxis tool. The % of cells that moved (a total accumulated distance of $>25 \mu\text{m}$) are presented as means \pm s.e. A univariate analysis of variance showed ¹ $P=0.014$ and ² $P=0.041$.

known to bind phorbol esters and DAGs (e.g., PKC δ (C1b), β 2-chimaerin, RasGRP1 and 3) and have been demonstrated to bind these molecules both *in vitro* and in transfected cells (Reuther *et al*, 2002; Yang *et al*, 2002; Johnson *et al*, 2007); the longer variant disrupts the binding motif and has been shown unable to bind lipid species (Johnson *et al*, 2007). We investigated public mRNA-seq data sets from mouse neutrophils for raw sequence information spanning the key region of the potential 15bp insert and found three independent sequence reads that spanned the splice boundary in a manner consistent with the shorter, and not the longer, variant. We concluded that the shorter, DAG-binding, variant of RasGRP4 is expressed in mouse neutrophils and that DAG could be a signal controlling RasGRP4.

DAG metabolism is complex, with a number of potential routes of synthesis and degradation spread across several membrane compartments. Both PLD activity, *via* hydrolysis of PtdCho and production and dephosphorylation of PtdOH, and PLC activity, *via* hydrolysis of PIP₂, can mediate receptor-stimulated increases in DAG. It is possible to preferentially monitor the pool of DAG produced by PLCs and thought to regulate signalling effectors like PKCs, by specifically measuring the levels of the molecular species of DAG that are enriched in PIP₂, particularly stearoyl/arachidonoyl-(C18:0/C20:4)-DAG (Pessin and Raben, 1989). Previously, this has been achieved by derivatization and HPLC (Pettitt

and Wakelam, 1993) or by lipidomic/mass spectrometry-based methods (Gorden *et al*, 2011), but in both cases demands significant input material. We have developed a method to quantify stearoyl/arachidonoyl-DAG in small numbers of neutrophils (0.5×10^6) based on use of an internal spike (ISD) of deuterated, D6-stearoyl/arachidonoyl-DAG to trace recovery of the endogenous lipid through a neutral lipid extraction, in-line chromatography on a C4 column and analysis by electrospray mass spectrometry using Multiple Reaction Monitoring. This assay revealed that fMLP stimulated a two-fold increase in stearoyl/arachidonoyl-DAG in mouse neutrophils (Figure 6B).

GPCR-stimulated increases in Ins(1,4,5)P₃, cytosolic-free Ca²⁺ and PKC activation are abolished in PLC β ^{-/-} \times PLC β 3^{-/-} mouse neutrophils (Li *et al*, 2000). We found fMLP-stimulated accumulation of stearoyl/arachidonoyl-DAG was also abolished in PLC β 2^{-/-} \times PLC β 3^{-/-} neutrophils (Figure 6B). This result indicates that PLC β 2/ β 3 are responsible for fMLP-stimulated DAG formation in mouse neutrophils.

To test the hypothesis that RasGRP4 is activated by DAG generated by PLC β 2/ β 3, we measured fMLP-elicited activation of Ras in PLC β 2^{-/-} \times PLC β 3^{-/-} neutrophils and found it much reduced compared with PLC β 2^{+/+} \times PLC β 3^{+/+} neutrophils (Figure 6A). Furthermore, acute treatment with the partially selective (Klein *et al*, 2011) PLC inhibitor U73122, but not the inactive analogue U73343, completely inhibited

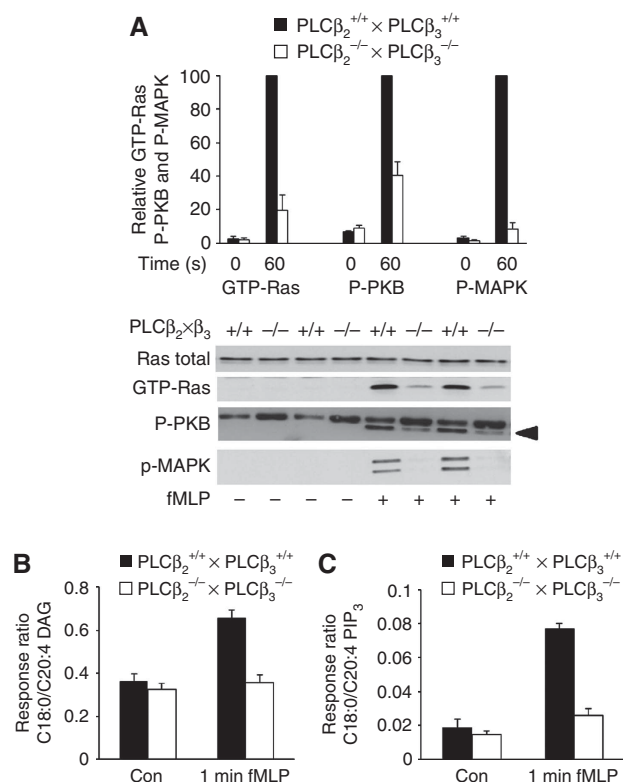


Figure 6 Genetic blockade of PLC β_2/β_3 signalling inhibits fMLP activation of Ras, p42/p44 MAPK, PKB and PIP $_3$ accumulation. Neutrophils were prepared from either PLC $\beta_2^{-/-}$ x PLC $\beta_3^{-/-}$ or PLC $\beta_2^{+/+}$ x PLC $\beta_3^{+/+}$ mice, stimulated with fMLP (10 μ M for 1 min), or its vehicle, and a range of responses were quantified. (A) Lower part shows representative immunoblots of Ras activation by pull down, and phosphorylation of S473-PKB (P-PKB) and T202/Y204-p42/p44 MAPK (P-p42/p44-MAPK) and total Ras (as a loading control) in aliquots of the lysates from which the pull downs were performed. The upper part shows quantification of data, like that below, from three independent experiments and are expressed as means \pm s.e., $n = 8$, as a % of the stimulated samples in that experiment, normalized *via* their respective loading controls. (B, C) Measurements of neutrophil stearoyl/arachidonoyl-DAG or stearoyl/arachidonoyl-PIP $_3$ as response ratios with the ISD recovered in that sample are shown. The data presented are means \pm s.d., $n = 8$, from three independent experiments. Figure source data can be found with the Supplementary data.

fMLP-stimulated activation of Ras in wild-type mouse neutrophils (see Supplementary Figure S4). A PLD1/2-selective inhibitor, VU0155056 (Scott *et al*, 2009), only inhibited the response weakly (see Supplementary Figure 4). Together, these results indicate that PLC β_2/β_3 -generated DAG can activate RasGRP4.

We tested that idea that PLC β_2/β_3 are required for full activation of MAPK and PIP $_3$ signalling in fMLP-stimulated neutrophils. We found that PIP $_3$ accumulation and phosphorylation of both PKB and p42/p44 MAPKs were substantially reduced in fMLP-stimulated PLC $\beta_2^{-/-}$ x PLC $\beta_3^{-/-}$ mouse neutrophils compared with their wild-type controls (Figure 6A and C). Furthermore, PIP $_3$ and PKB phosphorylation were also inhibited by U73122 (see Supplementary Figure 4). These results suggest that PLC β_2/β_3 powerfully regulate fMLP-stimulated PI3K γ and Raf/p42/p44 MAPK signalling.

We tested whether loss of RasGRP4 had an unexpected impact on other DAG effectors such as PKCs. We measured fMLP-stimulated, PKC δ -dependent phosphorylation of T154 in p40^{PHOX} (Chessa *et al*, 2010). It was not significantly reduced in RasGRP4^{kof/kof} compared with RasGRP4^{+/+} mouse neutrophils (Supplementary Figure 5) and hence concluded activation of other DAG effectors was normal in the absence of RasGRP4.

The simplest explanation for this body of results is that following GPCR activation of neutrophils PLC β_2/β_3 -generated DAG regulates RasGRP4 and hence activation of Ras and the PI3K γ and Raf/MAPK pathways. There are some details in our results that suggest this is not the complete story. The extent of the inhibition of PI3K γ by blockade of PLC β appears greater than that inflicted by removing RasGRP4, or knocking-in a Ras-insensitive version of PI3K γ , suggesting that although the major route by which PLC β s control this cassette is through RasGRP4 and Ras there may be an additional route by which PLCs modulate this pathway.

Discussion

The above results indicate that RasGRP4 is the major fMLP-sensitive RasGEF in mouse and human neutrophils and that it is regulated by PLC β_2/β_3 -derived DAG. They also indicate that Ras is the direct, dynamic regulator of the RBD domain of PI3K γ *in vivo* and, therefore, that PLC β signals shape class I PI3K responses in these cells (Figure 7). Many receptors are capable of simultaneously activating PLCs and class I PI3Ks and in some cells (e.g., B cells and mast cells) activation of PLC γ s can depend on class I PI3K signalling (Huber *et al*, 1998; Scharenberg *et al*, 1998). This is the first clear demonstration, however, that PLCs can regulate PI3Ks.

Previous work has suggested that the RBDs of PI3Ks are an important regulatory input but that they are likely to work in synergy with other signals, for example, with G $\beta\gamma$ subunits in the case of PI3K γ (Suire *et al*, 2006). This is consistent with the inability of phorbol esters to directly stimulate PIP $_3$ accumulation or PKB activation in neutrophils or neutrophil-like cells, although they can drive p42/p44-MAPK activation (Stephens *et al*, 1993; Poon and Stone, 2009), which is presumably a function of the fact that Ras activation is sufficient for Raf, but not PI3K γ , activation *in vivo*.

Given the restricted distribution of RasGRP4 message it is unlikely that RasGRP4 is a universal link between GPCRs and Ras activation, however, given the history of molecules that had been declared not to be expressed in tissues in which they were subsequently found to have important roles by genetic deletion, this is not yet completely clear. Superficially, it would seem that both RasGRP1 and 3 could be activated by GPCR receptors *via* PLC-generated DAG and associated PKC activity. However, it seems that although they are expressed in mouse neutrophils they do not fulfill this role. This apparent segregation in function could be a consequence of either the relatively weak expression of RasGRP3 in these cells or an unappreciated need for the enzymes to be associated with a phosphotyrosine-based signalling complex to organize efficient PKC-mediated phosphorylation. Similarly, there is strong evidence that RasGRPs can be activated by Ca²⁺ signals in brain; however, it is not clear whether they can respond to a pure GPCR stimulus.

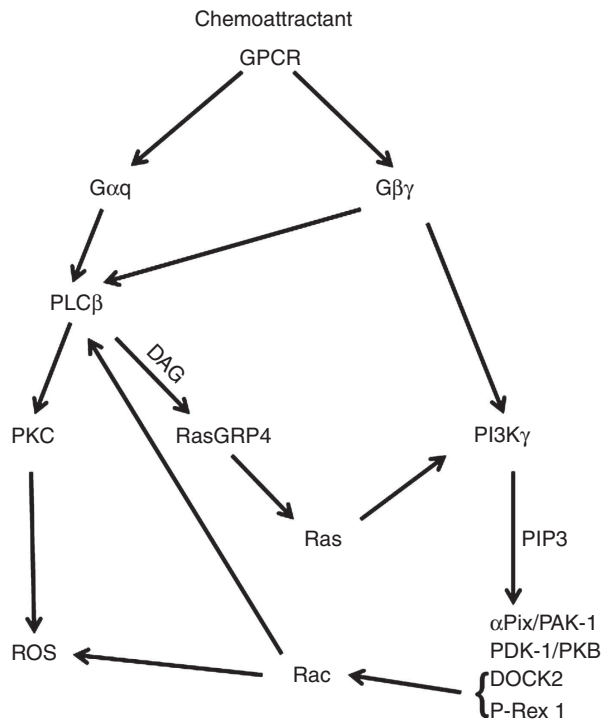


Figure 7 A schematic mapping RasGRP4 as an effector of PLC β -derived DAG and a regulator of PI3K γ upstream of ROS formation. There is strong biochemical and genetic evidence indicating that these connections are important in neutrophil function, with the possible exception of the idea that Rac can regulate PLC β , where, although the underpinning biochemical data are very clear, the genetic evidence is contradictory.

Our data also suggest that RasGRP4 can act as a GPCR-sensitive, DAG-regulated Rap1GEF. As RasGRP2 is a GPCR-sensitive, Ca²⁺-regulated Rap1GEF, this indicates that Rap1 activity is independently controlled *via* different signals, with contrasting spatiotemporal properties, that both emerge from activation of PLC β s. Perhaps, this is related to the range of GPCR-driven cell responses, spanning from sub-second to many minutes, with which Rap GTPases are associated in myeloid cells.

Given the above results, and the phenotypes of both p110 γ ^{-/-} and Ras-insensitive p110 γ -knock-in mice, we had expected that migration of neutrophils into an inflamed peritoneum would be reduced in RasGRP4^{kof/kof} compared with RasGRP4^{+/+} mice. Hence, we were surprised to find migration was unchanged. We note, however, that in the absence of PLC β 2/ β 3 neutrophil migration to sites of inflammation *in vivo* is, for unexplained reasons, increased (Li *et al*, 2000). We would argue that this presumably inhibitory role for endogenous PLC β 2/ β 3 in part involves RasGRP4 and this, combined with the fact neutrophil migration into the peritoneum depends on p110 γ in endothelial cells that do not express RasGRP4, may underlie the unexpected contrast in the phenotypic consequences of loss of PI3K γ versus RasGRP4 in this regard.

A recent study suggested, in contrast to this manuscript, that PLC β 2/ β 3 and PI3K γ operate on ‘parallel’ and not ‘serial’ signalling pathways downstream of GPCRs in mouse neutrophils (Tang *et al*, 2011). The only result in that study which appears inconsistent with our work is that fMLP-stimulated

activation of PKB was not reduced in the absence of PLC β 2/ β 3, where we find a 60% reduction (and larger reductions in fMLP-stimulated PIP₃ accumulation and Ras activation). We have no explanation for this difference as we imported both the PLC β 2^{-/-} × PLC β 3^{-/-} mice and their controls from the animal facility that supported the study, to Babraham for these experiments.

Previous work has shown that GPCR-mediated activation of Ras in neutrophils is conveyed by pertussis toxin-sensitive G proteins (Zheng *et al*, 1997). This result is in-keeping with the idea that PLC β 2/ β 3, like PI3K γ , are activated by relatively large amounts of G $\beta\gamma$ subunits released from these abundant G-protein species. PLC β 2/ β 3 can also be regulated directly by Rac GTPases and GTP-bound G α q-family subunits. It is unclear to what extent these different mechanisms contribute to how, where and when PLC β 2/ β 3, Ca²⁺ and DAG signals are delivered *in vivo* and may read-through to downstream targets such as PI3K γ . Previous work has indicated that the RBD of PI3K γ , but not G $\beta\gamma$ s/p101, are required for GPCR regulation of ROS formation and yet other PI3K γ -dependent neutrophil responses were similarly dependent on both routes of activation. It was concluded that this was likely based upon the principle that Ras regulation directed the synthesis of a spatially discrete pool of PIP₃ capable of controlling specific responses, for example, ROS formation (Suire *et al*, 2006). This work is consistent with that and supports the idea that the PLC β /DAG/RasGRP4/Ras pathway provides an input to PI3K γ that determines both the intensity and location, perhaps at Ras-centric nano-clusters, of its signals.

In conclusion, our results suggest that RasGRP4 is a central hub in GPCR-triggered pro-inflammatory pathways in both human and mouse neutrophils. They also reveal an unappreciated role for PLCs and Ras in the regulation of class I PI3Ks that could have wide spread implications, given that other RasGEFs in the RasGRP family can be activated by receptor tyrosine kinases and PLC γ in parallel with PI3Ks α , β and δ (Stone, 2011).

Materials and methods

Cell lines, antibodies and reagents

All materials used were of the lowest endotoxin level available and were purchased from Sigma unless stated otherwise. The polyclonal antibody against hRasGRP4 was raised in rabbit against 14-mer peptide (MNRKDSKRKSHQEC) found in the N-terminal end of the protein and affinity purified. The other antibodies used for western blots were commercially available: anti-pan-Ras (1:1000; Calbiochem); anti-P-PKB-S473 (1:2000; Cell Signalling); anti-Rap1 (1:750; Epitomics); anti- β -actin (1:10 000; Sigma); anti-P-p42/44 MAPK (T202/Y204) (1:2000; Cell Signalling), anti-Rac1 (1:3000; Millipore), anti-Rac-2 (1:300; Millipore), anti-phospho-p40phox (Thr¹⁵⁴) (1:1000; Cell Signalling) and anti- β cop (1:40, gift from Dr N Ktistakis). The PLD1/2 inhibitor VU0155056 (Scott *et al*, 2009) was synthesized at BI by JC and tested extensively in our laboratory (Norton *et al*, 2011). Deuterated and un-deuterated 18:0/20:4-DAG were made by JC/IN by modification of a published procedure (Chen *et al*, 1996). fMLP, PMA and C5a were from Sigma. Leukotriene B4 (LTB4) was from Enzo Life Sciences.

PLB-985 cells were cultured in RPMI supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C in 5% CO₂ in a humidified chamber. Prior to any cell assay, the PLB-985 were differentiated during 6–8 days in medium containing 0.5% N, N-dimethylfomamide without antibiotics.

HEK 293FT (Invitrogen) were cultured in DMEM, complemented with 10% FBS, 1% penicillin/streptomycin at 37°C in 5% CO₂ in a humidified chamber.

Reverse transcriptase PCR analysis

mRNA was extracted from PLB-985 cells using a QIAamp RNA Blood kit (Qiagen) according to manufacturer's instructions. The reverse transcription was performed with Omniscript Reverse Transcription and the amplification of the cDNA was performed with HotstartTaq DNA polymerase (Qiagen) using the following primers in order to check the expression of known RasGEFs in PLB-985 cells. The sequences of the relevant primers are shown in Supplementary data.

Production of lentivirus RasGRP4 shRNA

Using Dharmacon *siDESIGN* Center, we designed oligonucleotides (2–3 individual sets per RasGEF) of 19 mers of sense and antisense strands separated by a loop (flanked by a 5' *Bgl*III and 3' *Clal*/*Hind*III restriction sites) for the following RasGEFs: RasGef1c, RapGef2, RasGrf1, Sos1, Sos2, RasGRP3, sh2d3c and Bcar3, RasGRP4.

Once annealed, the respective double-stranded oligonucleotides were introduced into the bis-cistronic pCMS3-H1p-EGFP plasmid (via the *Bgl*III and *Hind*III sticky ends) and then cloned into a lentivirus expression vector plasmid pLVTH via *Eco*RI/*Clal* sites. Recombinant lentivirus were produced by co-transfecting 293FT cells with the above, pMD4-VSVG and pCMV-DR8.91 using standard procedures. Independent, stable, mixed, populations of cells expressing a single shRNAi were then selected by fluorescence activated cell sorting of GFP-expressing cells.

Generation of RasGRP4 knockout mice

We targeted mouse RasGRP4 using a vector created by the High Throughput Gene Targeting group at the Wellcome Trust Sanger Institute (RasGRP4^{tm2a(KOMP)Wtsi}), we abbreviate this to a 'knockout-first' (kof) allele; see Supplementary Figure 2. The vector was linearized with *Asi*SI and transfected into C57/Bl6-derived ES cells (Bruce 4) and appropriately targeted clones (from a total of 172) were identified by Southern blotting (5', 3' and internal [³²P]-oligonucleotide probes). Two correctly targeted, independent clones of ES cells (E09/AE1 and E09/AE12) were injected into C57/Bl6Tyr^{-/-}-derived blastocysts by the Gene Targeting Facility at Babraham and male chimeras were mated with C57/Bl6Tyr^{-/-} females. Southern blotting and PCR-based approaches were used to confirm germline transmission RasGRP4^{tm2a(KOMP)Wtsi/tm2a(KOMP)Wtsi} ('RasGRP4^{kof/kof}') and RasGRP4^{+/+} mice were derived from each ES cell line. Genotyping of the mice was routinely performed by PCR amplification of the area containing intron 6 and the adjacent lox-P site, generating a 315-bp fragment for Wt and 390-bp fragment from RasGRP4 targeted alleles. The results presented in this manuscript are derived from mice from both of the strains. We crossed RasGRP4^{kof/kof} × FlpE expressing strain (Farley *et al*, 2000, C57/bl-6 background) then progeny of this cross was mated with a Cre-deleter strain (Schwenk *et al*, 1995, C57/bl-6 background) to remove the drug selection and reporter cassettes and derive a RasGRP4^{-/-} strain with minimal heterologous DNA in the RasGRP4 locus (see Supplementary Figure 3 and result Figure 4B).

We obtained PLC β 2^{-/-} × PLC β 3^{-/-} mice (Li *et al*, 2000) and their colony-related wild-type controls in a C57/bl-6 background from Yale University. We included both imported PLC β 2^{+/+} × PLC β 3^{+/+} mice and wild-type C57/bl-6 mice from the Babraham Institute animal facility in experiments alongside imported PLC β 2^{-/-} × PLC β 3^{-/-} mice. We found neutrophils from these two types of 'control' mice responded indistinguishably in terms of fMLP-stimulated PIP₃ accumulation and phosphorylation of S473-PKB and hence pooled results from these strains to give rise to the data set described as 'PLC β 2^{+/+} × PLC β 3^{+/+}' in those assays in Figure 6.

All of the mice used in experiments were kept under specific pathogen-free conditions in Transgenics at the Babraham Institute. This work was performed under Home Office Project license PPL 80/2335.

Purification of mouse neutrophils

Murine neutrophils were isolated at room temperature from bone marrow using Percoll (62 and 55%; Anderson *et al*, 2008) (purity, as assessed by cytopsin and staining with May-Grumwald-Giemsa, was typically between 65 and 85% neutrophils). For Rac activation and chemotaxis assays, the neutrophils were purified

at 4°C (Ferguson *et al*, 2007) (typically between 55 and 75% neutrophils).

'Pull-down' assays

In experiments to measure Ras, Rac1 or 2 or Rap1 activation, neutrophils were stimulated while in suspension (4 × 10⁶/ml in HBSS, at 37°C) rapidly diluted with ice-cold PBS, sedimented by brief centrifugation, aspirated and solubilized into ice-cold lysis buffer containing 1% Triton X-100, 0.12 M NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM HEPES/NaOH pH 7.4 at 4°C. The lysates were sheared through a 25-gauge needle and centrifuged (12 000 r.p.m., 10 min, 4°C). Aliquots of the supernatants mixed with 4 × SDS-PAGE sample buffer and Ras, Rap1 and Rac 1/2 pull-down assays were performed using GST-Raf-RBD, GST-RalGDS and GST-PAK-CRIB proteins, respectively, freshly pre-bound to glutathione-sepharose beads (about 25 μ l of packed beads with 50 μ g of protein bait).

Western blot

After incubation with relevant primary and secondary, HRP-labelled, antibodies, membranes were incubated with ECL reagents (GE Healthcare) and exposed onto light-sensitive film. Protein levels were quantified by 2D densitometry using Aida Image Analyzer software v.3.27. Phosphorylation of T154-p40^{PHOX} was measured by immunoblotting with a phospho-specific antibody as described (Chessa *et al*, 2010).

In-vivo migration

The migration of murine neutrophils into the peritoneum was assessed after 3.5 h after intra-peritoneal injection of thioglycollate (0.25 ml of 3% thioglycollate) by flushing the peritoneal cavity with 2 × 8 ml PBS/5 mM EDTA. After lysis of the red blood cells, leucocytes were double stained for Gr-1 and Mac-1 and analysed by FACS. Neutrophils (double positive) were counted. Parallel, cytopsin were prepared and stained with May-Grumwald-Giemsa stain.

ROS assay

fMLP, C5a, LTB4 and PMA-stimulated ROS formation was measured with an HRP/luminol-based assay and a multiplate luminometer at 37°C (Anderson *et al*, 2008).

EZ-Taxiscan chamber assay

This assay was used to quantify migration of mouse bone marrow-derived neutrophils in gradients of fMLP (optimized to give a clear but submaximal response) as described (Ferguson *et al*, 2007). The cells were imaged every 20 s, with an exposure time of 50 ms, for 33.33 min (100 frames) using a × 10 objective (long-range, 0.3 NA) on an Olympus IX 81 microscope with a Hamamatsu Orca camera, Marzhauser SCAN-IM motorized stage and Till photonics Polychrome V illuminator. The system light path was configured in a reflection mode using incident light at 488/10 nm and reflected light acquired through a 483/32 emission filter. The glass coverslips were washed with concentrated H₂SO₄ and, after rinsing with H₂O, with 0.25 M NaOH before being washed with H₂O and stored under ethanol. Cleaned coverslips were used either directly or coated with 2.5 mg/ml fibrinogen in HBSS at 37°C for 1 h then washed with the buffer used in the chemotaxis experiments.

Analysis of migration movies

Movies taken of the EZ-Taxiscan chambers were converted into stacks of TIF files and analysed within the ImageJ plug-in 'manual tracking'. Only cells that remained within both the field of view and the bridge were tracked. The tabbed text files that were created were analysed within the ImageJ plug-in 'chemotaxis tool'. The proportion of cells that moved more than a total accumulated distance of 25 μ m was determined ('moved in response to fMLP') and was corrected for the purity of neutrophils in the cell preparation in each experiment. The population of motile cells was then used to calculate the velocity of the cells (using a velocity threshold of 0.006 μ m/s to make this an estimate of the velocity of moving cells and not a mixture of moving and stationary cells), the migratory index (a measure of how straight the paths taken by the cells are; distance from origin/total distance travelled) and mean total

accumulated distance. The data in Figure 5B and C are based on the following numbers of observations: on glass surfaces the number of cells that were tracked and moved >25 μ m were 141 for RasGRP4^{+/+} and 120 cells for RasGRP4^{ko/ko}, in 3 experiments; on fibrinogen-coated glass the numbers were 10 and 21 cells, respectively, in 2 experiments.

Quantification of phosphoinositides and DAG

Phosphoinositides in PLB-985 cells were quantified by [³²P]-Pi labelling of the cells followed by extraction, deacylation and anion-exchange HPLC (Condliffe *et al*, 2005). PIP₂ and PIP₃ in mouse neutrophils were quantified by mass spectrometry (Clark *et al*, 2011), but with adaptations to the procedure to allow both DAG and phosphoinositides to be measured in the same aliquots of cells. Aliquots of neutrophil suspensions (135 μ l, 0.5 \times 10⁶) were stimulated with fMLP (15 μ l, 100 μ M, 10 μ M final concentration) or vehicle alone. After an appropriate time, incubations were quenched with 750 μ l of a solvent mixture containing MeOH/CHCl₃ (2:1) that created a single homogenous phase. Two internal standards were then added to correct recoveries; D6-1-stearoyl-2-arachidonoyl-DAG (10 pg) and C16:0/C17:0-PIP₃ (1 ng). In all, 725 μ l CHCl₃ and 193 μ l H₂O were added, the samples mixed, and the resultant two phases (Folch *et al*, 1957) separated by centrifugation (5 min at 2000 g). Approximately 1 ml of the lower phase containing the neutral lipids, and none of the PIP₂ or PIP₃, was removed, dried under N₂, and resuspended in 100 μ l of methanol: water (4:1). These samples were further processed to quantify stearoyl/arachidonoyl-DAG as described below. The upper phase was acidified and resolved into two clear phases by the addition of 500 μ l MeOH/CHCl₃ (2:1), 500 μ l CHCl₃ and 170 μ l 2 M HCl, thorough mixing and centrifugation (5 min at 2000 g). The lower phase, now containing the PIP₂ and PIP₃, was derivatized using TMS-diazomethane, resolved by in-line HPLC and analysed by mass spectrometry, as described previously (Clark *et al*, 2011); values for endogenous C18/C20:4 PIP₂ and PIP₃ were corrected for recovery of the C16:0/C17:0-PIP₃ internal standard.

To measure 1-stearoyl-2-arachidonoyl-sn-glycerol (18:0/20:4 DAG) by mass spectroscopy, the samples in methanol/water (4:1) were injected (45 μ l) onto a Waters Acquity UPLC BEH300 C4 1.0 \times 100 mm column at 294 K and eluted with a 45–100% acetonitrile in water gradient with 0.1% formic acid added at a flow rate of 100 μ l/min over 20 min (see the gradient structure, below). The eluent was then passed into an AB Sciex 4000 QTrap mass spectrometer and the MRM transitions 645.6 > 341.4 for the 18:0/20:4-DAG and 651.6 > 347.4 for the deuterated internal standard were monitored, corresponding to loss of an arachidonate group (as the most sensitive MRM transition we identified) (mass spec machine settings are defined below). Both the labelled and unlabelled 18:0–20:4-DAG eluted at 10.94 min. Values for endogenous 18:0/20:4-DAG were corrected for the recovery of the internal standard.

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HPLC gradient for resolution of DAG species:

Time, min	Flow rate, μ l/min	% Water plus 0.1% formic acid	% Acetonitrile plus 0.1% formic acid	Curve
Initial	100	55	45	6
5	100	55	45	6
10	100	0	100	6
15	100	0	100	6
16	100	55	45	6
20	100	55	45	6

AB Sciex Instruments 4000 QTrap Mass spectrometer parameters are Scan Type: MRM; Polarity: Positive; Ion Source: Turbo Spray; Resolution Q1: Unit; Resolution Q2: Low; Dwell: 50 ms; CUR: 20; IS: 4500; TEM: 300; GS1: 18; GS2: 20; Ihe: ON; CAD: Medium; DP: 100; EP: 10; CE: 35; CXP: 10.

Statistics

Professional statistical support and advice was provided by the Bio-Informatics Facility at the Babraham Institute. Statistics tests are defined where applied.

Supplementary data

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

Acknowledgements

We would like to acknowledge Dianqing Wu (Yale university) for access to PLC β 2^{-/-} \times PLC β 3^{-/-} mice, Arnaud Deladrière (Babraham) for help with some experiments measuring neutrophil migration *in vivo*, the High Throughput Gene Targeting group at the Wellcome Trust Sanger Institute for help in obtaining the *RasGRP4* gene targeting vector, Felix Kreuger for help with analysis of mRNA-seq data bases and Anne Segonds-Pichon for help with statistics. This work was supported by a grant from the BBSRC, BB/D013593/1 and the British Lung Foundation.

Author contributions: SS performed experiments, analysed data and wrote the paper; CE, KA, GD, IN, HG and DP performed experiments and analysed data; KD and DP performed experiments; JC analysed data, wrote the paper and developed the DAG-assay methodology at all levels; PTH and LS planned work, performed experiments, analysed the data and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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