Chemokine signalling: pivoting around multiple phosphoinositide 3-kinases

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SUMMARY

The role of chemokines in mediating directional cell migration is well established, but more recently it has become evident that chemokines are able to couple to distinct signalling pathways that are involved in not only chemotaxis, but also cell growth and transcriptional activation. The signalling pathway controlled by the phosphoinositide 3-kinase (PI3K) family of lipid kinases has been the focus of much attention with respect to their role in chemokine-mediated functional responses. Indeed, there now exists convincing biochemical, pharmacological and genetic evidence that both CC and CXC chemokines stimulate PI3K-dependent chemotaxis of inflammatory cells such as eosinophils, macrophages, neutrophils and T lymphocytes. This review considers the role of individual PI3Ks (e.g. the p85/p110 heterodimer, PI3Kγ and PI3KC2α) as well their downstream effector targets in mediating chemokine-stimulated cell migration.

INTRODUCTION

The chemokine superfamily consists of low-molecular-weight proteins involved primarily in leucocyte migration. In recent years significant progress has been made in understanding the role of chemokines in inflammatory diseases, haematopoiesis, angiogenesis, metastasis, tumour rejection, T helper (Th)1/Th2 differentiation and human immunodeficiency virus-1 (HIV-1) infection. Members of the chemokine superfamily are classified by structure (according to the number and spacing of conserved N-terminal cysteine residues) into four major groups given the preferred names CC, CXC, C and CX_3C.[1–4] Chemokines engage seven-transmembrane G-protein coupled receptors (GPCR), and the heterotrimeric proteins are generally (but not exclusively) members of the Gαi subfamily of G proteins and are pertussis toxin sensitive.[5,6] The expression pattern of chemokine receptors is heterogeneous among leucocytes,[1–4] and engagement with their respective ligands regulates cytoskeletal rearrangement, integrin-dependent adhesions as well as binding and detachment of cells from their substrate. This occurs in a co-ordinated manner, with extension and retraction of pseudpods to execute co-ordinated directional migration.[7]

Despite substantial recent progress in our understanding of chemotaxis, the precise mechanism through which cells respond to a chemotactic gradient has yet to be determined. Leucocyte movement requires remodelling of the actin cytoskeleton, activation-induced changes in integrin affinity and integrin recycling at the leading edge of the cells. The signal transduction machinery implicated in these events has only recently begun to be elucidated. Most chemokines share the ability to activate G-protein sensitive phospholipase C (PLC) isoforms, resulting in inositol 3,4,5-trisphosphate generation and elevation of intracellular calcium.[8,9] However, the functional requirement for calcium is questionable as chemotaxis can be detected in situations where calcium mobilization cannot be detected,[10] suggesting that other

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Abbreviations: ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptors; MAP kinase, mitogen-activated protein kinase; PDK-1, 3′-phosphoinositide-dependent protein kinase-1; PH, pleckstrin homology; PLD, phospholipase D; PtdIns, phosphatidylinositol; PtdIns(3)*P*, phosphatidylinositol 3-monophosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; SH2, Src homology 2.

A new classification system has recently been proposed (see ref. 3). The present article will refer to individual chemokines by their classical as well as by their new nomenclature when first mentioned in the text. Thereafter, each chemokine will be referred to using their recognized classical names.

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biochemical events are probably more important. Several chemokines have been shown to inhibit adenylate cyclase and to activate mitogen/extracellular signal-regulated kinase (MEK)-1 and/or extracellular signal-regulated kinase (ERK)-1/2, stimulate tyrosine phosphorylation of focal adhesion complex components such as proline-rich tyrosine kinase (Pyk)-2, paxillin and Crk, and increase nuclear factor-κB (NF-κB) as well as signal transducer and activator of transcription (STAT)1 and STAT3 transcriptional activity in several cell models (reviewed refs 5 and 6). Thus, chemokines can couple to distinct signalling pathways that have been demonstrated to mediate not only migration, but also cell growth and transcriptional activation. One particular signalling pathway, namely that controlled by the lipid kinase phosphoinositide 3-kinase (PI3K) has been the focus of much attention with respect to its activation by chemokine receptors and the role it plays in regulating cell migration. Here, we review the emerging role of PI3K as a key mediator of chemokine-stimulated cell migration.

THE PI3K FAMILY

PI3Ks are a family of proteins that catalyse the phosphorylation of the 3-OH position of inositol head groups of phosphoinositide (PI) lipids, namely phosphatidylinositol (PtdIns), phosphatidylinositol(4)phosphate [PtdIns(4)*P*] and phosphatidylinositol(4,5)bisphosphate [PtdIns(4,5)P_2].[11] This results in the formation of PtdIns(3)*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respectively, collectively termed 3′-phosphoinositide lipids (Fig. 1). PtdIns(3)*P* is constitutively present in eukaryotic cells, its levels are largely unaltered upon cellular stimulation and it is thought to be involved in the regulation of membrane trafficking.[11] In contrast, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are generally absent from resting cells, but their intracellular concentration rises markedly upon stimulation via a variety of receptors, suggesting a second messenger function.

PI3Ks can be divided into three main classes on the basis of their *in vitro* lipid substrate specificity, structure and probable mode of regulation (Fig. 1). Hence, the class I PI3Ks can phosphorylate PtdIns, PtdIns(4)*P* and PtdIns(4,5)P_2. They also interact with Ras and form heterodimeric complexes with adaptor proteins that link them to different upstream signalling events. The prototypical class IA PI3Ks are heterodimers consisting of the 85 000-molecular weight (MW) regulatory/adaptor subunit and a catalytic 110 000-MW subunit.[11] The existence of multiple isoforms of both the regulatory (e.g. p85α/β, p55γ) and catalytic (e.g. p110α/β/δ) components means that there is considerable scope for specific variation between tissues as well as for coupling to different receptors and functional events. The class IB PI3K (PI3Kγ) is stimulated by G protein βγ subunits and associates with a unique p101 adaptor molecule.[12,13] Nevertheless, there is some evidence that GPCR, such as formyl-methionyl-leucyl-phenylalanine (fMLP) receptors, are also able to activate the p85/p110 PI3K.[14] The class II PI3Ks (e.g. PI3K-C2α/β/γ) are characterized by the presence of a C2 domain and utilize predominantly PtdIns and PtdIns(4)*P* as substrates. Recent evidence has indicated that clathrin functions as an adaptor for class II PI3K-C2α, binding to its N-terminal region and stimulating its catalytic activity.[15] The class III PtdIns 3-kinases utilize only PtdIns as a substrate.[11]

Numerous studies using PI3K inhibitors, overexpression of mutated forms of PI3K and, more recently, gene knockout experiments in mice, have implicated PI3Ks in the regulation of a diverse array of cellular responses, including cell survival, mitogenesis, membrane trafficking, glucose transport, neurite outgrowth, membrane ruffling and superoxide production, as well as actin reorganization and chemotaxis.[11] In addition, several 3-phosphoinositide-binding domains have been identified in a broad range of target molecules. For instance, the FYVE and PX domains bind PtdIns(3)*P* and are generally found in proteins involved in different vesicle trafficking events.[16–18] A number of proteins have been identified that directly bind PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 via pleckstrin homology (PH) domains, including PtdIns(3,4,5)P_3-dependent protein kinase-1 (PDK-1), protein kinase B (PKB/Akt), Bruton's tyrosine kinase, various PLC isoforms and several guanine nucleotide exchange factors[11] (Fig. 1).

EVIDENCE FOR A ROLE OF PI3K IN CELL MIGRATION

Elegant molecular and pharmacological evidence first suggested that PI3K and its lipid products might play an important role in platelet-derived growth factor (PDGF)-dependent actin polymerization and cell migration.[19–22] Moreover, selective activation of PI3K using constitutively active PI3K mutants or the addition of exogenous PtdIns(3,4,5)P_3 can initiate cell motililty and membrane ruffling.[23,24] However, the very first evidence for the involvement of PI3K in chemokine-stimulated cell migration was the demonstration that chemotaxis and polarization of T cells induced by Regulated on Activation, Normal, T-cell Expressed, and Secreted (RANTES)/CCL5 could be inhibited by PI3K inhibitors such as wortmannin and LY294002.[10] Subsequent studies by several groups have shown that other CC chemokines (e.g. macrophage inflammatory protein [MIP]-3α/CCL20 and monocyte chemoattractant protein [MCP]-1/CCL2) as well as CXC chemokines (e.g. interleukin [IL]-8/CXCL1 and stromal-cell-derived factor 1 [SDF]-1/CXCL12) stimulate wortmannin-sensitive chemotaxis of eosinophils, THP-1 cells, as well as neutrophils and T lymphocytes, respectively.[25–28] Thus, it seems probable that the production and degradation of 3′-phosphoinositide lipids is crucial in maintaining chemotactic signalling gradients. This interpretation was reinforced by subsequent evidence from mice deficient in SHIP (Src homology 2 [SH2]-containing inositol 5-phosphatase), an enzyme that hydrolyses PtdIns(3,4,5)P_3. These SHIP$^{-/-}$ mice suffer from lethal infiltration of the lungs by macrophages and neutrophils and therefore persistently high levels of PtdIns(3,4,5)P_3 and subsequent activation of its downstream effectors might lead to excessive inflammation.[29] Finally, elegant studies using green fluorescent protein-tagged PH domains that bind

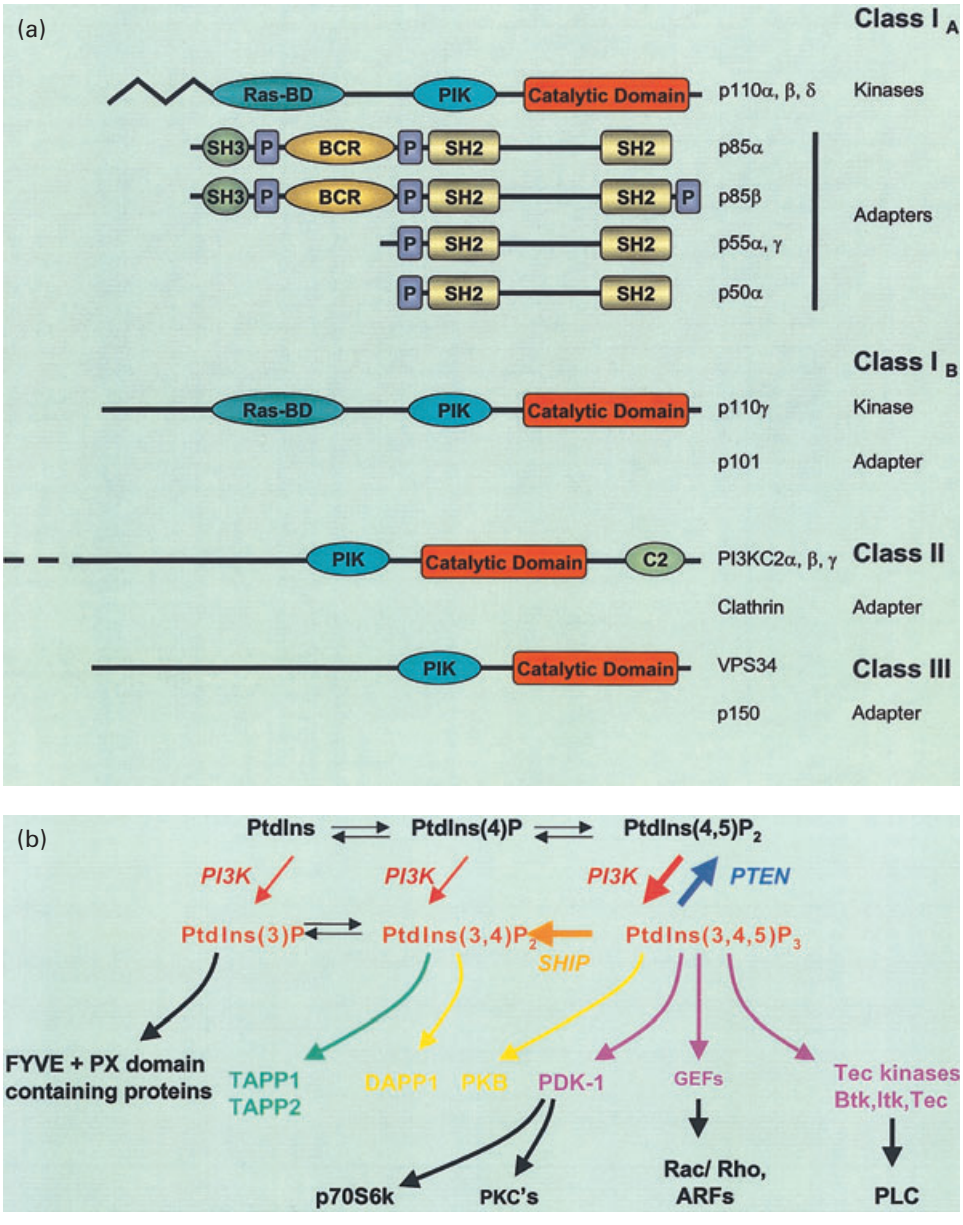

Figure 1. Classification of phosphoinositide 3-kinase (PI3K) family members and synthetic pathways for PI lipids. (a) PI3Ks have been divided into three classes, based on primary structure, substrate specificity and regulatory mechanisms; class 1 is further subdivided according to the associated adapter (regulatory) subunit. The protein domains are as follows: BCR, breakpoint-cluster region; C2, C2 domain; P, proline-rich motif; PIK, phosphatidylinositol kinase domain; Ras-BD; Ras-binding domain; SH2, src-homology domain 2; SH3, src-homology domain 3. (b) Routes of synthesis of known PI lipids: PtdIns 4-kinase and PtdIns(4)*P* 5-kinase mediate formation of PtdIns(4)*P* and PtdIns(4,5)P_2 from PtdIns, and all three lipids can potentially serve as substrates for different PI3Ks. The broad substrate specificity depicted is that of the p85/p110 heterodimer and PI3Kγ. PI3K phosphorylates only PtdIns, whilst PI3K-C2α specificity *in vitro* is restricted primarily to PtdIns and PtdIns(4)*P*. The thick arrows reflect the major route of accumulation of PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2 upon receptor stimulation and its subsequent conversion to PtdIns(3,4)P_2 by a selective 5-phosphatase. Other selective 3′- and 5′-phosphatases also help regulate the state of phosphorylation of the PI lipids. Representative proteins with specificities for particular 3′-phosphorylated PI lipids, as well as some downstream effectors of PtdIns(3,4,5)P_3-binding proteins, are shown. PTEN, 3′-phosphatase and tensin homologue deleted on chromosome 10 protein; ARF, ADP ribosylation factor; DAPP-1, dual adaptor for phosphotyrosine and 3- phosphoinositides; GEF, guanine nucleotide exchange factor; FYVE, Fab1, YOTB, Vac1 and EEA1 domain; PLC, phospholipase C; PX, phox homology; PKC, protein kinase C; TAPP, tandem PH-domain-containing protein.

selectively with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 revealed that PtdIns(3,4,5)P_3 accumulated at the leading edge of chemoattractant-stimulated HL-60 cells.[30] Similarly, PtdIns(3,4,5)P_3 localization at the leading edge of polarized cells was observed using a PtdIns(3,4,5)P_3-specific antibody (Ab).[31] This accumulation of PtdIns(3,4,5)P_3 at the leading edge correlates with the polarization of chemokine receptors that are involved in detecting a chemoattractant gradient.[7]

Chemokines stimulate accumulation of 3′-phosphoinositide lipids

One of the most extensively investigated chemokines with regard to signal transduction mechanisms is SDF-1 (CCL12). The advantage of studying this chemokine is that it binds exclusively to CXCR4 and therefore ligand promiscuity for other receptors is not an issue. Hence, it is possible to attribute signalling responses to one selective ligand–receptor interaction. CXCR4 is highly expressed on the leukaemic T-cell line, Jurkat, as well as on normal peripheral blood-derived T cells, which have both been used as models to study the effect of SDF-1 on PI3K activation. The most obvious marker of PI3K activation is accumulation of its lipid products within intact cells. SDF-1 and certain SDF-1 peptide analogues stimulate the transient accumulation of PtdIns(3,4,5)P_3 in leukaemic T-cell lines and peripheral blood-derived T lymphocytes.[26] In both cell types, the elevation of PtdIns(3,4,5)P_3 is rapid and transient, being detectable within 15 seconds after stimulation and returning to basal levels within 5 min after chemokine treatment. Other studies have investigated the effect of MCP-1 stimulation on PI lipid accumulation in the monocytic cell line THP-1. This model revealed that MCP-1 is also able to elicit rapid and transient accumulation of PtdIns(3,4,5)P_3.[27] It appears therefore that at least two different chemokines which bind to distinct receptors are able to activate PI3K in different cell systems.

PI3Kγ is a key component of chemokine-stimulated PtdIns(3,4,5)P_3 accumulation

The elevation of D-3 PtdIns lipids such as PtdIns(3,4,5)P_3, observed in response to either SDF-1 or MCP-1, may be the result of activation of more than one PI3K (e.g. the p85/p110 PI3K and PI3Kγ). Given that chemokine receptors are G protein coupled,[5,6] one might predict an involvement of the Gβγ-dependent PI3Kγ in mediating PtdIns(3,4,5)P_3 accumulation. Indeed, the accumulation of PtdIns(3,4,5)P_3 stimulated by SDF-1 and MCP-1 can be completely inhibited by pretreatment with pertussis toxin, strongly indicating that 3′-phosphoinositide lipid accumulation occurs via the G_i protein-coupled PI3Kγ.[26,27] Indeed, both SDF-1 and RANTES stimulate an increase in the *in vitro* lipid kinase activity present in anti-PI3Kγ immunoprecipitates derived from either Jurkat cells or natural killer (NK) cells, respectively.[26,32] Interestingly, the *in vitro* kinetics of activation of PI3Kγ in response to SDF-1 closely correlate with those observed for the accumulation of 3′-phosphoinositide lipids in intact cells.[26,32] Experiments using mice that have been genetically engineered to be deficient in PI3Kγ have revealed that leucocytes from such mice are unable to produce PtdIns(3,4,5)P_3 in response to the CXC chemokine IL-8,[33] suggesting once again that PI3Kγ is the key mediator of PtdIns(3,4,5)P_3 production. However, the effect of SDF-1 on PtdIns(3,4,5)P_3 was not examined in these PI3Kγ-deficient leucocytes.

Is PI3Kγ the whole story: what about the p85/p110 heterodimer?

Despite the strong biochemical and genetic evidence for activation of PI3Kγ by chemokines and its importance for PtdIns(3,4,5)P_3 accumulation, there is a body of evidence to suggest that PI3Kγ may not be the only PI3K activated by chemokines. Probably the most convincing evidence that other signalling molecules, in addition to PI3Kγ, are activated by chemokines is that in PI3K$\gamma^{-/-}$ mice there is incomplete (e.g. 50–70%) reduction in the capacity of neutrophils to migrate to a range of chemoattractants,[33–35] and PI3Kγ knockout does not prevent chemoattractant-induced actin polymerization.[34] One possibility is that other PI3K isoforms are activated by chemokinwde receptors. Certainly, *in vitro* assays of immunoprecipitated p85 subunits of PI3K indicate that the p85/p110 heterodimer is activated by SDF-1 and RANTES in T cells[10,26] and by MCP-1 in THP-1 cells.[27] Moroever, p85 has been reported to co-associate with anti-CXCR4 immunoprecipitates after SDF-1 stimulation of human peripheral blood T lymphocytes.[36] No mechanism has been proposed for these p85–CXCR4 associations and because there are no recognized p85-binding motifs within the CXCR4 cytoplasmic domains, the observed interaction may be via an indirect mechanism.

So, how do chemokine receptors couple to the p85/p110 heterodimer? Several studies have reported that GPCR activation of p85/p110 PI3K is dependent on βγ subunits.[11,14] However, the p85/p110 heterodimer is known to co-associate with phosphotyrosine-containing proteins[11] and many chemokines are able to stimulate tyrosine phosphorylation of proteins.[5,6] One route by which chemokine receptors might be able to regulate phosphotyrosine-dependent activation of p85/p110 is via the Gαi subunit. Recent elegant studies have revealed that GTP-bound Gαi subunits bind and activate Src and Hck in a saturable manner.[37] Although these results were obtained mainly with reconstituted systems, Gαi-mediated signalling downsteam of chemokine receptors can be assumed. Considering the high structural homology of the kinase domain (SH1) among Src-related enzymes, it is conceivable that Gαi subunits also activate other members of the Src kinase family (such as Fgr, Lck or Lyn) that are expressed in leucocytes. Stimulation of Src kinases by Gαi not only potentially links chemokine receptors to the class IA PI3K, but might also provide routes to Ras activation (via Shc, Grb-2 and Sos) and to FAK/Pyk2 activation.[38–40]

Is the p85/p110 heterodimer functionally significant?

Cell motility requires not only actin polymerization, but also the co-ordinated control of cell adhesion at the leading edge and contraction of the trailing edge. It is therefore unlikely that these complex processes are dependent solely upon PI3Kγ, but rather require the integration of several distinct signalling events, some of which may be provided by the p85/p110 heterodimer. In support of this, microinjection of antibodies to p110β and p110δ into a macrophage cell line resulted in reduced cell migration in response to colony-stimulating factor-1 (CSF-1), which binds to and activates a receptor tyrosine kinase.[41] In addition, several lines of evidence suggest that whilst p85/p110 does not contribute to SDF-1 or MCP-1-stimulated PtdIns(3,4,5)P_3 accumulation in the model systems examined to date,[26,27] the observed *in vitro* activation may still reflect the physiological relevance of this isoform and may account for the lack of complete inhibition of chemokine-stimulated cell migration of PI3K$\gamma^{-/-}$ macrophages and neutrophils.[32–35] For example, wortmannin inhibits MCP-1 stimulated THP-1 cell chemotaxis even though wortmannin has no effect on MCP-1-stimulated PtdIns(3,4,5)P_3 accumulation.[27] Indeed, overexpression of a constitutively active mutant of p110α has been demonstrated to induce intracellular adhesion molecule-3 (ICAM-3) redistribution in a T-cell line and is sufficient to increase ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1)-dependent firm adhesion of THP-1 cells under flow conditions. MCP-1 augmented this latter response under conditions where p85/p110, but not PI3Kγ, was activated.[42] Together, these data indicate that class 1A PI3K activation is not only necessary, but also sufficient to induce membrane receptor polarization and/or adhesion events, which are important processes during the chemotactic response.

The p85/p110 heterodimer may influence cell migration, making only a small, undetectable but nevertheless significant and important contribution to the overall pool of 3′-phosphoinositide lipids formed in response to chemokine stimulation. An alternative scenario is that the physiological role for p85/p110 may reside in the protein serine kinase activity of the catalytic subunit rather than its lipid kinase activity.[43] Interestingly, the leucocyte-specific δ isoform of class 1A PI3K exhibits unique protein serine kinase substrate specificity compared to p110α.[44] The possibility exists that each individual chemokine/chemokine receptor can achieve a degree of specificity of cellular responses by phosphorylating a distinct pattern of substrates (Fig. 2). It will therefore be important for future molecular strategies to establish whether the protein kinase activity of either p85/p110 or PI3Kγ has any role to play in chemokine signal transduction.

Activation of class II PI3Ks by chemokines

Studies with MCP-1-stimulated THP-1 cells revealed that PtdIns(3,4,5)P_3 accumulation in THP-1 cells is wortmannin resistant, yet entirely pertussis toxin sensitive. These surprising observations suggested the involvement of a novel PI3K-C2α which has recently been identified as displaying reduced sensitivity to wortmannin.[45] MCP-1 does indeed activate PI3K-C2α in THP-1 cells, as assessed by *in vitro* assays, and this activation exhibits the same resistance to wortmannin and sensitivity to pertussis toxin as the MCP-1-stimulated increases in 3′-phosphoinositide lipid generation.[27] So, the pertussis toxin-sensitive MCP-1-induced activation of PI3K-C2α may contribute solely or partly to the detectable changes in PtdIns(3,4,5)P_3 accumulation, although the exact mechanisms involved in receptor coupling, as well as the functional relevance of PI3KC2α, remain obscure. One caveat to this theory is that the *in vitro* substrate specificity of PI3KC2α is restricted to PtdIns and PtdIns(4)P,[45] which would apparently contradict its possible role in mediating MCP-1-stimulated PtdIns(3,4,5)P_3 accumulation. However, it should be noted that the substrate specificity of PI3K-C2α may be quite different in intact cells to that observed under *in vitro* conditions. At present there is no information as to whether activation of PI3K-C2α or the related PI3KC2-β occurs in response to other chemokines, or what the functional significance of class II PI3K activation is.

HOW DOES PI3K ACTIVATION REGULATE CELL MIGRATION?

Reorganization of the actin cytoskeleton is an important step in cell migration, and different chemokines are able to induce the polarization of lymphocytes with generation of specialized cell compartments. Given that Rho GTPases Rho, rac and Cdc42 are regulators of actin cytoskeleton and cellular polarity, there has been much interest in ascertaining the role of the Rho GTPases in chemokine signalling and their relationship with the PI3K-dependent signalling cascade.[46] Indeed, there is considerable evidence to indicate that Rho family kinases are regulated by PI3K in several systems. For instance, Rac and Cdc42 have been reported to associate with p85/p110,[47] whilst expression of active mutants of p110α in fibroblasts can induce actin reorganization in the form of Rac-mediated lamellipodia and focal complexes and Rho-mediated stress fibres and focal adhesions.[23] Similarly, reorganization of the actin cytoskeleton and membrane ruffling induced by overexpression of wild-type PI3Kγ or expression of an active mutant of PI3Kγ required Rac but not Cdc42,[48] whilst the PI3K homologue TOR2 controls Rho activation in *Saccharomyces cerevisiae*.[49] PI3K has been shown to be involved in the regulation of actin cytoskeleton by growth factors such as PDGF and insulin.[20–22,50]

Cdc42 appears to have a major role in the control of directional migration of leucocytes, as a dominant-negative mutant of Cdc42 displays a much more potent inhibitory effect on leukaemic T-cell line chemotaxis towards SDF-1 gradients than dominant-negative mutants of RhoA and Rac.[51] In addition, expression of Cdc42 mutants in monocytic cells demonstrated that rearrangement of the actin cytoskeleton in response to CC chemokines (MCP-1 and MIP-1α) is regulated via Cdc42.[51] Interestingly, MCP-1 and MIP-1α, but not Cdc42-stimulated cytoskeletal

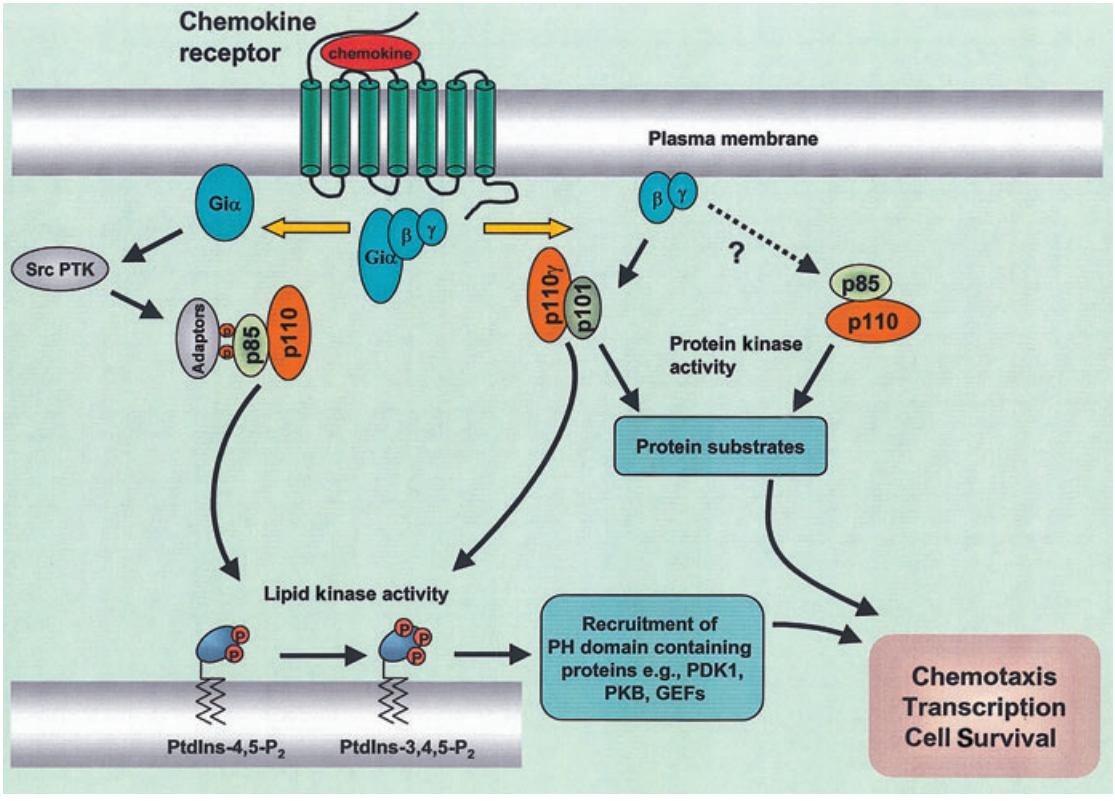

Figure 2. Potential role of lipid and protein kinase activity of phosphoinositide 3-kinases (PI3Ks). Proposed routes are shown by which PI3Kγ and p85/p110 PI3K may contribute to chemokine-stimulated functional events via 3′-phosphoinositide lipid dependent and/or protein serine kinase-dependent activity. PDK-1, 3′-phosphoinositide-dependent protein kinase-1; PKB, protein kinase B; GEF, guanine nucleotide exchange factor; PH, pleckstrin homology; PTK, protein tyrosine kinase.

reorganization, can be inhibited by wortmannin, indicating the involvement of PI3K upstream of Cdc42 in chemokine-stimulated cell migration.[52] There are also remarkable similarities between the phenotype of mice lacking the small GTPases Rac2 (which in mammals is usually restricted to expression in haematopoietic cells) and that of the PI3Kγ-deficient phenotype. Hence, Rac2-deficient animals have a higher leucocyte blood count, their leucocytes are less able to infiltrate the peritoneum in experimental inflammatory models and less able to migrate *in vitro* in response to chemoattractants such as fMLP and IL-8.[53] The overlap of phenotypes suggests that Rac2 may be in the same leucocyte signalling pathway as PI3Kγ. However, whilst the effect of chemokines on Rac activation was not assessed, it should be noted that Rac activation in response to the non-chemokine chemoattractant fMLP still occurs in PI3Kγ-deficient cells.[34] One probable explanation for this, however, is that fMLP stimulation of the p85/p110 heterodimer is able to sustain coupling to Rac in PI3Kγ-deficient mice.[14]

WHAT IS THE ROLE OF OTHER CHEMOKINE-STIMULATED PI3K-DEPENDENT EFFECTORS?

As mentioned above, a large number of downstream effector targets have been described for PI3K. The role of some of these targets in chemokine-stimulated cell migration is less than convincing, but they do provide routes by which chemokines can potentially influence events other than migration, such as transcription and cell cycle, and they will now be considered:

Protein kinase B

Protein kinase B (PKB, also termed Akt) is a 57 000-MW serine/threonine kinase that is the best characterized downstream effector of both PI3Kγ and p85/p110.[11,54] Indeed, several GPCR, including those activated by the chemokines SDF-1, RANTES and IL-8, have been shown to activate PKB in a PI3K-dependent manner.[26,55,56] Furthermore, IL-8 is unable to stimulate PKB in PI3Kγ-deficient neutrophils.[33] This latter observation is particularly interesting, as a recent study provided evidence that p110β is necessary and sufficient to stimulate PKB by GPCR, although it should be emphasized that chemokine receptors were not specifically investigated in this study.[57] In other settings, PKB is a key mediator of growth factor-induced cell survival and protection against c-Myc-induced cell death.[58–60] Recent evidence has implicated PKB to be required for efficient chemotaxis in response to chemoattractants in the slime mould *Dictyostelium*.[61]

In mammalian cells, however, molecular approaches have demonstrated that whilst cytoskeletal reorganization and lamellipodium formation are PI3K-mediated events, they occur independently of PKB activation.[48,62] A number of phosphorylation targets for PKB are now emerging. These include several transcription factors and it appears that PKB may be able to regulate activation of NFκB, although the exact mechanism remains a contentious issue.[63–65] Moreover, both PKB[54] and PI3Kγ[66] have been reported to translocate to the nucleus where they may influence transcription and the cell cycle.[67]

ERK1/2 MAP kinase as a target for the PI3K signalling cascade

The MAP kinases comprise a family of serine/threonine kinases, which include the extracellular signal-regulated kinases ERK1/2, the Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) and they represent a point of convergence for cell surface signals regulating cell growth and division.[68] Many receptors that couple to pertussis toxin-sensitive G_i (e.g. receptors for lysophosphatidic acid (LPA), bombesin, thrombin, α_2-adrenegic agonists) can activate MAP kinases via a Gβγ subunit complex-mediated pathway that is dependent upon tyrosine phosphorylation, $p21^{ras}$ activation and distal protein phosphorylation cascades.[68,69] Several studies have recently reported that CXC (e.g. SDF-1 and IL-8) as well as CC chemokines (eotaxin, MIP-3α, MCP-1) stimulate phosphorylation of MEK-1 and/or ERK1/2 in a number of cell systems.[25,26,28,70,71] Additionally, IL-8 has been reported to activate p38MAPK but not JNK.[28] This latter observation is somewhat surprising given our knowledge of the pivotal role of PI3Kγ in IL-8 signalling[33] and that PI3Kγ has been reported to mediate Gβγ-dependent JNK activation.[72] Several studies have now indicated that ERK1/2 activation in response to several chemokines can be inhibited by PI3K inhibitors.[25,26,71]

Inhibition of ERK activation by the use of MEK inhibitors such as PD98059 abrogates SDF-1, MIP-3α and eotaxin-induced actin polymerization and/or migration of T cells or eosinophils, respectively.[25,26,70] This would correlate with previous observations indicating a role for MAP kinases in amoeboid chemotaxis in response to cAMP and fibroblast chemotaxis in response to fibronectin.[73,74] However, it should be emphasized that chemotaxis of neutrophils in response to the chemokine IL-8,[28] or non-chemokine chemoattractants such as fMLP and C5a, is not blocked by the MEK inhibitor PD98059.[75–77] The different sensitivity to MEK inhibition of cell migration in response to chemokines and other chemoattractants, suggests that there are multiple pathways leading to leucocyte chemotaxis and highlights the fact that even though different chemokine receptors can share biochemical signalling pathways, there is sometimes a degree of redundancy with respect to the importance of those pathways in evoking a chemotactic response.

What comes first: Ras or PI3K?

Whilst chemokine-stimulated ERK1/2 phosphorylation is sensitive to PI3K inhibitors, it is unclear as to the relative proximal versus distal positioning of Ras and PI3K with respect to chemokine receptor signal transduction. Active GTP-bound Ras can directly interact with the catalytic subunits of PI3K and PI3Kγ and cells expressing constitutively active Ras have greater levels of PI3K products.[11] However, it is emerging that PI3K is not a universal Ras effector molecule in all cell types. In COS cells, both p110α/β and PI3Kγ are involved in Gβγ-stimulated activation of MEK and ERK1/2 at a point upstream of Ras activation.[78,79] What is the mechanism for this? In other systems Gβγ-stimulated Shc phosphorylation is sensitive to tyrosine kinase inhibitors and wortmannin,[80] whilst PtdIns(3,4,5)P_3 has been reported to bind with high affinity to SH2 domains of proteins such as Src.[81] So, PI3Ks may serve as a platform responsible for mediating activation of Shc-Grb-2-Sos-Ras pathway leading to increased MAPK activation. It is therefore tempting to speculate that PI3K may also lie upstream of Ras following activation of chemokine receptors, although this has yet to be formally shown.

Can PI3K mediate Ras-independent ERK activation?

Certain pertussis toxin-insensitive GPCR mediate ERK1/2 activation via a Gαq subunit pathway that is $p21^{ras}$-independent.[82] Several chemokine receptors are coupled to pertussis toxin-insensitive G proteins,[83,84] so there is the strong possibility that chemokine receptors may be able to mediate $p21^{ras}$-independent ERK1/2 activation. For example, in Chinese hamster ovary (CHO) cells transfected with IL-8 receptors, dominant-negative forms of Ras and Raf have no effect on IL-8-stimulated ERK1/2 activation,[85] supporting the existence of Ras-independent routes for activation of ERK1/2 by IL-8 and perhaps other chemokines. There is now some evidence to suggest that this Ras-independent ERK1/2 activation by chemokines may potentially involve PI3Kγ activation. Thus, whilst PI3Kγ has been reported to lie upstream of Ras during Ras-dependent activation of ERK1/2 in COS cells,[79] it has also been demonstrated to stimulate Ras-independent activation of both MEK and ERK1/2 in CHO cells.[86] The action of PI3Kγ on ERK1/2 in CHO cells may involve activation of the atypical ζ isoform of PKC.[86] Both PKCζ and PKCδ can be phosphorylated in the activation loop sites by PDK-1 in a 3′-phosphoinositide-dependent manner (Fig. 3).[87,88] There is also evidence that PKCζ may be involved in the signalling pathways leading to neutrophil adhesion and chemotaxis in response to IL-8.[89] One might therefore predict the reported Ras-independent ERK1/2 activation, stimulated by IL-8, to be wortmannin sensitive. However, in CHO cells transfected with the IL-8 receptors, Ras-independent ERK1/2 activation stimulated by IL-8 is not significantly inhibited by PI3K inhibitors.[85] The significance of these observations is not fully understood, although it is interesting to note that IL-8-stimulated MAPK activation is severely abrogated by PI3K inhibitors in human neutrophils.[28,71] These latter

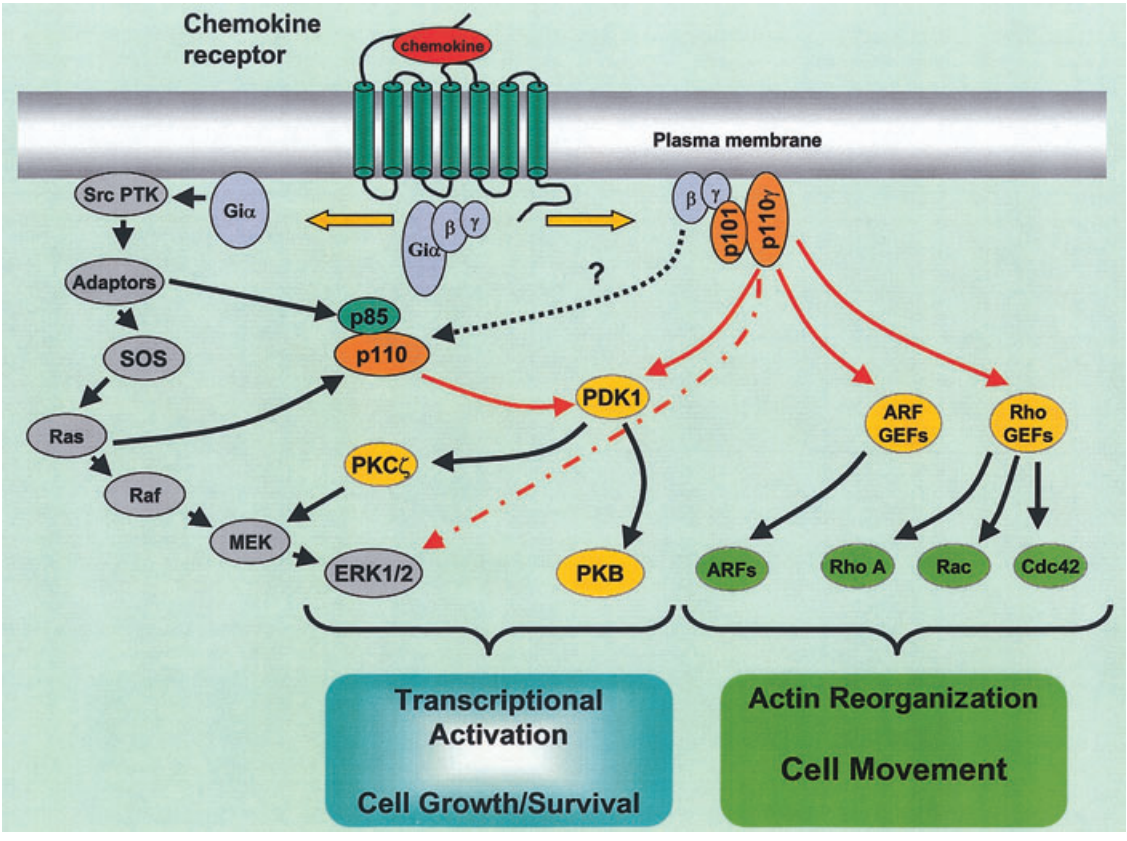

Figure 3. Schematic representation of phosphoinositide 3-kinase (PI3K)-dependent signalling pathways involved in chemokine-mediated functional responses. Following chemokine receptor engagement, both the $G\alpha_i$ and $G\beta\gamma$ subunits activate distinct subsets of signalling molecules. Activation of PI3Kγ by $\beta\gamma$ subunits has been established, but direct activation of p85/p110 PI3Ks by $\beta\gamma$ subunits is a contentious issue. GTP-bound $G\alpha_i$ can directly activate Src family protein tyrosine kinases (PTKs), leading to tyrosine phosphorylation of substrates and the recruitment and activation of Src homology 2 (SH2) domain-containing proteins. This potentially leads to the activation of numerous signalling molecules, including p85/p110 PI3Ks and Ras, which can activate both class IA and IB PI3Ks. Red arrows represent the lipid kinase activity of PI3K, but the relative contribution of individual PI3K isoforms in the activation of particular effectors has yet to be defined. As well as Ras-dependent activation of extracellular signal-regulated kinase (ERK)1/2, Ras-independent activation of ERK1/2 can occur via protein kinase Cξ (PKCξ)-mediated activation of mitogen/ERK kinase-1 (MEK), or by the activation of the mitogen-activated protein kinase (MAPK) cascade by the serine kinase activity of PI3Kγ (dashed red arrow). ARF, ADP-ribosylation factor; GEF, guanine exchange factor; PDK-1 3′-phosphoinositide-dependent protein kinase-1; PKB, protein kinase B; SOS, Son of Seven less homologue.

studies reinforce the considerable diversity in signalling characteristics of individual chemokine receptors that exists between cell models and which reflect a certain degree of redundancy in different systems.

WHAT IS THE POINT OF ACTIVATING MORE THAN ONE PI3K?

Pharmacological and genetic evidence strongly support a role for PI3Kγ as a major biochemical signal for chemotaxis in response to a number of chemokines. However, whilst several chemokines stimulate activation of p85/p110, there is currently no direct evidence to indicate that Class IA PI3Ks contribute to the 3′-phosphoinositide lipids that accumulate in response to these chemokines. It should be remembered that the relative contribution of PI3K isoforms to 3′-phosphoinositide lipid accumulation might vary depending on cell type and the chemokine receptor examined. In addition, there is considerable potential for heterogeneity of chemokine receptor isoforms and/or G protein subunits to facilitate differential regulation of PI3K isoforms and/or initiate signalling pathways that are independent of PI3K. For example, SDF-1 and RANTES induce receptor coupling to both pertussis toxin-sensitive G_i[83,90,91] and pertussis toxin-insensitive G_q[83,89–93] family members of G proteins. Similar heterogeneity in coupling to Gq and G_i proteins has been reported for the two IL-8 receptors CXCR1 and CXCR2.[84] Moreover, whilst both CCR2A and CCR2B can couple to the G_i-G$\beta\gamma$-PLCβ2 pathway, these receptors demonstrate an interesting specificity in their coupling to the α subunits of the Gq class. Hence, CCR2B couples to both Gα16 and Gα14, whereas CCR2A cannot couple to either.[9]

The diversity within both the G protein coupling mechanism as well as the PI3K family to which chemokine receptors are coupled, may be one way, in which chemokine receptors can exert control over multiple functional events such as adhesion molecule up-regulation, actin polymerization, lamellipipodia formation and shape change, as well as granule release and superoxide release.[94–96] Thus, although both the p85/p110 PI3K and PI3Kγ have been shown to be activated by thrombin, studies with PI3K inhibitors have indicated that only the p85/p110 PI3K complex is involved in regulating platelet aggregation.[97] It has also previously been proposed that phosphotyrosine-dependent activation of PI3K is responsible for phagocytosis, whereas G protein-mediated activation of PI3K gives rise to the respiratory burst.[98]

CONCLUSION

Whilst this review has focused on the role of PI3K-dependent events in regulating chemotaxis, it is important to remember that other functional events can be driven by chemokines, such as integrin-dependent cell adhesion, granule release and superoxide release. Some of these alternative chemokine-mediated responses are also PI3K-dependent: for example, treatment of phagocytes with PI3K inhibitors revealed that PI3K activity is required for stimulation of the respiratory burst, exocytosis and phagocytosis.[75,95] However, chemotaxis and shape change of neutrophils stimulated by agonists of GPCR (e.g. fMLP) are insensitive to PI3K inhibitors in certain cell models.[75,95] Hence, PI3K may not be involved in cell migration in response to all chemokines, but could still have a pivotal role in other chemokine-mediated functional responses. Nevertheless, there is strong evidence that PI3K-dependent signalling events do have an important role to play in cell migration stimulated by several chemokines. The challenge for the future is to identify whether all chemokine receptors can activate multiple or distinct PI3K isoforms. Just as important will be to understand how the PI3K-dependent signals are fine-tuned and integrated with other signals (e.g. inhibition of cyclic adenosine monophosphate elevation, intracellular calcium and tyrosine phosphorylation of multiple substrates) that are involved in sensing chemotactic gradients and co-ordinating chemotactic responses which lead to directional cell migration.

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