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Moving towards a Better Understanding of Chemotaxis

Review

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Eukaryotic cells are thought to move across supporting surfaces through a combination of coordinated processes: polarisation; extension of dynamic protrusions from a leading edge; adhesion-associated stabilisation of some protrusions; centripetal pulling against those leading adhesions; and de-adhesion at the rear. Gradients of extracellular ligands can be detected by cells and then used to guide them either towards the source (in the case of a chemoattractant) or away from the source (in the case of a chemorepellent) — such migration is termed chemotaxis. Recent work suggests that chemotaxis probably emerges from the ability of cells to spatially encode extracellular gradients of ligands, a process for which phosphoinositide 3′-kinase (PI3K) signals alone are insufficient, and to use that vectorial information to bias movement by enhancing the survival, and not the formation, of the protrusions that experience the greatest stimulation.

Introduction

Anybody who has watched the now famous movie, made by the late David Rogers in the 1950s, of a neutrophil 'chasing' its bacterial prey amongst red blood cells cannot fail to have been impressed by the ruthless efficiency of the predator despite its apparent 'senseless' simplicity. It is argued that the hunt was underpinned by the ability of the neutrophil to chemotax up gradients of formylated peptides released by the bacteria. As our general appreciation of the nature and detail of intracellular signalling has increased in the following decades, the particular properties of the signalling networks activated during chemotaxis have come under close scrutiny with the aim of identifying both the signals that coordinate the underlying movement as well as the compass mechanism that gives it a sense of direction. As always, progress has been made in unforeseen fits and starts. The emergence of high-resolution imaging of fluorescently tagged proteins combined with a leap in our understanding of phosphoinositide 3′-kinase (PI3K) signalling delivered a portfolio of results between 2000 and 2004 that seemed to provide a simple, intellectually pleasing and generalised explanation of the compass mechanism. Extracellular gradients of chemoattractants were translated into intracellular gradients of PI3K activation and the part of the cell privileged by intense PI3K signalling organised and pushed forward a so-called 'leading edge'. Some aspects of this model have subsequently been further validated whilst others have been adjusted in the light of further data. In addition, recent studies have implicated new signalling mechanisms in the compass mechanism. In parallel there have been important new conceptual advances in our understanding of the molecular machines that drive cell movement and the levels at which chemotaxis influences that process.

This review will not attempt to consider the whole field of chemotaxis but aims to put into context some recent advances in our understanding of cell movement and chemotaxis, concentrating particularly on two widely used model cell types that can exhibit very efficient chemotactic responses — mammalian neutrophils [1] and *Dictyostelium* amoebae [2]. These two model cell types undergo chemotaxis in very different circumstances. Mammalian neutrophils are normally found in the blood and are programmed to exit the circulation and chemotax towards epicentres of inflammation, guided by gradients of a variety of inflammatory mediators. In contrast, when starved, *Dictyostelium* amoebae begin to release cyclic AMP (cAMP) to which other cells respond, both chemotactically and by releasing more cAMP, via a process termed signal relay.

Morphological Polarisation of Moving Cells

Both neutrophils and *Dictyostelium* amoebae become polarised morphologically during chemotaxis. Their cell fronts are constantly protruding and retracting motile, membraneous structures and are consistently, but not perfectly, orientated in the direction of chemotaxis. The frontal structure, in its entirety, is commonly termed a pseudopod. The motile membrane sheets are sometimes called lamellipodia, by analogy with similar-looking structures on a variety of adherent cells, although this terminology glosses over some significant differences. Immediately behind the leading edge is a dense crescent of polymerised F-actin and then towards the cell body, in neutrophils at least, a pronounced density of recycling endosomes [3]. The rear of the cells is termed a uropod and often shows evidence of fine, trailing appendages that are apparently slow to detach. Two factors suggest that this structural front–rear polarisation is required for efficient, rapid movement (both cell types can attain speeds of 10–12 μm/min) but not chemotaxis *per se*: firstly, uniformly stimulated, randomly migrating cells display the same morphology; and, secondly, poorly polarised cells move more slowly but can chemotax with normal efficiency (for example, [4]).

Cellular Origins of the Forces Driving Movement

Concepts Emerging from Work with Cultured Cell Lines

The most influential force in moving cells is widely considered to be the creation of protrusions from the leading edge driven by the outward extension of actin filaments [5–8]. Recent work with cultured cell lines suggests that the actin-filament networks that support this protrusive activity are far more complex than previously envisaged and are composed of two distinct regions [9]. A peripheral network is manifest as a rapidly moving, morphologically dynamic, cycling structure that is largely unconnected to the substrate and poorly coupled to the actin networks within the body of the cell: in cultured cell lines, this can be accurately defined as the lamellipodium. Counterintuitively, this peripheral network does not seem to be important in driving protrusive advancement of the leading edge, which is instead mediated by the deeper actin network, termed the lamella in cultured

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cell lines. Critically this deeper network is physically associated, via a clutch-like series of contacts [10], with substrate–adhesion complexes at the cell surface (in cultured cell lines, these complexes are primarily focal adhesions). The primary force for advancement emerges from the addition of actin monomers to appropriately orientated filaments that are mechanically coupled to the substrate. In this context the lamellipodium can be envisaged to 'ride' on the front of the advancing boundary of the lamella.

The other major force driving cell movement is actomyosin-based contraction. The highest concentrations of contractile elements in the actin networks described above are within the lamella, and hence they are capable of pulling centripetally against points of adhesion to the substrate. These forces are also responsible for breaking substrate adhesions at the rear of the cell [11,12]. Often caricatured as being 'at' the rear of the cell, this process is actually active across most of a cell's footprint. Although this actomyosin-based mechanism of force generation is important, it is not essential for motility of amoebae [13], except through matrices that offer resistance [14].

Forces Governing Movement in Neutrophils and Amoebae

Recent work measuring traction stresses in neutrophils moving over polyacrylamide gel is difficult to reconcile with the above studies in cultured cells. The work in neutrophils showed that greatest forces were found under the uropod; furthermore, intense stress at the rear of the cell was correlated with the advancement of the leading edge along the same axis 1–2 minutes later [15]. These findings were interpreted to mean that actomyosin-mediated contraction at the rear of the neutrophil was a major factor in both initiating movement and forcing the cell forward.

A striking theme in studies of the cytoskeletal structures that potentially underpin the movement of cells like neutrophils and *Dictyostelium* is the detection of wave-like patterns of rearrangement [16,17], perhaps related to similar behaviours in cultured cells [9,18]. Some of this work analysed the distribution of a fluorescent-protein-tagged version of Hem-1/Nap1 (a ubiquitous regulator of actin polymer formation [19]) in the vicinity of the plasma membrane in neutrophil-like cell lines. This study led to the suggestion that Hem-1 is recruited to the plasma membrane from the cytosol in self-propagating waves that move in the plane of the surface [16] and are mechanistically coupled to waves of actin polymerisation. Although an attractive idea, it is not yet clear how these waves are related to either cyclical protrusive activity at the leading edge or the deeper, force-generating actin networks.

Despite these outstanding studies of cytoskeletal structure in neutrophils and *Dictyostelium*, we do not have a detailed understanding of their actin dynamics and architecture that is equivalent to that gained in cultured cell lines. Furthermore, there are some clear differences between neutrophils/*Dictyostelium* and cultured cells, such as a lack of conventional focal adhesions. It seems reasonable to assume currently that the relentlessly and randomly protruding and retracting structures at the front of moving neutrophils are supported by a wave-like propagation of polymerisation and rearrangement in peripheral actin networks, as seen in lamellipodia and described above. Further, it is likely that these structures can either retract or be consolidated by the advancement of an actin network that is mechanically coupled to the substrate and has contractile properties. Clearly, the decision to consolidate or retract/collapse protrusions from the leading edge is central in the determination of the direction of movement.

Membrane Dynamics during Movement

The process of extending pseudopods during phagocytosis or movement leads to an increase in the effective surface area of neutrophil-like cells. A recent study indicated that the surface area of motile *Dictyostelium* amoebae can fluctuate by 20–30% in a manner that is not compensated by assembly/disassembly of filopodia [20]. Given that the plasma membrane ruptures if it is stretched beyond 2–3% of its starting area [21], it is clear that motile cells must manage this problem. Some past work has been interpreted as indicating that there is rapid, vectorial recycling of plasma membrane, associated with net membrane flow from the leading edge and endocytic retrieval at the rear [22,23]. These ideas were developed with the aim of defining mechanisms driving cell movement. The concept of membrane flow as a force for movement is no longer accepted widely. However, the underlying data demonstrating high rates of endocytic recycling of membrane in *Dictyostelium* have been confirmed and extended and used as the basis of a proposal that this membrane flux may accommodate rapid changes in surface area associated with shape change and movement [20]. Potentially related to this idea, an analysis of phagocytosis revealed that the dramatic increases in surface area associated with the uptake of large particles were dependent on PI3K activity [24].

Signalling Networks Engaged by Chemoattractants

The effects of both extracellular cAMP on *Dictyostelium* and chemoattractants on neutrophils are mediated via G-protein-coupled receptors (GPCRs) and heterotrimeric G proteins. In *Dictyostelium*, Gα2$\beta\gamma$ (there are only single copies of the Gβ and Gγ genes in *Dictyostelium*) and the cAMP receptor (cAR1) are specifically responsible for conveying the chemotactic signals from cAMP [25,26]. Many would further accept that the G$\beta\gamma$ subunits are probably necessary and sufficient for transmission of the cAMP signal. Neutrophils express many more combinations of G proteins; nevertheless, chemotactic signals from GPCRs for ligands like fMLP, C5a and IL-8 are mostly mediated by pertussis-sensitive Gαo/i proteins that are similarly thought to signal via G$\beta\gamma$ subunits [27,28]. However, some work indicates that Gα12/13 may also play a role in chemotactic signalling by stimulating the activation of Rho GTPases [29]. A large and growing family of intracellular signalling effectors engaged by G-protein activation has been demonstrated to have a role in chemotaxis, including Ras- and Rho-family GTPases, PI3Ks, phospholipase C (PLC) and phospholipase A_2 (PLA_2). Most relevant for the discussion in this review are the PI3Ks.

PI3Ks can phosphorylate one or more of the lipids, PtdIns, PtdIns4P and PtdIns(4,5)P_2 at the 3′ position. There are three classes of PI3K [30], but only the class I PI3Ks are thought to be relevant to chemotaxis in neutrophils and *Dictyostelium* (some data suggest that class II PI3Ks may be involved in migration of cultured cell lines [31,32]). The most characteristic feature of class I PI3Ks is their ability to be activated rapidly by many types of cell-surface receptor to produce PtdIns(3,4,5)P_3, primarily in the inner leaflet of the plasma membrane.

Table 1. Class I PI3K effectors in neutrophils.

Class		Names	Function/effector domains/comments
Protein kinases	Serine/threonine kinases	PDK-1	Phosphorylates $PtdInsP_3$-bound PKB on T308.
		'PDK-2'	Phosphorylates S473 of PKB in PI3K-dependent manner; likely to be TORC2 complex but PI3K regulation is unclear.
		PKB/AKT	$PtdIns(3,4)P_2$/$PtdInsP_3$-regulated AGC kinase; targets include GSK3ß and $p70^{S6K}$; three forms (1, 2, 3), not clear which are in neutrophils. PH domain is an effective reporter.
	Tec-family tyrosine kinases	BTK	$PtdInsP_3$-regulated kinase involved in a variety of antibody receptor signalling pathways.
		ETK/Bmx	Similar to BTK.
Adaptor	PI3K/tyrosine kinase signalling	DAPP1/Bam32	SH2- and PH-domain-containing effector (binds both $PtdIns(3,4)P_2$ and $PtdInsP_3$) involved in Src signalling.
GAPs	Rho and/or Arf GAPs	ARAP3	$PtdInsP_3$- and Rap1-regulated RhoA GAP and Arf6 GAP.
		Centaurin 1α	Arf GAP.
	Cdc42 GAP	Cdc42 GAP	SEC14 -domain-containing Cdc42 GAP.
GEFs	Rac GEF	PRex1	$PtdInsP_3$- and Gβγ-regulated Rac GEF critical in the formation of reactive oxygen species but less important for movement.
		DOCK2–Elmo complex	$PtdInsP_3$ regulation of this Rac GEF distribution is critical for polarisation of F-actin; may bind through both subunits.
		Vav1/3	Vav1 and Vav3 are functionally important in neutrophils possibly regulated by PI3Ks indirectly.
	Cdc42 GEF	PIX	A Gβγ-regulated Cdc42 GEF, the distribution of which is regulated by $PtdInsP_3$, critical for chemotaxis.
	Arf GEF	Cytohesin 4	$PtdInsP_3$-binding Arf GEF, Arf selectivity *in vivo* is unclear.
Adaptor	Cytoskeleton	WAVE2	$PtdInsP_3$-binding shown to be important for its role in regulating actin polymerisation.
Others	Unconventional myosin	Myosin IF	Poly-basic region appears to confer $PtdInsP_3$ binding. [1]NFV
	Cytoskeletal, ERM family	Ezrin	$PtdInsP_3$-binding cytoskeletal protein can bind other phosphoinositides and RhoGEFs. [1]NFV
	Protein tyrosine phosphate phosphatase	MEG2	SEC14 domain seems likely to colocalise and interact with phosphoinositides, important roles in vesicle fusion through its substrate NSF. [1]NFV

[1]NFV: Not fully validated as a PI3K effector. For references see text.

PI3K Signalling in Neutrophils

Four class I PI3Ks are expressed in neutrophils and all of these are heterodimers of regulatory subunits and catalytic subunits (α, β, δ and γ, after which the PI3K is named, with approximate relative abundancies of 4%: 4%: 90%: 2%, respectively, in neutrophils; [33] and Marcus Thelen, personal communication). These four class I PI3Ks are further divided into two classes: class IA PI3Ks (α, β and δ) have regulatory subunits from one of a family of three genes (p85α, p85β and p55) that contain Src homology 2 (SH2) domains and can be activated by phosphotyrosine-based mechanisms [30]. The single class IB PI3K (γ) can bind to Gβγ-sensitive regulatory subunits (p84/p87PIKAP [34,35] or p101 [33]) and can be activated by relatively abundant pertussis-sensitive Gαo/i proteins *in vivo*.

All of the catalytic subunits of class I PI3Ks contain Ras-binding domains (RBD). Studies of Ras-insensitive knock-ins of PI3Kγ and PI3Kα in mice and Dp110 (the class I PI3K in *Drosophila*) in flies have shown that these class I PI3Ks are regulated, probably by Ras–GTP, via their RBDs *in vivo* [36–39]. Given that chemoattractants can activate the N- and K-Ras isoforms extremely rapidly in neutrophils (although via an unknown mechanism), it appears that these dynamic Ras signals act synergistically with Gβγ subunits to stimulate PI3Kγ and hence $PtdIns(3,4,5)P_3$ production. The unique characteristics of this form of regulation have been predicted to be important in the creation of $PtdIns(3,4,5)P_3$ gradients.

The majority of neutrophil chemoattractants activate $PtdIns(3,4,5)P_3$ accumulation through Gαi/o-containing G proteins. Consistent with the above, PI3Kγ is the dominant class I PI3K in this context [40–42]. There are subtleties to this regulation, however; the kinetics of accumulation of $PtdIns(3,4,5)P_3$ in response to fMLP is bi-phasic, and different ligands, although all acting through Gαi/o, have very different time courses ([43] and H. Guillou, L.S., P.T.H., unpublished data). Detailed analysis of the response to fMLP revealed that the second phase of $PtdIns(3,4,5)P_3$ accumulation in human neutrophils is also dependent on PI3Kδ and, to a lesser extent, PI3Kβ [43]. It would not be surprising if the difference in the kinetics of the various ligand responses was partly attributable to the differential involvement of the class IA PI3Ks. The molecular mechanism underpinning the recruitment of the class IA PI3Ks in these G-protein-dependent pathways has not been fully established, but evidence suggests a requirement for signalling through Src-family kinases. In keeping with this idea, studies of related signalling in neutrophil-like cell lines have revealed complex patterns of $PtdIns(3,4,5)P_3$ accumulation and an involvement of class IA and B PI3Ks that are quite distinct from those seen in neutrophils [44]. Chemoattractant-stimulated $PtdIns(3,4,5)P_3$ accumulation in neutrophils is transient due to the actions of two phosphatases — SHIP-1, a phosphoinositide 5-phosphate phosphatase that leads to the delayed accumulation of $PtdIns(3,4)P_2$, and PTEN, a phosphoinositide 3-phosphate phosphatase [30].

$PtdIns(3,4,5)P_3$ signalling is transduced by a large family of effector proteins that can bind selectively to the head groups of $PtdIns(3,4,5)P_3$ and/or $PtdIns(3,4)P_2$. In many cases these protein–phosphoinositide interactions are mediated by a sub-family of pleckstrin homology (PH) domains with a characteristic cluster of basic residues [45]. Typically, but not universally, these PH-domain-containing effector proteins

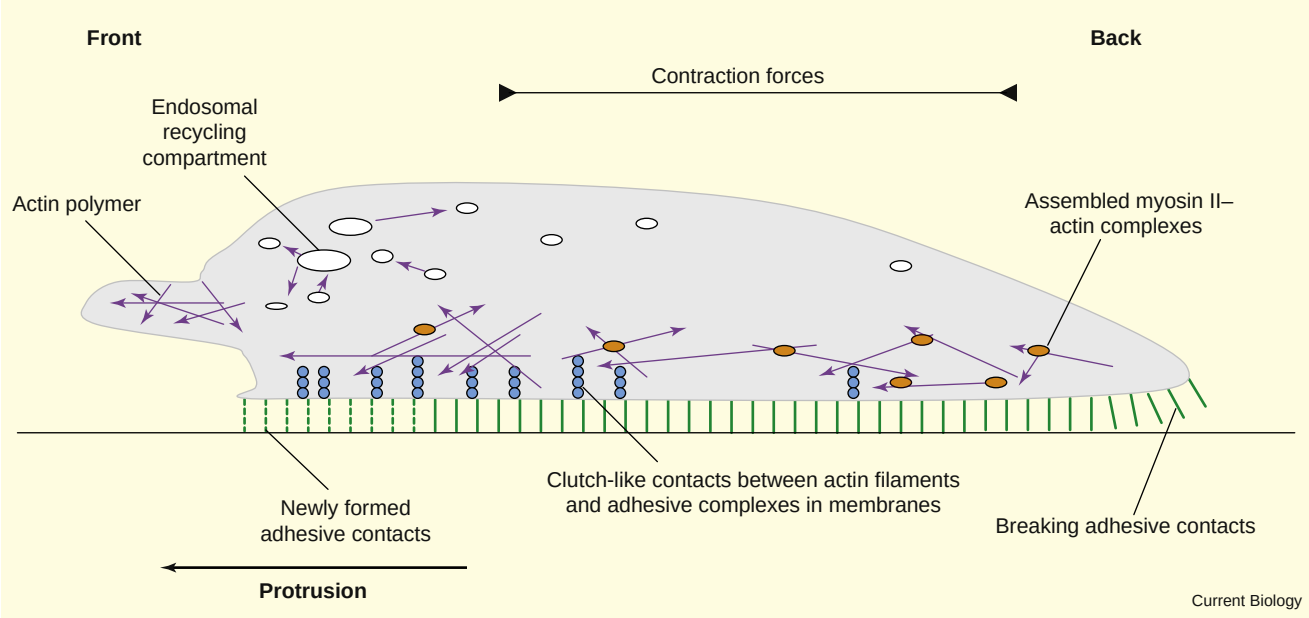

Figure 1. Schematic representation of a side view of a motile neutrophil.

Actin filaments (purple) grow at their barbed ends (depicted as arrowheads) through polymerisation. Filaments can be associated through clutch-like complexes of proteins (blue) to adhesion complexes (green) at the base of the cell, against which actin-polymerisation-driven protrusive forces can operate. In neutrophils it is not clear where the primary forces driving advancement of a leading edge are located. (In other cells this appears to be the lamella, a zone immediately behind the highly motile, but unadhered, lamellipodium.) The endosomal recycling compartment is located near to the leading edge. Net gain of adhesive contacts occurs at the front of the zone of adhesion to the substrate and net loss at the rear. Myosin II complexes (orange), capable of generating a contractile force in concert with actin filaments, are mostly found at the rear of the cell but are probably active throughout the actin network.

translocate to the plasma membrane upon activation of class I PI3K signalling. The translocation propagates further signalling through recruitment and/or allosteric modulation of additional enzymatic or protein–protein interaction modules involving the effector proteins [30].

A targeted proteomic screen for PtdIns(3,4,5)P_3-binding proteins in neutrophils identified 16 proteins [46]. Taken together with data showing that other known PtdIns(3,4,5)P_3-binding proteins are present in neutrophils (such as protein kinase B (PKB), 3-phosphoinositide-dependent kinase-1 (PDK-1) and P-Rex1 [47]) and the fact that a number of proteins were isolated in the above screen but remained unidentified, it becomes apparent that neutrophils contain around 25 PtdIns(3,4,5)P_3 effectors (Table 1). These proteins would be expected to translocate to sites of class I PI3K activation and PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 accumulation, but to different extents and with different kinetics, on the basis of their distinct lipid-binding selectivities and interaction constants. The collection of PtdIns(3,4,5)P_3 effectors in neutrophils thought to be recruited to sites of PtdIns(3,4,5)P_3 accumulation includes proteins with a range of signalling capabilities potentially relevant to cell movement (Figure 1 and described in more detail below).

PI3K Signalling in Dictyostelium

Dictyostelium has six class I-like PI3Ks: DdPI3K1–5 each contain an RBD and DdPI3K6 contains a PH domain but not an RBD. To date, none of these PI3Ks has been shown to bind to a regulatory subunit. DdPI3K1 and 2 are responsible for most of the production of PtdIns(3,4,5)P_3 in response to cAMP [48]. A genetic knock-in of a Ras-insensitive point mutant of DdPI3K1 (in a constitutively active Ras background) has shown that Ras is the prime activator of DdPI3Ks at early timepoints after stimulation with cAMP [39]. Although a large family of Ras guanine nucleotide exchange factors (RasGEFs) and Ras proteins could potentially act upstream of PI3Ks in *Dictyostelium*, study of a collection of knockout mutants suggests that the GefR–RasG and GefA–RasC cassettes are most likely to act within cAR1- and G-protein-regulated pathways controlling PI3Ks [49]. Interestingly, genetic analysis only connects the GefR–RasG cassette to regulation of migration. The consequence of engaging this pathway is a rapid accumulation of PtdIns(3,4,5)P_3 (i.e. within 5–10 seconds). Simultaneous signals eliciting the creation of a local F-actin network further enhance PtdIns(3,4,5)P_3 accumulation at later timepoints (60 seconds) through the ability of class I PI3Ks to bind to F-actin and hence become concentrated at sites enriched in cortical F-actin [50] (Figure 2). Furthermore, a recent study has indicated that an additional positive-feedback loop, in which F-actin-driven accumulation of PI3Ks enhanced local activation of Ras, may operate within this apparently simple pathway [51].

Increases in PtdIns(3,4,5)P_3 levels in *Dictyostelium* are very transient as a consequence of PTEN-like 3-phosphatases [52,53]. The *Dictyostelium* genome contains four relatives of mammalian phosphoinositide 5-phosphatases, Dd5P1–4 and, while only Dd5P2 has been reported to have PtdIns(3,4,5)P_3 5-phosphatase activity, loss of Dd5P2 has only a minor effect on cell migration [54]. PTEN appears to be held at the membrane through a number of interaction mechanisms in which PtdIns(4,5)P_2 appears to be important. Some recent work suggests that PLC, through its ability to degrade PtdIns(4,5)P_2, can regulate PTEN distribution by preferentially driving the loss of PTEN from the leading

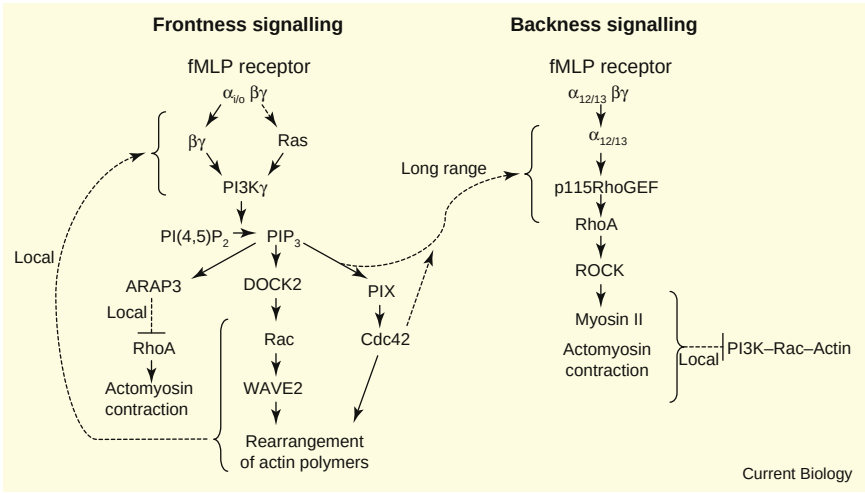

Figure 2. Spatial resolution of signalling and structure during chemoattractant-stimulated polarisation.

A model attempting to map the core signalling processes in neutrophils that enable the creation of stable polarity in response to a single uniformally applied chemoattractant, fMLP. GPCR and G-protein signalling is thought to remain homogeneous in contrast to downstream events. Additional mechanisms may contribute in some contexts (e.g. localised autocrine signalling at the leading edge or polarisation of receptor distribution). Key features are the local incompatibility of the two networks manifest through reciprocal suppression based on key local cytoskeletal structures and a long-range reinforcing signal from the front favouring domination of backness signals at the rear providing stability to the system. Many molecular details are missing, such as signals translating an active Rho–myosin II cassette into suppression of the frontness, and the PI3K–Rac–actin axis supplying positive feedback from Rac and actin polymers to PI3Ks. Dashed lines signify undefined pathways.

edge [55]. This mechanism appears critical for the formation of gradients of PtdIns(3,4,5)P_3.

Dictyostelium has only three known class I PI3K effectors; cytosolic regulator of adenylate cyclase (CRAC) [52], the PH-domain-containing protein PhdA [48] and PkbA (a PKB-related kinase) [56]. Significantly, CRAC is required for the process of signal relay, in which extracellular cAMP stimulates further production and release of cAMP [57]. Loss of either PhdA or PkbA has distinct consequences, leading to reductions in the levels and kinetics of actin polymerisation or to defects in cell polarisation, respectively.

Polarisation of Signalling in Response to Chemoattractants

The majority of studies of eukaryotic chemotaxis had assumed that eukaryotic cells, unlike prokaryotes, can detect differences in chemoattractant concentration across their surface and do not rely on movement and/or time to decipher a gradient. The evidence underpinning this assumption had been weak until the publication of a recent study that used micro-fluidics to rapidly establish defined, complex gradients around neutrophil-like cells [58]. This work showed that, under conditions in which the cells had not moved significantly and/or the concentration gradient around the cell was unchanged, the cells could still move off in the orientation of that gradient. The simplest interpretation of this result is that cells can detect differences in the concentration of chemoattractant between points on their surface [58], presumably by translating the extracellular gradient into an intracellular derivative, encoded by the signalling networks engaged by the chemottractant.

Gradient-based stimulation of both neutrophils and *Dictyostelium* leads rapidly to profound polarisation aligned with the gradient. Interestingly, when the gradient of chemoattractant is relatively shallow (such as 1–4% change over a cell's length — corresponding to a fall from 100% at the front to 99–96% at the rear), cells can become far more polarised, in terms of % change per unit distance, than the external gradient; this phenomenon led to the concept of polarity amplification. Studies of both neutrophils and amoebae indicate that neither the distribution of chemoattractant receptor nor the distribution of G-protein activation becomes substantially polarised but instead follows the gradient of chemottractant [59–63]. (Work in neutrophils has shown that ATP can be released from developing leading edges and that this can activate P2Y2 purinergic receptors locally and also be degraded to adenosine, which can stimulate A3 adenosine receptors externalised at the leading edge; however, this process can only operate in the context of substantial pre-existing polarity [64].) Use of biosensors composed of fluorescent proteins fused to PH domains, which are capable of binding selectively to different phosphoinositides [65,66], has revealed that, in contrast to the distribution of chemoattractant receptors and G proteins, the levels of PtdIns(3,4,5)P_3 become remarkably polarised in amoebae, neutrophils and neutrophil-like cell lines, with high levels closest to the leading edge [4,56,62,67–70].

Molecular Basis of the Polarisation of PI3K Signalling during Neutrophil Chemotaxis

The localised accumulation of PtdIns(3,4,5)P_3 was quickly appreciated to be a potentially critical signal co-ordinating chemotaxis or movement because of its ability to concentrate PI3K effectors at a leading edge where they could contribute to remodelling of that domain.

In mouse neutrophils the PtdIns(3,4,5)P_3 accumulation is clearly dependent on PI3Kγ and SHIP-1 (and not PTEN) activities, but the mechanistic details have yet to be resolved [4,43,68]. A number of molecular mechanisms that could contribute to the steep polarisation of PtdIns(3,4,5)P_3 signalling have been considered. Firstly, PI3Kγ may be regulated *in vivo* by the synergistic action of G$\beta\gamma$ subunits and Ras [36]. Modelling studies of eukaryotic chemotaxis have revealed that introduction of the assumption that PI3Kγ activity is controlled by a coincidence circuit requiring simultaneous activation by Ras–GTP and G$\beta\gamma$ subunits (a specific form of synergy) [71] has powerful effects in accentuating the local accumulation of PtdIns(3,4,5)P_3 at the leading edge (as suggested previously in [72]). It then becomes unnecessary to propose the existence of undefined global inhibitors,

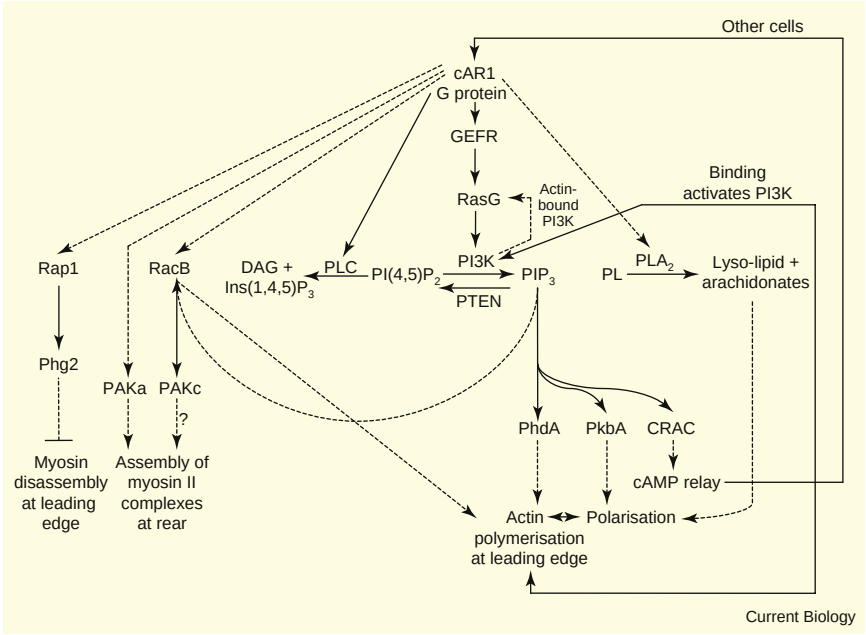

Figure 3. Signalling regulating chemotaxis in *Dictyostelium*.

cAMP binding to cAR1 leads to activation of a large collection of signalling cassettes that have roles in chemotaxis. The PI3K network takes a central position in coordinating assembly of leading edges. Key events in zones of protrusion are: the ability of PI3Ks to bind, and be further activated by, accumulating actin polymers; activation of PLC driving a local reduction in PtdIns(4,5)P_2 (PI(4,5)P_2) levels, dissociation of PTEN from the membrane and thus reduced suppression of PI3K activity; and Rap1- and Phg2-kinase-mediated local disassembly of myosin [100]. The identity of the arachidonate derivatives and their effectors that control actin polymerisation are unknown. Some evidence, from studies using combined application of inhibitors and $InsP_3$R- and PLC-deficient cells, suggests Ca^{2+} may also have a role [95]. PL, phospholipid substrates of PLA_2. Dashed lines signify undefined pathways.

required in the majority of 'local-excitation, global-inhibition' models [73]. Secondly, although it has not been established whether SHIP-1 is regulated directly by chemoattractants, the phenotype of SHIP-1-deficient neutrophils — extremely flattened, spread and with weakly polarised morphology and PtdIns(3,4,5)P_3 levels [4] — and the known regulatory mechanisms, binding partners and structure of SHIP-1, mean that this phosphatase might 'actively' drive the polarisation of PtdIns(3,4,5)P_3 through changes in its distribution and/or activity. Thirdly, a large body of work has indicated that Rac proteins, and/or components of F-actin networks that are dependent on Rac activity, may form part of a positive-feedback loop capable of stimulating PtdIns(3,4,5)P_3 production preferentially at the leading edge [29,50,74,75]. Fourthly, mechanisms involving local release of chemoattractants at the leading edge that are capable of stimulating PI3K activation (e.g. ATP and adenosine [64]) have been proposed to contribute. Finally, substantial and diverse data support the concept that PI3Kγ can drive activation of class IA PI3Ks [43,75,76]. Little is known, however, about the molecular details of such activation, beyond a possible involvement of Src-family kinases and PI3Kδ.

Molecular Basis of the Polarisation of PI3K Signalling during Dictyostelium *Chemotaxis*

In *Dictyostelium*, activated Ras proteins can be concentrated at a leading edge and are the most rapid chemoattractant-elicited signals that stimulate class I PI3K activity [39,50]. Together with the dissociation of PTEN from the plasma membrane at the leading edge, driven by PLC-mediated depletion of PtdIns(4,5)P_2 [55], and a positive-feedback signal delivered by the recruitment of PI3Ks to F-actin formed in response to activation of RacB at the leading edge [50,74], this activation of Ras drives polarisation of PtdIns(3,4,5)P_3 (Figure 3).

Polarisation in Response to Uniform Stimulation with Chemoattractants

A number of authors have argued that the molecular mechanism responsible for the generation of polarity amplification is likely to have a central role in the process by which cells detect the vector of shallow gradients of chemoattractants. However, both neutrophils and amoebae can polarise in response to uniform stimulation with chemoattractants. Indeed, *Dictyostelium* mutants that lack G proteins can spontaneously polarise both their PI3K signalling and their morphology [51]. In both cell types, PI3Ks are activated uniformly around the cell initially and, after a few minutes, clear leading edges emerge, with concentrated PI3K signalling. Clearly, cellular polarity can develop without the need to be seeded by an external gradient; furthermore, as described above, cells with very weak morphological polarisation are still able to detect a gradient of chemoattractant and chemotax efficiently (Figure 4).

Other signalling systems can also become polarised in response to chemoattractants. The data describing a gradient of Rho activity along the axis of polarised neutrophils (low at the front) [77] are convincing and this gradient is considered to drive myosin-based contraction at the rear [29,78], in part due to signals mediated via Gα12/13, probably through p115RhoGEF, Rho, its effector kinase ROCK and myosin II [29]. The authors of this work have also provided evidence and persuasive argument that this 'backness' pathway is mutually exclusive (in spatial terms) [29,77], but partially dependent upon a 'frontness' network [79], driven by the same chemoattractant and mediated via Gαi, PI3K (probably γ), Rac and F-actin (Figure 2). Although many of the key molecular details are missing, this concept could potentially explain spontaneous polarisation and, indeed, has formed the basis of a recent mathematical model of chemotaxis [71].

Role of Polarised PtdIns(3,4,5)P_3 Signals

A collection of data came together in around 2002 that suggested that PtdIns(3,4,5)P_3 signals were a part of a compass mechanism, sensing and relaying the vector of extracellular gradients of chemoattractants. Firstly, a large number of studies showed that PI3K inhibitors or genetic loss of PI3Ks cause neutrophils and amoebae to fail to respond in a variety of *in vitro* and *in vivo* chemotaxis assays that macroscopically assess whether cells reach a destination, such

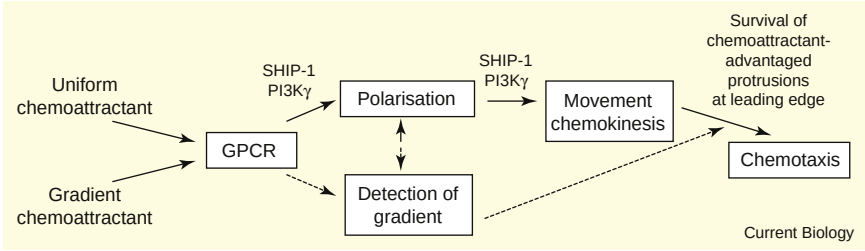

Figure 4. The components of neutrophil chemotaxis.

Uniform levels of chemoattractant can stimulate neutrophils to polarise. This profound structural polarisation is probably made possible by the creation of spatially incompatible frontness and backness signalling domains that resolve into leading and trailing edges to the cell (refer back to Figure 1). The PI3Kγ, PtdIns(3,4,5)P_3 and SHIP-1 signalling cassette is involved in organising some aspects of polarisation. The outcome is that the cell gains the potential for very efficient, rapid movement, as a result of the random generation of protrusions at the front. If the chemoattractant was present in the form of a gradient the cell is able to detect the vector of that gradient without needing to move or experience progressive changes in chemottractant concentration. The molecular mechanisms involved are unclear but must involve, at least, local polarisation of signalling. However, this is not dependent on the complete polarisation programme, because substantially unpolarised cells can still chemotax efficiently. Signals convey information about the direction of the gradient through an undefined mechanism, perhaps operating locally at the leading edge, and act at the leading edge to favour the survival or adhesion of those randomly formed protrusions that receive the strongest stimulation by chemoattractant. Chemotaxis emerges from the integration of random motility and gradient-biased signalling.

as the lower well in a transwell filter assay, the target zones of an under-agarose assay or the peritoneum in a murine model of peritonitis. Secondly, a smaller number of reports examined the paths of individual cells during chemotaxis and some of these described reductions in chemotactic efficiency (measured in terms of, for example, migratory index) in the presence of PI3K inhibitors or in the absence of PI3Ks. Thirdly, further evidence is provided by some previously mentioned data demonstrating the rapid emergence of steep gradients of PtdIns(3,4,5)P_3 in chemotaxing cells. Finally, additional data suggested that these PtdIns(3,4,5)P_3 gradients were only reduced, and not abolished, by inhibitors of the accumulation of F-actin, such as latrunculin, and were therefore unlikely to be caused by the polarisation of the actin cytoskeleton.

This body of work has been re-evaluated recently through the publication of a series of papers that further dissect some of these issues. In many cases this additional work has confirmed the earlier findings, whilst enabling a more complete interpretation, although some discrepancies remain unresolved [68]. The outcome is that models putting PI3Ks at the heart of a compass mechanism that is conserved between amoebae and mammals are no longer widely held.

Several high-quality studies have resolved multiple roles for PI3Ks (particularly PI3Kγ and δ) in neutrophil migration *in vivo* [80,81]. Collectively, this work showed that PI3Kγ, in the neutrophils and not the endothelium, was important for emigration of neutrophils from the circulation in response to brief stimulation with chemokines; after longer stimulation, emigration was dependent on PI3Kδ. In contrast, when challenged with TNFα, a role for PI3Kγ in the endothelium in the interaction between neutrophils and endothelial cells could be discerned [80]. Hence, these results showed that inhibition of PI3K activity resulted in fewer cells exiting the circulation and therefore fewer cells being in a position to chemotax to a site of inflammation. In fact work that focused on migration within tissue compartments revealed that neutrophils moved faster in the presence of PI3K inhibitors because of the repression of PI3K's adhesive functions [82].

Work examining migration of neutrophils through transwell filters either in the presence of chemoattractant gradients or uniform chemoattractant concentrations showed that genetic loss of PI3Kγ or selective PI3K inhibitors caused reductions in the chemokinetic responses of the cells that could explain the apparent reductions in chemotactic migration [68] (confirming the earliest work on this issue [83]). Similarly, some experiments examining the effects of either genetic loss of PI3Ks or selective PI3K inhibitors on the chemotactic efficiency of both neutrophils and *Dictyostelium* amoebae revealed no specific deficiencies [4,68,84–86]: in one study it was clear that a PI3Kγ-selective inhibitor caused a reduction in the migratory index of neutrophils but a precisely equivalent effect was seen with the inhibitor on PI3Kγ KO neutrophils, indicating that these effects were probably off-target [68], possibly explaining some reported effects of PI3Kγ-selective inhibitors on neutrophil navigation [79].

The concept that PI3K signalling becomes sharply polarised to the leading edge during chemotactic or random movement has received substantial further support, although, as argued above, the molecular mechanisms sustaining this response have not been fully clarified [4,68,87]. The primary functions of this PtdIns(3,4,5)P_3 signal also remain poorly understood. Suppressing the accumulation of PtdIns(3,4,5)P_3 at the leading edge in neutrophils has identified a number of potential targets. Initially the neutrophils showed a less complete and less stable polarisation of their F-actin network, suggesting that some aspects of polarisation are being led by PI3Ks [4,68]. Part of this effect may be mediated via the ability of PtdIns(3,4,5)P_3 to regulate the distribution of key regulators of the location and extent of actin polymerisation, such as the RacGEF DOCK2, WAVE2, WASP (in *Dictyostelium*, WASP is a target of Cdc42, which controls Arp2/3 in parallel with WAVE [88]) and PIX–CDC42GEF complexes [89–91], similar to the situation in *Dictyostelium* described previously [74]. Although a molecular mechanism is missing, these details dovetail with evidence that PI3K activity might control the rate, but not the location, of protrusion formation driven by actin polymerisation from the leading edge [84].

There is also evidence that PI3K signals may dictate integrin-mediated adhesive events that are relevant to neutrophil movement [68,92,93]. On fibrinogen-coated surfaces, where fMLP causes a dramatic chemokinetic response, there is a coincident rise in the overall strength of adhesion of neutrophils to this substrate and upregulation of the surface levels of a major integrin, $\alpha_M\beta_2$, involved in binding fibrinogen. Blockade of PI3Ks resulted in reductions of both the upregulation of $\alpha_M\beta_2$ and the increased strength of adhesion and movement [68]. The implication of these findings is that the significant body of work suggesting that PI3K activity has an important role in controlling various aspects of integrin

function may, unsurprisingly, be relevant in chemoattractant-stimulated movement (Figure 4).

Some past work has suggested that PI3Ks are involved in regulating the rapid delivery of new membrane to the cell surface to accommodate the demands of phagocytosis of large particles [24]. Furthermore, a number of PI3K effector proteins, such as ARAP3 or centaurin α1, can act as GTPase-activating proteins (GAPs) for Arf GTPases and seem well poised to fulfil roles relevant to such processes, given that cycling of Arf GTPases can control endosomal recycling. Collectively, these results point to the possibility that PI3K signals in chemotaxing cells may also co-ordinate delivery of new membrane required to enable the changes in cell shape associated with movement [20].

Within the framework of models envisaging frontness and backness signalling networks that are, in spatial terms, mutually exclusive, it is clear that a key role of the PtdIns $(3,4,5)P_3$ gradient is to shape a reciprocal Rho pathway [77]. Potentially consistent with this model, and with work in other cells indicating the importance of localised inhibition of Rho function to enable lamellipodia extension [94], ARAP3 is a molecule in neutrophils that is recruited and activated by PtdIns$(3,4,5)P_3$, presumably at the leading edge, and also acts as a RhoA GAP, capable of suppressing Rho function.

Role for PI3Ks in Orientation?

Some workers have reported that in *Dictyostelium* the sensitivity of chemotaxis to PI3K inhibitors is a function of the cAMP concentration to which the cells are responding: at low concentrations of cAMP, either distant to a micropipette or in a micropopulation assay, scoring gradient-biased accumulation, the response was PI3K dependent [95,96]. Furthermore, two independent studies have revealed roles for PLA_2 activity acting in concert with PI3Ks in *Dictyostelium* to regulate chemotaxis [95,97]. Both groups suggest the two signalling pathways are operating in a 'redundant' parallel manner (although it is clear that these pathways are very unlikely to represent molecular replacements for one another), such that in the absence of PLA_2 activity chemotaxis becomes PI3K dependent. Together these results have been interpreted to suggest that, under conditions where the chemotactic abilities of *Dictyostelium* are challenged, an underlying role for PI3K signalling in the orientation of movement is revealed. Both of these very interesting results are difficult to reconcile with work in which *Dictyostelium* amoebae have been shown in an under-agarose assay format to chemotax towards similarly low concentrations of cAMP in a PI3K independent manner [84] and chemotax perfectly efficiently in the complete genetic absence of all PI3Ks and PTEN (in contrast to all previous studies in which only subsets of PI3Ks were deleted, although this work used relatively high concentrations of cAMP) [85]. The quality of the latter results implies that the 'redundancy' between PI3K and PLA_2 signalling must be very near complete; the potential purpose/advantage of this type of redundancy for a wild-type cell is totally unclear. Perhaps both phenomena are a result of a reduction in the effective responsiveness of cultures and/or individual amoebae with diminished PLA_2 or PI3K activity to cAMP in specific assay contexts. In the case of cells with reduced PI3K activity, this reduced responsiveness might be a consequence of a decline in cAMP signal relay, a process known to be dependent on the PI3K effector CRAC, and this effect would possibly be more significant when assays are conducted in the presence of low concentrations of exogenous cAMP.

Mechanism by Which the Gradient-Encoding Apparatus Biases Cell Movement

Currently there is no single signalling system that appears to be responsible for encoding an intracellular representation of extracellular gradients of chemoattractants that is used to guide chemotaxis. Such a system may only become apparent after the combined analysis of the entire signalling network engaged by chemoattractants. However, a consensus is emerging (or re-emerging [98]) that the critical sampling and comparison of chemoattractant concentration occurs at points of protrusion across the leading edge [84,99], contrasting with many analyses of eukaryotic chemotaxis, which have assumed that the gradient is sampled across the length of the cell. Two models have been proposed to explain how this sampling becomes linked to an increased likelihood that a protrusion becomes firmly attached to the substrate and represents a point of advancement up a gradient. The first suggested that stronger signalling at a particular point will drive the local advance of firm attachment to the substrate, but noted the volatility of comparisons of receptor activation between two points close to one another in a gradient, and hence suggested that chemotaxis emerged from the biasing over time of very noisy protrusive activity [99]. The second model was based on data that suggested that protrusions are created cyclically, but in a spatially random manner, within the confines set by both the structure of the leading edge and the site of the preceding protrusion. These authors argued that directional bias emerged because those protrusions that received, in their lifetime, the strongest direct stimulation by chemoattractant, survived by becoming firmly connected to the substrate [84].

Conclusions

Eukaryotic cells are capable of spatially encoding extracellular gradients of chemottractants and using this vectorial information to guide chemotaxis. These processes probably occur within the region of the leading edge and can occur in the absence of PI3K activity. They act to bias the co-ordinated but underlyingly random process of cell movement by favouring the survival of the protrusions from the leading edge that receive the strongest stimulation. PI3K signalling can become polarised and aligned with the vector of gradients of chemoattractants and then appears to drive a variety of aspects of cellular polarisation, at least some of which are relevant to facilitating the chemokinetic response of the cells.

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