GPCR activation of Ras and PI3K\(\gamma\) in neutrophils depends on PLC\(\beta\)2/\(\beta\)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\(\beta\)2/\(\beta\)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\(\gamma\) in its effects on PI3K\(\gamma\)-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.

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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 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\(\gamma\)), PLC\(\beta\)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\(\gamma\).

PI3K\(\gamma\) 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\(\gamma\) 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 comprises a regulatory (p110\(\gamma\)) and a catalytic (p101\(\gamma\)) subunits (Stoyanov et al, 1995; Stephens et al, 1997; Suiré et al, 2005; Voigt et al, 2006). Full activation by G\(\beta\)\(\gamma\)s depends on p101 (Suiré et al, 2006). The p110\(\gamma\) 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; Suiré et al, 2002). The RBD is crucially important for activation of PI3K\(\gamma\) by GPCRs (Suiré et al, 2006). It is unclear, however, whether Ras proteins regulate the RBD of PI3K\(\gamma\) 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-g, but not GDP-g, 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\(\gamma\) (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\(\beta\)2 and PLC\(\beta\)3 are expressed in neutrophils and can be activated by a range of GPCRs, via both G\(\beta\)\(\gamma\) and G\(\alpha\)q subunits (Smrcka et al, 1991; Taylor et al, 1991; Camps et al, 1992), to hydrolyse PIP\(\delta\) and drive accumulation

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of its products, diacylglycerol (DAG) and Ins(1,4,5)P_3. DAG can activate DAG/phorbol ester-binding, C1 domain-containing effectors, such as the conventional or novel PKCs, and Ins(1,4,5)P_3 activates intracellular Ca^{2+} mobilization, both of which are important regulators of ROS formation and cell migration (Li et al., 2000; Shi et al., 2007). Although PI3K and PLCβ2/β3 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 PI3Kγ is crucial for GPCR-stimulated accumulation of PIP_3 (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 Dicyostelium (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 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 PI3Kγ 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 PIP_3 accumulation, indicating that the GTP-Ras which we were assaying was relevant to activation of PI3Kγ (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 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 FceRI 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

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**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 ± s.e. Figure source data can be found with the Supplementary data.
RasGRP4 is required for fMLP-stimulated activation of Ras and PI3K\gamma pathways

fMLP failed to stimulate significant activation of Ras in neutrophils from RasGRP4\textsuperscript{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\textsuperscript{kof/kof} neutrophils also reduced activation of predicted Ras effector pathways. We found fMLP-stimulated phosphorylation of p42/p44 MAPKs (T202/Y204), PIP\textsubscript{3} accumulation and phosphorylation of PKB (S473) were severely reduced in RasGRP4\textsuperscript{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\gamma/PKB pathways.

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

Rac GTases are central regulators of neutrophil responsiveness and can be rapidly activated by GPCRs via the RacGEFs carrying the targeted RasGRP4 allele in their germline. RasGRP4\textsuperscript{+/+} and RasGRP4\textsuperscript{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\textsuperscript{kof/kof} strains. RasGRP4\textsuperscript{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\textsuperscript{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\textsuperscript{kof/kof} mice with FlpE- and Cre-expressing strains sequentially to derive a strain, RasGRP4\textsuperscript{−/−}, 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\textsuperscript{−/−} mice were viable and fertile and their neutrophils responded indistinguishably to those from RasGRP4\textsuperscript{kof/kof} mice in specific experiments (see below).

Figure 2  fMLP-stimulated activation of Ras, p42/p44 MAPKs, PKB and PIP\textsubscript{3} accumulation is reduced in RasGRP4\textsuperscript{kof/kof} mouse neutrophils. Neutrophils were prepared from either RasGRP4\textsuperscript{kof/kof} or RasGRP4\textsuperscript{+/+} 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 P-42/44 MAPK\textsuperscript{−/−} via their respective loading controls. Figure source data can be found with the Supplementary data.

RasGRP4\textsuperscript{−/−} and two independent experiments for RasGRP4\textsuperscript{kof/kof}, like that shown in the upper 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-PIP\textsubscript{3}, as a ratio with the recovered PIP\textsubscript{3}-ISD, in fMLP- (10 μM) or vehicle (plotted at 0 s) -stimulated neutrophils, from the experiment shown in (A). The lower part shows quantification of phosphorylation of S473-PKB 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 were from the experiment shown in (A). The lower part shows quantification of phosphorylation of S473-PKB from three independent experiments for RasGRP4\textsuperscript{+/+} and two independent experiments for RasGRP4\textsuperscript{kof/kof}, like that shown in the upper part, as means ± s.e., n = 3 for RasGRP4\textsuperscript{+/+} or as means ± s.d., n = 2 for RasGRP4\textsuperscript{kof/kof} and are expressed as % of the samples stimulated for 1 min (100%), normalized to their respective loading controls. Figure source data can be found with the Supplementary data.
DOCK2 (Kunisaki et al., 2006) and PXre-1 (Welch et al., 2005). PI3K is thought to regulate the intracellular distribution of both RacGEFs but the activity of only PXre-1. We measured activation of Rac1 and 2 by fMLP using a PAK-CRIB pull-down assay and found that in RasGRP4kof/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 PI3P 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 PI3P-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 RaLGD5-RBD and found it to be reduced in RasGRP4kof/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 Rac 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, LTβ4, 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 RasGRP4kof/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 RasGRP4kof/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 PI3Kγ or knock-in of a Ras-insensitive mutant PI3Kγ 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 PI3Kγ. 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 RasGRP4kof/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 EZtaxisin chamber. These experiments revealed that RasGRP4kof/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 RasGRP4kof/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...
The data are means ± s.e., from four independent experiments (fMLP and PMA for RasGRP4kof/kof) or three experiments (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, RasGRP4kof/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 ± s.e., from four independent experiments (fMLP and PMA for RasGRP4kof/kof) or three experiments (C5a, LTB4 and RasGRP4−/− data).

Figure 4 ROS responses displayed by neutrophils from RasGRP4kof/kof and RasGRP4+/+ mice. (A) Neutrophils were prepared from either RasGRP4kof/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, RasGRP4kof/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 ± s.e., from four independent experiments (fMLP and PMA for RasGRP4kof/kof) or three experiments (C5a, LTB4 and RasGRP4−/− data).

remarkably similar to the phenotype of PI3Kγ-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 RasGRP4kof/kof and RasGRP4+/+ mice, despite the fact that a number of mouse models with loss of PI3Kγ 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 PI3Kγ 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, PI3Kγ-independent role for RasGRP4 in migration of neutrophils into the peritoneum (see below, possibly involving PLCβ2/β3).

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.
known to bind phorbol esters and DAGs (e.g., PKCδ (C1b), β2-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 15 bp 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 × 10⁶) 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 PLCβ₂⁻/⁻ × PLCβ₃⁻/⁻ mouse neutrophils (Li et al., 2000). We found fMLP-stimulated accumulation of stearoyl/arachidonoyl-DAG was also abolished in PLCβ₂⁻/⁻ × PLCβ₃⁻/⁻ neutrophils (Figure 6B). This result indicates that PLCβ₂/β₃ are responsible for fMLP-stimulated DAG formation in mouse neutrophils.

To test the hypothesis that RasGRP4 is activated by DAG generated by PLCβ₂/β₃, we measured fMLP-elicited activation of Ras in PLCβ₂⁻/⁻ × PLCβ₃⁻/⁻ neutrophils and found it much reduced compared with PLCβ₂⁺/⁺ × PLCβ₃⁺/⁺ 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 5](image-url)
We tested whether loss of RasGRP4 had an unexpected impact on other DAG effectors such as PKCs. We measured fMLP-stimulated, PKCδ-dependent phosphorylation of T154 in p40<sub>G<sub>TP</sub></sub>20<sub>NCX</sub> (Chessa et al., 2010). It was not significantly reduced in RasGRP4<sup>+/−</sup> 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 PLCβ2/β3-generated DAG regulates RasGRP4 and hence activation of Ras and the PI3Kγ 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 PI3Kγ by blockade of PLCβ appears greater than that inflicted by removing RasGRP4, or knocking-in a Ras-insensitive version of PI3Kγ, suggesting that although the major route by which PLCβs 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 RasGRP is the major fMLP-sensitive RasGEF in mouse and human neutrophils and that it is regulated by PLCβ2/β3-derived DAG. They also indicate that Ras is the direct, dynamic regulator of the RBD domain of PI3Kγ in vivo and, therefore, that PLCβ 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 PLCγs 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 Gβγ subunits in the case of PI3Kγ (Suire et al., 2006). This is consistent with the inability of phorbol esters to directly stimulate PI3 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 PI3Kγ, 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 Ca<sup>2+</sup> signals in brain; however, it is not clear whether they can respond to a pure GPCR stimulus.
activation of PKB was not reduced in the absence of PLCβ2/β3, where we find a 60% reduction (and larger reductions in fMLP-stimulated PIP2 accumulation and Ras activation). We have no explanation for this difference as we imported both the PLCβ2−/− × PLCβ3−/− 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 PLCβ2/β3, like PI3Kγ, are activated by relatively large amounts of Gβγ subunits released from these abundant G-protein species. PLCβ2/β3 can also be regulated directly by Rac GTPases and GTP-bound Gαq-family subunits. It is unclear to what extent these different mechanisms contribute to how, where and when PLCβ2/β3, Ca2+ and DAG signals are delivered in vivo and may read-through to downstream targets such as PI3Kγ. Previous work has indicated that the RBD of PI3Kγ, but not Gβγ5/p101, are required for GPCR regulation of ROS formation and yet other PI3Kγ-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 PIP2 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 PLCβ/DAG/RasGRP4/Ras pathway provides an input to PI3Kγ 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 PLCγ 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 (MNRRDSKRRSSHQEC) 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-actin (1:1000; Sigma); anti-P-p42/44 MAPK (T202/Y204) (1:2000; Cell Signalling); anti-Rac-2 (1:300; Millipore); anti-P-PKB (1:1000; Cell Signalling); anti-Rap1 (1:3000; Calbiochem); anti-P-PKB-S473 (1:2000; Cell Signalling) and anti-P-PKB (1:1000; Cell Signalling) and anti-β2-adrenergic receptors (1:40, gift from Dr N Kistakis). 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/N 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-dimethylformamide 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.

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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 HotstartTag 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 DHarnaco 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' BglII and 3' Clal/HindIII restriction sites) for the following RasGEFs: RasGei3c, RapGei2, 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 BglII and HindIII sticky ends) and then cloned into a lentivirus expression vector plasmid pLVTH (6 background) then progeny of this cross was mated with a Cre-RasGRP4 targeted alleles. The results presented in this manuscript are derived from mice from both of the strains. We crossed these strains to give rise to the data set described as ‘PLC b+’ in those assays in Figure 6.

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 x 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, cytospins were prepared and stained with May-Grumwald-Giemsa stain.

ROS assay
IMLP, 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 IMLP (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, Marbachaus Scan-DRM 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 μm was determined (‘moved in response to IMLP’) 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 0.06 μm/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

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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 μm were 141 for RasGRP4+/− and 120 cells for RasGRP4AlkoAlko 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 adaptions to the procedure to allow both DAG and phosphoinositides to be measured in the same aliquots of cells. Aliquots of neutrophil suspensions (135 μl, 0.5 × 10⁶) were stimulated with fMLP (15 μl, 100 μM, 10 μM final concentration) or vehicle alone. After an appropriate time, incubations were quenched with 750 μl of a solvent mixture containing MeOH/CHCl3 (2:1) that created a single homogeneous 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 μl CHCl3 and 193 μl 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 (5 min at 2000 g), acidified and resolved into two clear phases by the addition of 2 M HCl, 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-PiP2 internal standard.

To measure 1-stearoyl-2-arachidonoyl-sn-glycerol (18:0/20:4 DAG) by mass spectrometry, the samples in methanol/water (4:1) were injected (45 μl) onto a Waters Acquity UPLC BEH300 C4 1.0 × 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 μl/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.

HPLC gradient for resolution of DAG species:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (μl/min)</th>
<th>% Water plus 0.1% formic acid</th>
<th>% Acetonitrile plus 0.1% formic acid</th>
<th>Curve</th>
</tr>
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<tbody>
<tr>
<td>Initial</td>
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<tr>
<td>20</td>
<td>100</td>
<td>55</td>
<td>45</td>
<td>6</td>
</tr>
</tbody>
</table>

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; the: ON; CAD: Medium; DP: 100; EP: 10; CE: 35; CXP: 10.

Statistics
Professional statistical support and advice was provided by the Bioinformatics 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).

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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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