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REVIEW

Chemotaxis: Movement, Direction, Control

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Abstract—This review focuses on basic principles of motility in different cell types, formation of the specific cell structures that enable directed migration, and how external signals are transduced into cells and coupled to the motile machinery. Feedback mechanisms and their potential role in maintenance of internal chemotactic gradients and persistence of directed migration are highlighted.

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The ability to move is a common feature of virtually all cells. In the absence of external stimuli and open spaces only some cells remain rounded and immobile, whereas others constantly move around by extending membrane projections and shifting the bodies in random manner. The overall distances covered by the cells are rather small because they cannot keep direction of movement. They often stop, mark time as if oscillating, and move inconsistently. The motile behaviour changes abruptly upon application of external stimuli. Cells respond by activation of surface receptors and engage a number of internal signaling molecules to transmit the external signal to cell cytoskeleton and instigate changes in the cell shape and morphology. They acquire a front-to-tail polarized morphology and move up the gradient of the external stimulus, continuously sensing its direction and biasing motility toward its source. This behaviour is commonly referred to as directional migration, or chemotaxis if the external stimulus is a soluble substance.

Chemotaxis is a fundamental phenomenon and has many physiological and pathophysiological implications. It plays a central role in reproduction, development, organ patterning, mobilization, and homing of stem and progenitor cells. In the grown body it is critical for lymphocyte-mediated immune and inflammatory responses, angiogenesis by endothelium cells, vasculogenesis and

Abbreviations: Arp, actin-related protein; DOCK, dedicator of cytokinesis, a group of guanine nucleotide exchange factors for Rho-family proteins; EGF, epidermal growth factor; Epac, guanine nucleotide exchange protein directly activated by cAMP; Erk, extracellularly regulated MAP-kinase; FAK, focal adhesion kinase; FH1/FH2, formin homology (domain) 1/2; GBD/CRIB, GTPase-binding domain/Cdc42 and Rac interactive binding (domain); GbpC, cGMP-binding protein C in *Dictyostelium* cells; GPCR, trimeric G-protein-coupled receptor; Grb2, growth factor receptor-bound protein 2; IL-1β, interleukin-1β; LEGI, local excitation and global inhibition model; LIM-kinase, protein kinase containing the LIM-domain encoded by the *lin-11*, *isl-1*, and *mec-3* genes of *Caenorhabditis*; LPA, lysophosphatidic acid; MLCK, myosin II light chain kinase; NAD(P)H, reduced nicotinamide adenine dinucleotide (phosphate); NPF, nucleation-promoting factor; PAK, p21-activated kinase; PDGF, platelet-derived growth factor; PDK, phosphoinositide-dependent kinase; PH, plekstrin homology (domain); PI3K, phosphatidylinositol-3-kinase which phosphorylates phosphatidylinositols at 3′-position of the inositol ring; PIP2, PI(3,4)P2, and PI(4,5)P2, phosphatidylinositol bisphosphate with two phosphate groups attached to the indicated positions of inositol ring; PIP3 and PI(3,4,5)P2, phosphatidylinositol 3,4,5-trisphosphate; PIX, PAK-interacting exchange factor; PKB/Akt, protein kinase B, *aka* Akt; PLA2, phospholipase A2; PTEN, phosphatase and tensin homolog deleted on chromosome ten that dephosphorylates phosphatidylinositols at 3′-position of the inositol ring; ROCK, Rho-associated, coiled-coil containing protein kinase; ROS, reactive oxygen species; RPTK, receptor protein tyrosine kinase; Scar, suppressor of cAMP receptor in *Dictyostelium* cells; sGC, soluble guanylyl cyclase; SH2, Src-homology 2 (domain); SHIP1, Src homology 2 domain containing inositol-5-phosphatase 1 that dephosphorylates phosphatidylinositols at the 5′-position of the inositol ring; Src, Rous sarcoma tyrosine kinase; TorC2, target of rapamycin complex 2; WASP, Wiscott–Aldrich syndrome protein; WAVE, WASP and verprolin homologous protein in mammalian cells; WH1/WH2, WASP homology (domain).

vessel growth, axon guidance, and nerve growth. Chemotaxis is a critical component of inflammation, tissue regeneration, and reparation through wound healing and tissue remodeling. It is also involved in pathogenesis of the infectious and allergic diseases, asthma, and atherosclerosis. The central role of chemotaxis in tumor metastasis and dissemination of cancer cells has been well established.

A variety of biological compounds serve to attract or repel cells, although no strict classification has been developed so far. They include small chemical or peptide molecules, *en bloc* classified as chemoattractants or chemorepellents, and larger polypeptides or proteins of the cytokine superfamily, the name reflecting their ability to induce cell (*cyto*-) movement (-*kinos*). A subfamily of chemokines is a group of *chemo*tactic cyto*kines* that are usually small peptides. These soluble molecules serve as ligands to activate chemotactic cell surface receptors, which further signal to chemotaxis. In contrast, the movement of cells directed by the insoluble components of extracellular matrix is known as haptotaxis. This type of movement is controlled by distinct cell surface receptors that are involved in cell–substrate and cell–cell interactions and include integrins, cadherins, selectins, and ephrin receptors and their ligands.

The coupling of chemotactic receptors to the cell motile machinery occurs via specific signal transduction pathways that control cell movement and steer it towards an external stimulus. This review deals with molecular mechanisms of cell motility and their directional control.

COMMON DEFINITIONS

Cells move using protrusions commonly called pseudopods – the membrane projections of various shape and size extended at the cell front, the region of cell that faces the direction of movement. The pseudopods fasten to the surrounding matrix either by forming substrate adhesions, or by filling up gaps in 3D matrix. In the first case the cell body is then translocated by means of contractile forces applied to the points of adhesions, which is called traction, whereas in the second case the cytoplasm is squeezed into the front protrusions, which is blebbing motility.

Polarization is a key event to initiate cell migration, which results in morphological changes in the cell shape and ensures the motile behaviour. This process is sketched in Fig. 1. The cells are shown to balance, in a dynamic equilibrium, between basic states of different shapes and motility. While the transitions are formally reversible, in reality they are shifted to one of the predominating states depending on the extent of polarization. Considering these states as discrete intermediates, an analogy can be drawn with a traditional for biochemists formalization of multistep enzymatic conversions, for instance as those modeling the actomyosin cross-bridge cycle [1]. It is the ability to develop polarity that drives the transition between the A and B states in unstimulated cells and contributes dramatically to the equilibrium between the C and D states in stimulated cells, but becomes less important in the already polarized cells (Fig. 1). The intracellular mechanisms underlying the initial cell decision of whether or not polarize and which direction to choose remain a mystery, and some of them are discussed in the last section.

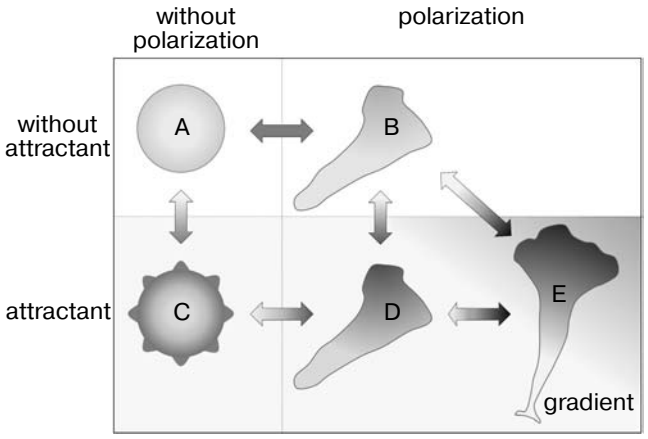

Fig. 1. Morphologic transitions induced by polarization and chemoattractants. Successive changes in the shape of unpolarized (A, C) and initially polarized (B, D, E) cells are shown schematically as the reversible transitions of basic states in the absence (A, B) and in the presence (C-E) of a chemoattractant; E) the state when chemoattractant emanates from the top right corner and forms a gradient shown in the grayscale shading.

Stimulation promotes polarization, and nonuniform stimulation exerts much stronger effects (Fig. 1); this depends on responsiveness of a particular cell and is called *directional sensing* [2]. The ability of polarized cells to extend protrusions and keep moving preferentially in one direction in chemoattractant gradients is defined as *persistent motility*. It can be determined experimentally [3]. Stimulating receptors at the front side of a cell, attractants induce *intracellular signaling* that converts external gradient into internal gradient of signaling molecules and localizes the protrusive activity to the leading edge. It is *amplified* there by an elaborate combination of signaling networks defined as the *local excitation and global inhibition* (LEGI) mechanism [2, 4]. These signaling pathways also target remodeling of cytoskeleton and locomotion responses, orienting them in the direction of the external gradient.

EXPERIMENTAL MODELS

According to the basic type of movement, chemotactic reception, and signal transduction, cells can be broad-

ly arranged into four large groups. The *prokaryotes* use fundamentally different mechanism of gradient detection and movement than eukaryotic cells. Although it may be viewed as a prototype chemotaxis to some extent [5], it will not be reviewed here. Two eukaryotic cell types, the free-living amoeba *Dictyostelium* and mammalian leukocytes are purely *amoeboid* by movement. To detect chemotactic signals they primarily use cell surface receptors coupled to the trimeric G-proteins (GPCRs). The term "amoeboid" is a conventional morphological definition rather than distinct mechanical principle [6]. Although evolutionarily distant, *Dictyostelium* cells and neutrophils are morphologically similar and share common principles of chemotactic signaling [7-9]. Instead, *fibroblasts* and smooth muscle cells are intrinsically polarized and move in phases of protrusion, standstill, and withdrawal [10, 11]. They use receptor protein tyrosine kinases (RPTKs) to recognize chemoattractants. Although their signaling and motility mechanisms differ from those in amoeboid cells, many general features are still in common [12-16]. This group also includes many other chemotactic cells such as epithelial cancer cells [17, 18], somatic follicle border cells [19, 20], and mesenchymal stem and progenitor cells [21-23]. The fourth group is *neural cells*, whose chemotactic behaviour is much more complex. While not translocating their cell body, these cells use both GPCRs and RPTKs on neurites to recognize and respond to attractive and repulsive stimuli of different classes of molecules [9, 24]. Because mechanisms of chemotactic signaling and motility of these cells obey common principles exemplified above, they will not be detailed here.

Dictyostelium

First discovered in 1935, the social amoeba *Dictyostelium discoideum* is now probably the most favorite experimental model for studying chemotaxis [25, 26]. It inhabits forest soil, has a small size (10-20 μm in diameter), and two stages of the life cycle. In the vegetative state it is an independent single amoeboid cell that hunts bacteria and yeast by chemotaxis, consumes them by phagocytosis, and divides by mitosis. These cells migrate with a speed up to 20 μm/min and divide every 4-12 h. When food runs out, the individual cells initiate a differentiation program and enter the reproductive state. They synthesize and use cAMP as a chemoattractant to communicate with each other and gather at one point to aggregate into a single body. The central cells release radial waves of cAMP with one in 5 min frequency [27]. The neighbor cells are exposed to these waves at the front and in turn release pulses of cAMP at the back. The surrounding cells encounter an increasing cAMP gradient in the front of the relayed wave for about 1.5 min. They rapidly polarize and move toward the source of cAMP. When its gradient becomes shallow and finally plateaus at the peak of the wave, the cells switch off intracellular chemotactic signaling, depolarize, and stop moving. As the wave passes over, the cells are exposed to the declining gradient of cAMP in the back of the wave for the next 90 sec, and to no chemoattractant in between waves in the remaining 2 min. During this period the movement is inhibited and the cells activate extracellular phosphodiesterase to hydrolyze cAMP and prevent turning away and chasing passing chemoattractant. Such a behavior maintains chemotactic relay and effectively works over long distances to the periphery of the colony causing aggregation of thousands of the cells [28, 29].

The cell aggregate can crawl along the substrate as a slime mold. Because it looks like a small garden slug, it is called identically. Although the slug is composed of rather individual cells that retain physical independence, it behaves as a multicellular entity and moves directionally along external gradients of light, temperature, and chemoattractants. They guide the slug to the surface where it creates an upright fruiting body consisting of stalk and sorus, which produces and spreads spores. Afterwards the stalk cells undergo programmed cell death and the spores give rise to new amoeba and life cycle. The entire cycle of *Dictyostelium* takes about 24 h and can be easily manipulated in the laboratory by starvation conditions so that the lengths of the unicellular and slug states are controlled by the nutrient supply [25].

An advantage of *Dictyostelium* as an experimental model is its genome simplicity and ease of genetic manipulations. Its six chromosomes have been sequenced [30], and the information made publicly available, along with a variety of genetic and biochemical techniques as well as cell strains at the stock center (see http://dictybase.org [31]). This model allows to easily assess individual protein function, and results of these studies serve as a guideline to learn mechanisms of chemotactic motility in other eukaryotic cells. The *Dictyostelium* cells detect external signals over a wide range of concentrations and display remarkable sensitivity. They amplify external gradients into internal ones of spatially localized signaling molecules and respond by morphologic polarization, directionality, and speed of movement. They adjust sensitivity, adapting to changes in gradient in a spatiotemporal manner. These properties are the hallmark of small chemotactic cells including mammalian leukocytes; in essence they are conserved in larger fibroblasts and cancer cells.

Leukocytes

In the mammalian immune system, the function of leukocytes relies completely on chemotaxis. The most thoroughly studied are neutrophils, the polymorphonuclear leukocytes [7]. They are among the fastest and most sensitive mammalian cells with speeds of migration close

to *Dictyostelium* cells (10-20 μm/min), and similar sentience to as little as 1% difference in chemoattractant gradient over the cell length of about 10 μm [32]. Both these cells use GPCRs as chemotactic receptors and almost identical intracellular pathways to transmit and process chemotactic signals. All the same, leukocytes are different from *Dictyostelium* in two important aspects.

First, neutrophils are inactive and almost immobile without stimulation; their chemotaxis is fully induced by chemoattractant gradients. Otherwise, chemotaxis of these cells has a typical amoeboid signature: a uniform stimulation causes appearance of random membrane protrusions, whereas the protrusive activity is biased towards higher concentrations of chemoattractant in gradients [17, 32] (Fig. 1). The steeper the gradient, the more efficient cells orient and move along it.

Second, leukocytes are capable of discriminating between several gradients of different chemoattractants [33]. They are selectively guided to various sites of infection and inflammation in an organism by a complicated array of about 50 different chemokines, which usually form superimposed gradients. To successfully reach correct destinations without being stuck between the opposed gradients, these cells chiefly respond to "end target chemotactic factors", rather than to "intermediary endogenous chemotactic factors" [9]. While the precise mechanisms of such selectivity are not completely understood, they are likely to involve different sensitivity to the mean concentrations of chemoattractants, to steepness of their gradients, as well as different receptors and internal signaling pathways engaged by different chemotactic factors [34], which are also subject to cross-desensitization [35]. Persistence of neutrophil chemotaxis increases with concentration of chemoattractant up to some level, above which it becomes inhibited, like in *Dictyostelium* cells [28, 29, 32]. Thus, secondary gradients can still guide neutrophil chemotaxis when the concentration of the primary factor has reached saturation. Such a mechanism, defined as multistep navigation, is thought to allow the leukocytes to travel directionally over long distances to particular target areas in the organism [33].

Maintenance of stable gradients of soluble molecules over long distances is unlikely *in vivo*. In addition to multistep navigation, another hypothesis has been proposed that, like in *Dictyostelium* cells, neutrophils may also detect signals in the form of a relayed wave to be attracted over long distances [28, 29]. Indeed, they respond to pulses of chemoattractant in a remarkably similar way, at least *in vitro*, first by activating the chemotactic signaling and migration at the front of the wave, then by adapting and decreasing motility as the wave passes, and switching it off in between waves [28]. It is still unknown whether such waves exist in the organism and how far they can spread in the blood. However it might be that in small niches enriched in several types of cells the neutrophils may only respond to the wave, whereas other "signaling" cells relay it. In places such as the bone marrow there are possible candidates where hematopoietic precursor cells can be gradually guided out during mobilization.

Fibroblasts

Fibroblasts are clearly different from amoeboid cells in morphology, motility, and chemotaxis [11, 15, 16, 36, 37]. They are much larger mesenchymal cells (50-200 μm in a flat shape) having intrinsic polarity and elaborate cytoskeletal architecture. Fibroblasts adhere stronger to substrate via multiple integrins than do amoeboid cells, and therefore they move more slowly with net speeds of about 0.25-1 μm/min. They extend broad lamellipodia enriched in polarized dendritic actin network at the leading edge and use microtubule network to regulate cell polarity and migration [38, 39]. Fibroblast movement in the 3D context is even more complex. It involves interaction with and degradation of the extracellular matrix by secreted metalloproteinases [40, 41]. In addition to chemoattractants, components of the extracellular matrix also serve as external cues to guide fibroblasts through the tissues by integrin-mediated haptotaxis.

Migration of fibroblasts is an important event in wound healing and tissue repair. It is guided by growth factors and their receptors (RPTKs) on the cell surface. PDGF is the primary chemoattractant that recruits fibroblasts into wounds from adjacent tissues; it is released by the earliest first aid cells recruited there such as platelets, neutrophils, and macrophages [14, 16, 42]. Fibroblasts are situated around the wound and thus do not need to locate it over long distance or to use any form of relayed signaling to be attracted. The attractant comes from the wound in relative excess, its stable gradient being formed and maintained close to the front of the fibroblast population with aid of receptor-mediated endocytosis and consumption of PDGF by the cells [12]. This physiological context may determine the way fibroblasts respond to PDGF. They detect gradients in a simpler way than amoeboid cells [15, 16] and are less persistent in keeping straight movement. They are less sensitive and respond to rather steep stable gradients of PDGF that naturally occur around the wounded area [12]. Their chemotactic responses are also bell-shaped; they have optima at intermediate concentrations and are inhibited at high concentrations of PDGF [43]. However this may be handy for the wound healing because PDGF appears to discriminate between the migration and proliferation of fibroblasts in a concentration-dependent manner [44]. Thus, fibroblast chemotaxis is stimulated by low concentrations of PDGF without increase in proliferation, but at the higher levels PDGF completely switches migration to proliferation [44].

Although intracellular signaling pathways leading to fibroblast chemotaxis downstream of PDGF receptors

are largely common to those in amoeboid cells, there are notable differences. There is neither significant amplification nor adaptation because of the lack of appropriate feedback loops in chemotactic signaling [15]. Localization of internal signaling molecules occurs differently, exhibits slower kinetics, and is more sustained than in amoeboid cells [12, 16]. Because of this fibroblasts are often regarded as a stripped-down system [2, 45].

Fibroblasts have additional and specific mechanisms involved in migration and possibly in chemotaxis. Thus, the directional movement of fibroblasts involves localized protein synthesis at the leading edge [46]. It is not observed in *Dictyostelium* or neutrophils, but is also present in the neuronal growth cone [9]. Fibroblasts also use receptor-dependent endocytosis both for keeping PDGF gradients [12] and to link the activation of PDGF receptors to the Rac1-dependent actin machinery [47]. In addition, the microtubules, substrate adhesions, and asymmetric distribution of actin and myosin II also contribute, to different extents, to amplification of the external signals and motility of fibroblasts [11, 36, 38, 39, 48].

BASIC MOTILITY

Cell locomotion is a complex cyclic process; it can be observed following seeding cells on the plain surfaces, either coated or not with a matrix. Under these unrestricted conditions, most cells move in continuous protrude-stick-pull-and-retract fashion [10]. This behavior is clearly detectable in fibroblasts and manifests itself by a jerky, fibroblast-type movement [11]. In contrast, cells like *Dictyostelium* and neutrophils display amoeboid gliding motility when the transitions between individual phases of locomotion are blurred and obscure. The cells are able to switch between different types of motility according to the 3D surrounding and matrix architecture, but they still use basic principles of motility and common migration strategy [6, 37, 40].

Polarized cells successively alternate cycles of locomotion, each of which can be typically dissected into four sequential steps (Fig. 2) [49, 50]. The *first step* is the forward movement of the leading membrane, which is defined as protrusion. Although the term "pseudopodia" now mostly refers to those of *Dictyostelium* and leukocytes, it is common to all protrusions [6]. In most cases protrusions are driven by actin polymerization resulting in the formation of lamellipodia, filopodia, and other protrusions [51]. The lamellipodia are flat, sheet-like structures that contain lattice-like branched meshwork of actin filaments [52], whereas the filopodia are cylindrical and finger-like projections enriched in tightly bundled actin filaments running alongside [53]. At the *second step*, protrusions attach to the substrate through adhesion assemblies that link actin cytoskeleton to extracellular matrix and provide for productive advance of the cell leading edge [54]. Adhesion structures are highly dynamic. They serve as platforms for actin stress fiber assembly and respond to mechanical load [48, 54]. The *third step* of movement is the actual translocation of the cell body and nucleus. It requires contractile forces generated by the myosin II molecular motor incorporated into bundles of actin filaments [55, 56]. Lastly, the *fourth step* involves rear detachment and tail retraction, the two spatially and functionally separated events [55, 57].

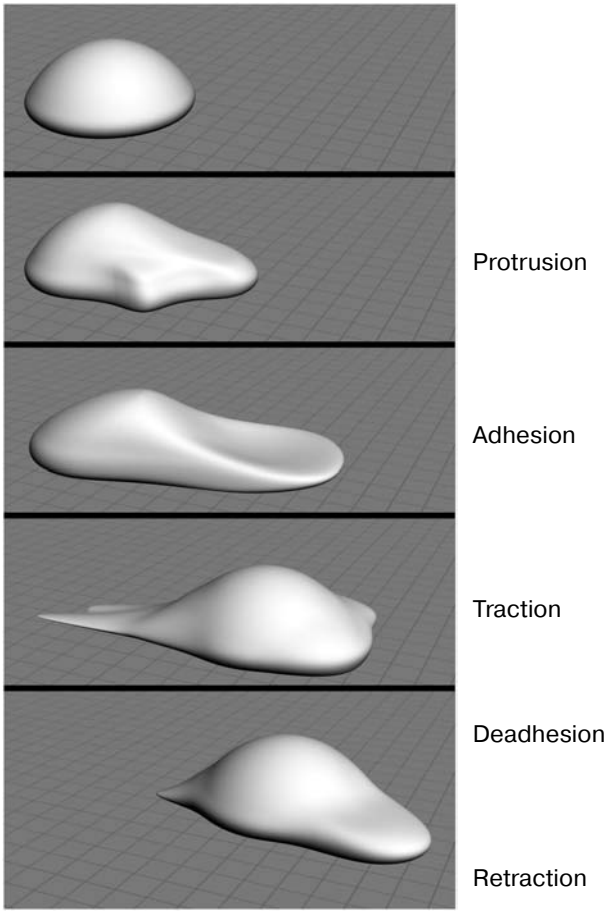

Fig. 2. The cell cycle of locomotion. Schematically shown are an initially unpolarized cell on the top and its movement below, which includes the indicated consecutive subtypes of motility [49, 50]. In amoeboid cells such as *Dictyostelium* and neutrophils these steps occur simultaneously and intermingle into a continuous process such that the changes in shape and morphology are not as distinct as in moving fibroblasts.

The speed of cell movement is not only determined by the protrusive activity, but also depends on the strength of adhesion and contractility. Early experiments and theoretical studies established that maximum speed of migration occurs at intermediate traction forces and adhesion strengths ([58], see also [49] for more details). Fast migrating amoebae and leukocytes display low adhesion and intermediate traction forces, whereas strongly adher-

ent fibroblasts generate forceful traction and move slowly. An optimum is because the protrusive, adhesive, and contractile activities depend on each other and are tightly balanced [6].

Protrusion

The mechanism of dendritic actin polymerization currently dominates in explaining protrusive activity at the leading edge of crawling cells [52, 59]. It underlies different membrane activities and forms of motility; however cells may also engage other types of motility, such as myosin-mediated bleb formation [60] and membrane flow by polarized endocytic recycling [61]. The actin motility is regulated at different steps by more than a hundred of actin-binding proteins.

The first mechanical step in cell crawling is the membrane protrusion at the cell front. It occurs via an ATP-dependent polarized turnover of actin filaments, the process known as treadmilling [59, 62, 63]. It was first observed *in vitro* [64] and later found to take place in most cells. Actin filaments grow by self-assembly unidirectionally with the barbed ends toward the plasma membrane (Fig. 3). They have an inherent polarity, i.e. the rates of actin association and dissociation are different at the opposite ends. The ATP-bound actin monomers are preferentially added to the barbed ends of the filaments near the membrane and hydrolyze ATP within filaments. This destabilizes the opposite pointed ends of the filaments, causing the ADP-bound actin monomers to dissociate distal to the membrane. The barbed end elongation and pointed end depolymerization are carefully balanced. The local concentrations of the available actin monomers and activity of regulators of actin polymerization and depolymerization determine the rate of treadmilling and protrusive activity.

Three major groups of cytoplasmic proteins have been identified that bind and maintain a pool of actin monomers, releasing them upon activation by migratory stimuli. These proteins exert distinct and often several activities each, contributing dramatically to actin dynamics [63]. Profilin promotes filament elongation and indirectly activates nucleation; cofilin promotes depolymerization of actin at the pointed ends and may act as a filament cutter; β-thymosin provides the source of actin monomers. Cofilin is inactivated by phosphorylation with LIM kinase and activated by regulated dephosphorylation with Slingshot and Chronophin phosphatases [65]. It has been suggested that migratory stimuli first trigger activation of cofilin at the leading edge, which induces rapid actin polymerization by severing actin filaments and creating multiple barbed ends; later the Arp2/3 complex (see below) is recruited to amplify the initial effect [66].

Actin polymerization enables pushing the plasma membrane by the barbed ends of the growing actin filaments (Fig. 3). Among several models suggested, the elastic Brownian ratchet mechanism is perhaps more realistic for most of the motile phenomena [67]. It suggests that a growing actin filament contacts plasma membrane that constantly undergoes local fluctuations and forms gaps between the filament end and membrane. When space

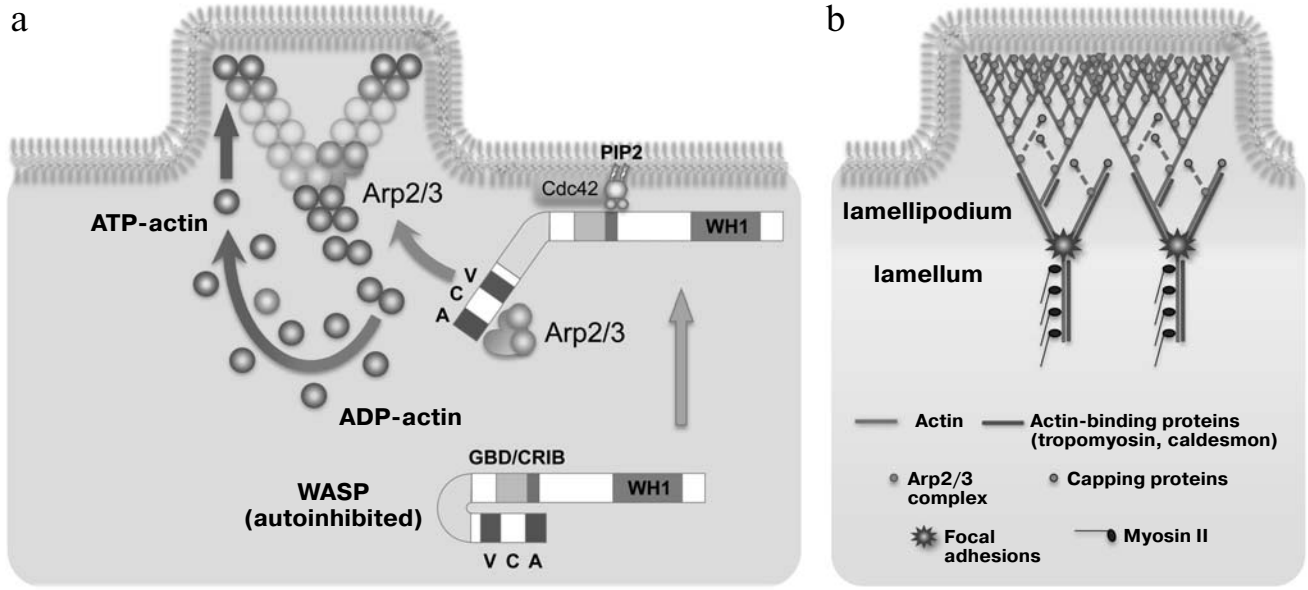

Fig. 3. Actin assembly at the leading edge. a) Polymerization and branching of actin filaments. The scheme shows treadmilling of actin filaments, their branching by the Arp2/3 complex, and sequential activation of Arp2/3 by Cdc42, PIP2, and WASP as an example [63, 77]. b) Actin organization in lamellipodia and lamellae. The structure and composition of the actin network between the leading edge of lamellipodium and internal lamellum; focal adhesions mark the border between these compartments [62].

allows, the next actin monomer is introduced into the gap, attaching to the tip of the filament and setting the membrane into a new distal position. This model provides a basic mechanism for membrane extension driven by actin polymerization that is consistent with most of the experimental data accumulated so far [67].

Dynamic actin filaments form a continuous network (Fig. 3b) that is regulated by a plethora of actin-binding proteins [68-70]. As a result, diverse structures of different shape and architecture emerge at the cell periphery [51]. Most characterized is the lamellipodium/lamellum compartment. The lamellum borders the cell body and transforms into the outer lamellipodium at the periphery. The lamellipodium is just a few microns in depth, thinner than the lamellum, and much more dynamic. It is also distinguished by faster actin treadmilling, whereas the lamellum exhibits slower retrograde flow of actin and stronger adhesions [71, 72]. The floppy lamellipodium can produce short-lived ruffles that do not adhere to the substrate, often bend up and move rearward on the upper side of the lamellipodium. The sheet-like configuration of lamellipodia and ruffles is supported by dendritically branched network of actin filaments oriented with barbed ends toward the membrane edge.

Individual actin filaments laterally associate within lamellipodia to form related finger-like microspikes and filopodia [11, 69]. The filopodia are enriched in the parallel actin arrays, which are bundled by fascin, espin, and fimbrin and join the dendritic actin at the filopodia base. At the other end the bundles attach to the tips of filopodia by the formins mDia and vasodilator-stimulated phosphoprotein VASP, which are responsible for elongation of actin filaments and filopodia growth [53]. Filopodia are flexible structures by which a cell is thought to explore the surroundings.

Extracellular stimuli that modulate speed and direction of cell migration act via signaling cascades targeting actin-binding proteins and altering their activities to evoke changes in actin dynamics and architecture [73]. A key function of small Rho-family GTPases in this regulation is well established [74, 75]. Thus, Rac and related Cdc42 GTPases are responsible for regulation of lamellipodia and filopodia, while Rho functions proximal to the cell body to regulate stress fiber assembly and actomyosin contractility in the rear of the cell.

The shape, area, and overall rate of protrusions clearly depend on geometry of the actin network, the number of barbed ends, and actin treadmilling activity. The number of barbed ends increases by nucleation and *de novo* formation of actin filaments, by regulated uncapping and severing of the existing filaments. The nucleation of actin filaments is unfavorable, being a rate-limiting step in polymerization; thus nucleation factors are needed to initiate actin assembly. Three classes of such proteins are presently known, including formin, Spire, and Arp2/3 proteins [51].

Formins are processive capping proteins that bind actin filaments at the growing end, prevent their capping by capping proteins, and allow for polymerization [76, 77]. They are homodimers composed of profilin-binding (FH1), actin-binding (FH2), and regulatory domains, including the Rac/Cdc42 binding domain [77]. The formin-bound profilin simultaneously interacts with an actin monomer, tethers it to the end of a growing filament, and may accelerate the filament elongation. FH2 domains form a donut-like structure that encircles the barbed end and mediates nucleation [76]. Importantly, formin remains bound to the growing filament during polymerization.

The Spire proteins nucleate by longitudinally bringing together four actin monomers and likely dissociate afterwards [78]. These proteins share a common composition: four actin-binding WH2 domains are flanked by the KIND and FYVE modular domains at the protein termini. KIND (kinase noncatalytic C-lobe domain) is an evolutionarily separated noncatalytic C-lobe domain of the active center of many protein kinases [78] that may mediate interaction of Spire with signaling molecules. The FYVE (zinc finger found in Fab1p, YOTB, Vac1p, and EEA1 proteins) domain binds PIP3 generated by some PI3K isoforms and predominantly localizes to endosomes [79]. It may enable Spire to link the actin polymerization machinery to endocytic pathways.

In contrast to formin and Spire that nucleate the unbranched actin filaments, the Arp2/3 complex nucleates by sitting on the side of a pre-existing filament and thus initiating a branch (Fig. 3). The Arp2/3 complex consists of seven subunits. The two actin-related proteins, Arp2 and Arp3, are stabilized by the other five subunits, from ARPC1 to ARPC5 [52, 77, 80, 81]. All components appear to interact with actin filament, and the Arp3 and Arp2 subunits form the first pseudo-actin dimer with the barbed end facing out of the parent filament. They are brought together into nucleation-competent contact by the other subunits with the help of specific nucleation-promoting factors (NPFs) such as WASP, Scar/WAVE, cortactin, and others [52, 62, 73, 81]. Due to precise positioning of the proteins, the daughter filaments always grow at a regular 70° angle to the mother filament, facing the plasma membrane with their barbed ends. In addition, some biochemical evidence suggests that Arp2/3 can branch directly at the barbed ends [82]. It states that monomeric actin, Arp2/3 proteins, and NPFs such as WASP assemble at barbed end where the Arp2/3 nucleates a lateral branch [63].

The Arp2/3 complex is intrinsically inactive and becomes activated by interaction with WASP or Scar/WAVE proteins that transmit signals from chemotactic receptors. These NPFs are the most extensively studied modular domain proteins [80, 83-85]. WASP contains several domains having different functions. The GBD/CRIB and WH1 domains receive regulatory inputs, and the VCA domain mediates interaction with the

Arp2/3 complex (Fig. 3). The VCA domain incorporates the verprolin-homology region (V) that binds actin monomer, cofilin-like (C), and acidic (A) regions that bind together to the Arp2/3 complex and activate it. Activation of the WASP proteins occurs via release of autoinhibition following binding of Cdc42 and PIP2 to the GBD/CRIB and WH1 domains, respectively (Fig. 3). It is therefore confined to the plasma membrane, where WASPs initiate the barbed end filament branching in close proximity to the membrane [62]. In addition, other SH3-domain containing proteins (WISH, Ash/Grb2, Nck, and profilin) may activate WASP by binding to its proline-rich motif and relieving the autoinhibition [85]. In contrast, WASP-interacting proteins (WIP) bind the WH1 domain and interfere with WASP activation, suppressing its activity [77, 83].

The mammalian WAVE and *Dictyostelium* Scar homolog proteins contain characteristic WAVE/Scar homology domain (WHD) and VCA domain, which confers ability to activate Arp2/3 and nucleation of actin branches. In contrast to WASP, they do not possess the GBD/CRIB domain and are not autoinhibited; they seem to be activated within a larger protein complex that involves four other constituents. One of those binds Rac through a Rac-binding (RCB) domain and the other principal one belongs to the Abi protein family of c-Abl (Abelson) protooncogene tyrosine kinase targets [80, 85]. The regulatory mechanisms of WAVE/Scar activation remain poorly understood.

Thus, NPF proteins are the key signal transducers and activators of the Arp2/3-mediated actin polymerization machinery. Their number is growing and new members are being found including cortactin, which synergizes with WASP and stabilizes the branches, and WASH, WHAMM, and JMY proteins [80, 83]. Actin filament turnover is additionally regulated by a variety of other proteins that cap barbed ends and protect them from growth, limit the filament length, anneal and protect filaments from severing, cross-link and bundle filaments, and alter the steady-state level of monomeric actin [63]. Many of them exert several activities and are in turn regulated by signaling molecules, providing a means for the tight control of actin dynamics by the receptor-dependent signaling cascades initiated by external stimuli.

Adhesion

To translocate the body, cells must to anchor to the surrounding substrate and generate traction forces. In the simplest case, such as moving on flat 2D-surfaces, the load-bearing adhesions are set between front protrusions and the matrix (Fig. 4; see color insert). They include transmembrane integrins that couple the actin cytoskeleton to the matrix and enable the traction forces generated by actomyosin contractility to be applied [69, 86]. The strength of adhesions is spatially regulated inside moving cells so that stronger attachments are maintained at the front and weaken toward the rear where they disengage [87].

The adhesion contacts undergo cycles of creation, maturation, growth, and disassembly [54, 57, 88]. *Nascent adhesions* are formed in lamellipodium as microclusters of several integrin molecules that are mobile and disassemble in minutes unless they are captured by vinculin and talin, which trigger the matrix engagement by integrins (Fig. 4a). Then paxillin and α-actinin are recruited and promote further clustering of integrins and actin binding [89]. This marks the transition of nascent adhesions into *focal complexes* and *focal adhesions*, which occurs at the lamellipodium/lamellum border [71] and is largely independent of the myosin II motor activity [89].

For subsequent maturation the mechanical tension needs to be applied to the focal adhesions by myosin II through the attached actin filaments [48]. The amount of force applied later to the focal adhesions determines the fate of the earlier nascent adhesions and focal complexes. At low activity of myosin II, the nascent adhesions are prone to disassembly rather then maturation [89, 90]. Vice versa, tension is increased upon strengthening the adhesions as the outside forces stimulate polymerization and growth of actin filaments attached to the focal adhesions [48] (Fig. 4a).

The focal adhesions created at the lamellipodia/lamellum border mature within the lamellum as long as the cell rolls over and grow into long stable *fibrillar adhesions* under the cell body [54, 57]. The major signal to mature is the increased tension. It allosterically reinforces the focal adhesions by switching the $\alpha_5\beta_1$-integrin from its relaxed to the tensioned state [91]. In this state the integrin engages a synergy site on fibronectin, which is required to induce phosphorylation of and recruitment of focal adhesion kinase (FAK), which initiates the secondary signaling. It phosphorylates the integrin associated proteins paxillin and $p130^{cas}$, leading to further recruitment of Src and other SH2-domain containing adaptor molecules. The increased tension is also thought to alter arrangement of the adhesion proteins by their localized unfolding and unmasking new integrin binding sites [92].

The adhesion dynamics are primarily regulated through the Rho family small GTPases. Rac and Cdc42 control the actin polymerization machinery in front protrusions, and Rho functions at both the front and the rear to regulate adhesion assembly primarily by activation of myosin II [93]. Rho-activated kinases, ROCKI and ROCKII, increase the activity of myosin II by directly phosphorylating the critical Ser19 on its regulatory light chain [94]. In parallel, ROCKs activate myosin II indirectly by phosphorylating and inhibiting the myosin-binding subunit of phosphatase that dephosphorylates this site [94]. The two ROCKs appear to bifurcate the Rho signaling toward the front and the rear compart-

ments of cell. ROCKI has been shown to regulate stress fiber assembly and focal adhesions, whereas ROCKII controls microfilament assembly [95].

A classic Ca^{2+}/calmodulin-dependent pathway of phosphorylation and activation of myosin II by dedicated myosin light chain kinase (MLCK) [96] contributes to the adhesion control in cell-type and matrix-dependent context. An intracellular biosensor for active MLCK reveals its activation in the leading lamellum of epithelial cells, but not in the trailing tail [97]. Consistent with this, specific inhibitor of MLCK impairs maturation of the peripheral focal adhesions and pseudopod stability in fibroblasts, leading to inhibition of their movement [98]. However, a biosensor reporting the level of myosin II phosphorylation shows it is enhanced in the tail compartment of fibroblasts, but not in the front lamellum [99]. Inhibiting this phosphorylation or removing the cytosolic Ca^{2+} impaired the tail retraction and neutrophil migration on the adhesive substrates, but had no such effect on non-adhesive surface [100]. Thus, the data accumulated so far suggest an auxiliary role for Ca^{2+}/MLCK in the regulation of adhesion dynamics and cell migration as compared to the Rho/ROCK pathway.

For cells moving in a more complex environment such as uneven surfaces or 3D extracellular matrix, creation of strong focused cell–substrate contacts may not be essential. Instead, cells may use protrusions to fill up pores and gaps in the nearby matrix, stick to them by inflating protrusions with cytoplasm, and drag the cell body along [101]. As demonstrated for amoeboid leukocytes and *Dictyostelium* cells, the same cells can adaptively switch the motility between integrin-dependent and independent modes of migration [102, 103]. These observations led credit to a hypothesis that cells use common principles for amoeboid crawling and shift between the adhesive and blebbing modes of motility [6]. Compared to the actin polymerization driven motility outlined above, the blebbing motility appears to use the opposite sequence of events. Initially, the cortical actomyosin-mediated contractility creates hydrostatic pressure that drives bleb formation at the cell front and squeezes in there the cytoplasm fluid. Subsequently, the blebs are supplied with the actin machinery that produces characteristic lamellipodium actin network [103].

Traction

Traction is probably much less understood than the protrusion and adhesion components of motility. Clearly, it involves adhesion and contractility as the major contributors, as well as the intuitive mechanism that uses actin and myosin to generate pulling forces on front adhesions to translocate cell body [54]. Perhaps its molecular mechanism is similar to that described for muscle contraction and widely known as the filament-sliding model [104, 105]. However, the major questions remain incompletely understood as to how contractile units are organized and which proteins they are composed of, how they couple to and coordinately function with the adhesion contacts, and whether the cell body is differently translocated in the front and the rear [97, 98].

The actomyosin contractile network, which contributes to the cell body advancement, localizes to the lamellum and is distinct from the protrusive machinery in lamellipodia. Although molecular details of actin dynamics in these two compartments are incompletely understood and debated [106, 107], it is clear that the myosin II molecular motor is localized in lamellum and absent in lamellipodia. Besides, strong focal adhesions form at the border of these two compartments and link extracellular matrix to actin cytoskeleton of the lamellum [71, 86]. These focal adhesions mediate coupling of the protrusions at the leading edge to translocation of the cell body by a mechanism known as regulated "molecular clutch" (Fig. 4b) [54, 57, 63]. When the clutch is engaged, actin filaments are fixed immobile at the adhesion point. Polymerization at the barbed ends of actin filaments drives forward protrusion of the leading edge, whereas centripetally generated actomyosin tension is converted via the focal adhesion into traction on the extracellular matrix (Fig. 4b, left). If the clutch disengages, the connection between the adhesion and actin filament becomes lost, resulting in the conversion of actin treadmilling into myosin-mediated retrograde flow of filaments and no protrusion or traction (Fig. 4b, right). While currently little is known how the molecular clutch is regulated, vinculin and talin are proposed as the best candidates to mediate this regulation [54].

Non-muscle myosin II has been identified by inhibitory analysis and isoform-specific knockdown as the intracellular motor responsible for traction [89, 108-111]. It also best suits this function among other molecular motors [112]. It polymerizes into filaments containing at least several motor units, which amplifies their individual forces. It also allows moving the actin filaments along without letting them slip back, because each myosin II molecule contains two relatively independent motor domains working in non-processive manner. Thus, both the enzymatic activity and assembly of myosin II into filaments contribute importantly to focal adhesion dynamics and chemotaxis in different cells [90, 113].

Electron microscopy of myosin II in isolated form (see [114] for details) and *in situ* [115, 116] shows that it forms filaments with the motor domains oriented to both ends. The sarcomere-like myosin II oligomers are abundant in the lamellum of fibroblasts [115] where they organize into ribbon-like structures possibly needed for the stress fiber assembly [116]. Incorporation of these bipolar myosin II oligomers (or their alternative structures with side polarity [114]) into actin fibers has to result in formation of actomyosin bundles (stress-fibers)

with mixed polarity. However, these structures pull on both ends when they contract, thus producing little or no net traction force for translocation of the cell body. This suggests that myosin II unlikely incorporates, but rather laterally associates, in the form of oligomers or polymers, with the polar actin filaments growing from the integrin sites in lamellum. These filaments form dorsal stress-fibers, which anchor to integrins by one end and project to the upper cell surface by the other [55, 117]. Thus polar by actin the dorsal stress fibers may provide traction vector when the integrin clutch is engaged.

The dorsal stress fibers merge with the short myosin II-containing stress fibers known as arcs that are attached from inside to the upper cell surface [117]. Alternatively, myosin II oligomers may directly connect the free ends of dorsal stress fibers. In any case, the resulting assemblies, "stitched" by myosin II as shown in Fig. 4b, have mixed actin polarity. They build up the ventral stress fibers [117], which are largely responsible for contractility.

Thus, the contractile lamellum network is different from that in the rest of the cell. It consists of the actin bundles that are less dense than the inner stress fibers; displays different isoform content of actin [119] and non-muscle myosin II [89, 90, 109-111]. Whereas myosin IIA is abundant in the lamellum where it mediates cell adhesion, spreading and tension applied to cell adhesions, myosin IIB is localized in the posterior region and largely contributes to cell rear retraction. Differential regulation of these myosin II isoforms [56, 98] supports their asymmetric pattern and greatly contributes to cell chemotactic behavior. Although a role of myosin II in cell migration is accepted, the data reported on distribution of its active (phosphorylated) and inactive (unphosphorylated) myosin isoforms inside cells [99, 108, 118] and contribution to the directional persistence [90, 113, 118] are still variable and not completely consistent.

Detachment and Retraction

Inasmuch as adhesion and traction are intimately coupled, retraction of trailing tail is linked to the rear detachment. While the detachment is basically a reversal of cell adhesion, the retraction and traction are not; they work altogether by similar mechanism to contribute to translocation of the cell body in a contraction-dependent manner. The structures that mediate retraction are also stress fibers that are built similar to the contractile acto-myosin bundles in the lamellum, but they are denser, and they have different myosin IIB isoform and its regulation. They mediate contractility in the cell body that likely serves other purpose than in the lamellum. It generates powerful forces in both directions along the stress fibers to pull and detach the adhesions, whereas contractility in the lamellum provides traction forces directed towards the leading edge.

The ventral stress fibers are the major structures responsible for pulling off substrate adhesions. They are built by annealing dorsal stress fibers that are firmly anchored to mature adhesions by one end and elongate by the other while incorporating myosin II [55]. Zyxin recruitment, which is a marker of mature adhesions [88], initiates ventral stress fiber assembly from the dorsal stress fibers and incorporation of myosin II [55, 117]. It is important that they contain actin filaments of mixed polarity, more active myosin IIB isoform, and β-isoform of non-muscle actin, although functional specialty of the latter is not known [119]. This composition enables the ventral stress fibers to generate centripetal forces and pull together the attached mature adhesions.

All the same, the forces produced by these stress fibers are asymmetric, and their cumulative vector is directed toward the cell center due to function of yet unclear mechanisms that stabilize internal adhesions while the peripheral weaken [57]. Focal adhesion kinase (FAK) is a key regulator of focal adhesions involved at several steps of their disassembly. Acting directly or via Src family tyrosine kinases, it increases tyrosine phosphorylation of the adhesion proteins and contributes to disassembly and turnover of the adhesion contacts by multiple and poorly defined mechanisms [54, 57, 88, 120]. Other important mechanisms of the adhesion contact disassembly include calpain-mediated proteolysis of β-integrins, talin, and other associated proteins [121], and asymmetric disruption of the adhesions mediated by microtubules [86, 87]. Partially disassembled rear adhesions are usually taken in by endocytosis and their components are transported along the upper side of the cell to the leading lamellum, to provide building material for assembly of new adhesions ([61] and references therein).

Time-lapse microscopy of *Dictyostelium* and neutrophil cells often shows that they move effectively by a single leading protrusion while dragging the tail. This implies that protrusion activity and asymmetric adhesion turnover are more important for motility than tail retraction. Indeed, knockout of myosin II does not produce severe defects in motility of *Dictyostelium* cells, but it affects speed of migration in the manner dependent on strength of adhesion [122]. The latter also suggests a role for detachment of the rear adhesions; however, this component is not clearly detectable in the weakly adherent cells. In contrast, it is noticeable in highly adhesive cells such as in moving fibroblasts, which tend to leave trails of integrin complexes behind, "ripping" off the rear membrane along with adhesions [87].

DIRECTIONAL MOTILITY

Directional motility requires polarization, and maintaining polarity determines persistence with which a cell moves directionally. Morphological polarization is a

consequence of an internal asymmetry in distribution of signaling molecules and cellular structures, primarily cytoskeletal. The external stimuli are not necessarily required to trigger and maintain polarity (Fig. 1, states A and B), although they contribute considerably and shift equilibria in favor of polarized states (Fig. 1, state D). Polarized cells retain alternative to redirect movement and do it more often in the absence than in the presence of chemotactic gradients. The gradients serve as external guides by keeping up cell polarity and reducing the probability of changing direction (Fig. 1, state E). In addition to polarization governed by gradients, contribution of the intrinsic, stimulus-independent polarity may not be neglected because the transitions are coupled (see Fig. 1). In some cells such as leukocytes, this contribution is likely minor (these cells have mostly round morphology in the absence of stimuli), but in the other cells like fibroblasts it must be substantial as they display high degree of intrinsic polarity. Chemotactic behavior of fibroblasts requires steep external gradients [15], presumably because of the need for chemoattractants to counteract the intrinsic polarity to convert and align it with the external gradient. How such a conversion is achieved remains uncertain, but an emerging hypothesis is that the behavior of the existing pseudopods is biased to the direction of gradient rather than new pseudopods being turned on [45].

Pseudopod Behavior

Known for more than 50 years, an ability of many cells to move persistently in the absence of external cues is due to a correlated random walk [3, 123, 124]. In this behavior cells usually generate one pseudopod at a time and tend to project it in a direction close to the previous one, i.e. correlate orientations of the successive protrusions. Usually, new pseudopods form at the base of the previous, alternatively changing the sides, and grow perpendicular to cell membrane in a manner reminiscent of "ice skating" [123, 124]. When the daughter pseudopod becomes dominant and parent one is retracted, the directional persistence of cell locomotion is increased. In the absence of gradient, the intrinsic polarization supports the ice-skating behavior by reducing the frequency of the *de novo* (lateral) pseudopods and by stabilizing splitting pseudopods in the front [45, 125].

In the presence of shallow gradients cells largely keep their basic behavior and generate pseudopods spontaneously. Gradients act permissively, but not instructively, to bias the origin of new pseudopods to the cell front and reorient cells to the new polarity axis [45, 124, 125]. In sidewise gradients cells prefer changing direction smoothly by gradually turning the front toward the attractant source. This is achieved by increasing the probability to generate a few successive pseudopods at the up-gradient side of the parent protrusion, which resembles the pacing of a short track speed ice-skater making a circumferential turn.

In steep gradients cells visibly change chemotactic behavior. Such gradients become instructive and direct formation of new pseudopods. The front pseudopods are stabilized and new protrusions appear as their continuation in the direction of the gradient. As a result, cells move persistently in one direction. If the gradient swerves, the major turns occur by formation of new lateral pseudopods rather than by splitting the previous ones. To what extent this behavior overcomes the biasing mode depends on the strength of the stimulus versus the relative cell polarity [45, 124, 126].

Regulation Strategy

Currently two hypotheses are proposed that are based on either instructive function of chemoattractant in directing cell chemotaxis [2, 4, 127] or its permissive effect on the intrinsic pseudopodial activity [45, 125]. Each is well justified and best explains a particular aspect of the same phenomenon; the first explains cell movements in steep gradients, the latter in shallow gradients.

The first model extends the earlier theory of biological pattern formation (see [128] for more details) with respect to probability for generation of successive pseudopods in a finite region of the membrane by local excitation and global inhibition, known as the LEGI model [2, 4, 127]. A key hypothesis is that two signals exist: the strong one is formed locally by self-enhancing regional excitation; the second is weak but long-ranged. The former shows up when it exceeds a threshold level imposed by the second. The remaining cut-off excitatory signal is amplified in nonlinear fashion by a positive feedback loop to produce a highly localized sharp response [128].

In the context of chemotaxis, it is proposed that cells have an internal compass that allows them to navigate in gradients. Chemoattractants act as a primary signal to activate cognate receptors on the up-gradient side of a cell, which excite secondary signaling inside cells. While the local internal signals initiate pseudopod formation, the long-range signals act as a global inhibitor [2, 4, 126, 127]. Clearly, the inhibitor molecules must have faster diffusion rate and/or longer half-life. So far, the LEGI model has been tested in unpolarized cells; however, the polarity component is likely to be an important contributor to cell responses [2] (see Fig. 1).

In *Dictyostelium* cells chemotactic GPCRs initiate generation of both activatory and inhibitory signals. Cyclic GMP produced by the soluble guanylate cyclase (sGC) suppresses formation of lateral pseudopods, suggesting it acts as a global inhibitor [129]. In mammalian cells the cGMP function is apparently taken up by cAMP and Rho protein that regulate myosin II and cell adhesion. Phosphatidylinositol-3,4,5-trisphosphate (PIP3), a

short-lived lipid compound generated by PI3K upon activation of chemotactic receptors, acts as a key activatory molecule at the leading membrane in virtually all cells [130-133]. Together with sGC and cGMP, PIP3 mediates orientation responses of *Dictyostelium* cells in gradients [129]. In addition, the target of rapamycin complex 2 (TorC2) and phospholipase A_2 (PLA2) are also involved in *Dictyostelium* and leukocyte chemotaxis, serving apparently the excitatory function [134, 135]. Blocking these pathways causes a complete collapse of the *Dictyostelium* chemotactic system [129]. Although the LEGI model gives a reasonable explanation of what are the signaling molecules and how they may provide for the spatiotemporal control of chemotaxis [2, 4], it still lacks unequivocal experimental evidence.

Other models [136, 137] suggest that correlated random walk may explain chemotactic behavior of cells in gradients. Recently a "pseudopod-centered" model has been proposed, which is based on basic pseudopod behavior in the absence of or in shallow gradients of attractants [45, 125]. It describes chemotactic motility as biased by external gradients and considers pseudopod splitting as the primary mechanism. In essence it takes into account the intrinsic polarity component (Fig. 1, b and d) for cell behavior in gradients (Fig. 1e). In situations when it contributes considerably (no or shallow gradients), it dominates and external stimuli exert permissive biasing effects. In steep gradients, the external stimulation is dominant and becomes instructive. By overcoming constraints imposed by the intrinsic polarity, it makes cells create lateral pseudopods and new axes of locomotion. Signaling aspects of chemotaxis have not been worked out in this new model yet. Perhaps a combination of the LEGI and "pseudopod-centered" models is more realistic to describe signaling and motile behavior both in the absence and in the presence of gradients.

CHEMOTACTIC SIGNALING

Based on the knowledge gained so far, a few concepts of chemotactic signaling can be defined. (1) Cells detect external cues by surface *receptors* that transmit these signals into cells. (2) The external gradient is ultimately translated into a separate, *internal* one of signaling molecules. It is *amplified* downstream of receptors. (3) There are *several* signaling cascades leading to chemotaxis and organized into an interdependent redundant network; there is no one dominant pathway. (4) The purpose of chemotactic signaling is *polarization* of signaling molecules and, consequently, the cytoskeleton. (5) Mechanisms must exist to *amplify* chemotactic signaling, control its *duration*, and *adapt* cell behavior to the spatial and temporal changes in the external gradients.

Signal transduction from chemotactic receptors to motile responses is remarkably similar in most cells and uses common components. Receptors perceive extracellular stimuli and transduce their signals through the membrane to Ras GTPase and PI3K, the two major routers of chemotactic signal transmission inside cells [8, 138, 139]. In amoeboid cells the signal is split thereafter into three critical cascades involving PIP3, sGC, and TorC2 (Fig. 5; see color insert). PIP3 functions as the major amplifier, sensitizer, and orchestrator of the chemoattractant-induced pseudopod machinery; it also determines high sensitivity to changes of gradient [26, 131]. Most of the signaling cascades target small Rho-family GTPases, the key regulators of actin polymerization and myosin II activation. These proteins distribute asymmetrically and govern polarization and direction of cell movement (Fig. 5) [73, 140].

The other less characterized routes include MAP-kinases, PLA2 (Fig. 5a), and phospholipase Cγ (Fig. 5b). They may be cell-type specific and activated by chemotactic receptors in parallel to Ras and PI3K. Albeit simpler, the chemotactic signaling in fibroblasts also involves PIP3 as a key regulator [16]. In addition, fibroblast chemotaxis also involves activation of soluble tyrosine kinases Src and FAK because the turnover of focal adhesions critically contributes to the mesenchymal type of motility [6]. They are activated downstream of RPTKs (Fig. 5b) and integrins; crosstalk between these receptors [141] is also imperative for fibroblast chemotaxis. Phospholipase Cγ regulates initial shape changes and traction in fibroblasts [142]. Regardless of the cell type, this pathway produces both the local lipid (diacylglycerol) and global cytosolic (inositol trisphosphate and Ca^{2+}) second messengers that may contribute to chemotaxis via the LEGI mechanism to locally regulate Rac and myosin II (Fig. 5b).

Keeping the direction of movement requires temporal control and maintenance of the internal signaling gradients. This is achieved by engagement of positive feedback mechanisms. A well-known feedback loop that prolongs and enhances PIP3 production operates in *Dictyostelium* cells and leukocytes (Fig. 6; see color insert), but its existence in fibroblasts is questioned [15, 16]. Instead, endocytic signaling and traffic of chemotactic receptors is critical to fibroblast chemotaxis [19, 44, 47, 143]. A number of feedback loops have been proposed to control the actin cytoskeleton and focal adhesion dynamics [144]. Perhaps they will help to provide an insight into mechanisms that maintain internal gradients and position of new protrusions.

Receptor Level

Cells use different receptors to recognize different chemotactic stimuli. The receptors can be conventionally classified into two major groups by their structural and functional properties. One group includes the serpentine

receptors coupled to the trimeric membrane G-proteins (GPCRs). The other integrates various polypeptides with a single transmembrane domain, which assemble upon activation and either possess intrinsic tyrosine kinase activity (RPTKs), or serine/threonine kinase activity (TGFβ receptor), or recruit cytosolic tyrosine kinases to further transmit the signal (GM-CSF receptor for granulocyte macrophage colony-stimulating factor). In addition, different cells appear to have a preference for a particular type of chemotactic receptors and probably downstream signaling. Thus, *Dictyostelium* cells, mammalian leukocytes, and hematopoietic cells use mainly GPCRs [9, 131], but RPTKs are the major chemotactic receptors in fibroblasts, mesenchymal stromal cells, *Drosophila* border cells, and smooth muscle, epithelial, and cancer cells [17-23, 145]. The guidance receptors used by axons for pathfinding are also mostly RPTKs [9]. The common feature of all guidance receptors is that they signal through small G-proteins of the Ras superfamily and PI3K to increase PIP3 level at the leading membrane (Fig. 5).

In *Dictyostelium*, chemotactic GPCRs are coupled to the Gi family of G-proteins and signal to chemotaxis via the βγ-subunit complex. Out of at least 11 α-subunits identified in these cells, mainly Gα2 couples to chemotactic receptors and only one β- and one γ-subunit are found in this organism [2, 26, 126]. In neutrophils, chemotactic GPCRs are linked to Gαi and Gα12/13 [146]; they transmit signals to the cell front and the rear, respectively. Gαi seems to signal via βγ to PIP3 and Rac at the leading edge, whereas Gα12/13 may directly signal to Rho and formation of myosin II filaments in the rear [26, 146]. The βγ-subunit complex is absolutely required for chemotaxis in neutrophil and *Dictyostelium* cells ([147] and references therein).

Chemoattractant receptors remain uniformly distributed along the cell surface in chemotaxing or polarized *Dictyostelium* cells [148] and neutrophils [149]. The ligand occupancy of the receptors reflects shallow external gradients; there is no modulation of the receptor affinity; phosphorylation of chemotactic receptors has little impact on *Dictyostelium* and neutrophil chemotaxis (see [2, 26] for further details). The activated receptors diffuse rapidly in the plane of the plasma membrane and there is no specific localization or clustering of bound ligand [150]. The GFP-tagged β-subunit of the trimeric G-protein revealed shallow polarity aligned with the external gradient in *Dictyostelium* cells [147]. FRET experiments also showed that activated trimeric G-protein and its βγ-subunits are distributed in shallow manner reflecting the receptor occupancy and the external gradient [151, 152]. This indicates that chemotactic signaling is only partially polarized at the receptor level, but the major amplification occurs downstream of the surface receptors and their trimeric G-proteins.

During growth factor-induced chemotaxis the ligand binding results in dimerization and autophosphorylation of the receptors. This creates docking sites for binding of signaling and adaptor molecules. PDGF-BB is the primary chemoattractant in fibroblasts that binds to β-receptors of PDGF and activates their autophosphorylation at Tyr857. This leads to recruitment and activation of Ras and PI3K, generation of PIP3, and activation of Rac, Src, Ras-GAP, and PLC-γ (Fig. 5a) [14, 152, 153]. Ras and PI3Ks have central roles in both the RPTK and GPCR signaling [153]. This means that PDGF activates the same critical downstream effectors as chemotactic GPCR ligands. The other signaling molecules appear to be specific for RPTK signaling. Many of them mediate proliferation responses but also have an auxiliary role in regulating migration [44, 154].

Little is known whether RPTK and related receptors redistribute to leading membrane in the directionally migrating cells. The epidermal growth factor (EGF) receptor was evenly distributed on the plasma membrane of adenocarcinoma cells chemotaxing towards EGF [155], but PVR, a PDGF/VEGF related receptor in *Drosophila* border cells, was found to accumulate in the front [20]. This indicates that the internal gradient may be at least partially created at the receptor level. Still, the major external–internal conversion of gradient occurs downstream of receptors.

Membrane Level

The signaling cascades that control cell polarization downstream of chemotactic receptors make up two groups. One stimulates actin dynamics and adhesion formation at the leading edge (Fig. 5a), and the other activates myosin II and assembly of the contractile stress fibers, weakening adhesions in the back of the cell (Fig. 5b).

The major route of chemotactic signaling passes through Ras GTPase, which acts as a common regulator and signal distributor along the front-to-back axis. Ras activates the membrane PI3K/PIP3 circuit and cytosolic TorC2 and MAP-kinase cascades in the front. In addition, the membrane lipid PIP3 locally amplifies and distributes the signal, and PLA2 modifies the membrane lipids, alters the physical properties of membrane, and generates secondary signaling molecules (Fig. 5a).

Ras GTPase. The Ras superfamily contains a large number of conservative small GTPases, which are divided into six families on the basis of structural homology and functional similarity [156]. The Ras family includes Ras itself, Rap, Ral, and Rheb that act as signaling switches in the cells. The largest family of Rab proteins is responsible for vesicle traffic, sorting of endosomes, and formation of secondary signaling complexes associated with them. The Arf-proteins regulate endocytic and secretory vesicle transport as well as that between the reticulum and Golgi complex. The Rho-family contains

regulators of the actin cytoskeleton Rho, Rac, and Cdc42. Ran GTPases regulate transport of RNA and proteins between nucleus and cytoplasm. The sixth family includes atypical Miro GTPase, which has an EF-hand Ca^{2+}-binding domain, localizes to mitochondria, and regulates integrity of these structures [156]. Interestingly, *Dictyostelium* has multiple Ras, Rab, and Arf proteins, including Rap and Rac, but no Ral, Rho, or Cdc42 homologs [30]. More than 10 Ras proteins were identified in these cells that are homologs to three major H-, N-, and K-Ras proteins in mammals. Perhaps these multiple Ras proteins perform functions that were taken over by Rho and Cdc42 in mammalian cells later in evolution. This may account for differences in *Dictyostelium* signaling as compared to that in leukocytes [138].

Influencing signaling via Ras strongly impacts chemotaxis of *Dictyostelium* cells [157] and fibroblasts [158]. In most cases upregulation of Ras results in increased motility, whereas dominant-negative mutants of Ras suppress cell migration. Curiously however, the hyperactivation of Ras causes directional defects in motility [130, 157, 158]. A key role of Ras in chemotaxis of neutrophils has not been explicitly demonstrated. Although it is expected, it is also possible that some functions of Ras have evolutionarily passed on to Rac and Rho, which are activated in neutrophils by the Gi and G12/13 coupled receptors, respectively [146].

Signal transduction to activation of the Rho-family GTPases is probably the primary function of Ras. This however occurs indirectly and involves PI3K/PIP3 as an intermediate step to amplify the activatory signal in the cell anterior [138] (Fig. 5a). That amplification occurs downstream of Ras is suggestive from uniform distribution of the Ras protein along the plasma membrane of chemotaxing cells, which mirrors localization of the receptors. Although active Ras tends to gather at the leading edge, the steepness of its gradient also reflects that of external gradients and of the active receptors [157].

PI3K/PIP3 module. A large family of PI3K enzymes produces PI(3,4,5)P3 from PI(4,5)P2 by phosphorylating the 3′-position of the inositol ring. There are three classes of PI3Ks, among which the class I PI3Ks are activated by cell surface receptors [153]. These PI3Ks are heterodimers containing one of the four possible p110 catalytic subunits and one of the p50-55/p85 regulatory subunits. Depending on the catalytic subunit, PI3Ks are subdivided into class IA (p110α, p110β, or p110δ) and class IB (p110γ), and often are called according to the type of their catalytic subunit, i.e. PI3Kα, β, δ, and γ [153].

The Ras-dependent mechanism of PI3K activation is backed up by the direct activation of PI3K on Gβ subunit of GPCRs, or on the receptors as in the case of RPTKs. PI3K translocates to the up-gradient sites on the plasma membrane forming a steeper internal gradient [130, 159]. There it is activated by binding of the regulatory subunit to Ras and Gβ-subunit followed by interaction with the catalytic subunit, which independently anchors to the membrane through a characteristic N-terminal motif [130]. In the case of RPTK, the p85 regulatory subunit of PI3K uses its SH2-domain to bind to activated receptors and interacts there with Ras via the Ras-binding domain (RBD), which upregulates PI3K activation. Then the regulatory subunit binds the p110 catalytic subunit to form active holoenzyme [153].

Out of five class I PI3Ks present in *Dictyostelium* cells, PI3K1, PI3K2, and PI3K3 are most important, and the first two are essential for chemotaxis [130, 132, 160]. A triple PI3K1/2/3 knockout results in severe defects both in the speed and polarity of *Dictyostelium* cells [132]. In mammalian cells, the γ-isoform of PI3K is most relevant to chemotaxis; however, the PI3Kβ and PI3Kδ isoforms also contribute [131]. Macrophages and neutrophils isolated from p110γ knockout mice do not generate PIP3 when stimulated by chemoattractants, cannot stabilize and maintain leading edge, and consequently are less motile [161, 162].

An ability of the exogenous membrane-permeable analogs of PIP3 to induce polarization and cell movement provides direct evidence for a key role of PIP3 in chemotaxis [7, 163]. PIP3 recruits from the cytosol and binds specifically proteins that contain the pleckstrin homology (PH) domains. Many guanine nucleotide exchange factors of Rac, the critical regulator of actin dynamics, contain such a domain, as well as do the proteins that directly regulate actin polymerization (Fig. 5a). Other proteins important for chemotaxis also contain this domain, but their mechanisms are not yet completely known.

PIP3 gradients are usually observed in the living cells by expressing chimeras of the GFP-like fluorescent proteins fused to PH-domains of different proteins. Thus PIP3 gradients have been visualized in directionally moving *Dictyostelium* cells [160, 164], neutrophils [162, 165], and fibroblasts [13]. They always align with the external chemotactic gradients and demonstrate that amplification and distribution of chemotactic signal occurs at the level of PIP3 generation. The internal gradients of PIP3 are much steeper than those of external stimuli, active receptors, or Ras in cells. The PIP3 signal is amplified by about an order of magnitude in neutrophils and *Dictyostelium* cells, which requires a feedback loop involving PI3K and the actin cytoskeleton [8, 146, 163]. This loop is thought to be absent in fibroblasts; their internal PIP3 gradients are not that steep and depend only on the receptor-activated PI3K [13, 15].

The lipid phosphatases PTEN and SHIP1 are functional antagonists of the PIP3 signaling that play an important role in migration of amoeboid cells and fibroblasts [130, 166-169]. While PTEN removes phosphate from the 3′-position of the inositol ring using mainly PIP3 and, to a less extent, PIP2 as a substrate, SHIP1 cleaves the 5′-phosphate. The same as PI3K, PTEN is a

dual specificity enzyme, i.e. it uses both lipids and proteins as substrates [170].

Pten is one of the most frequently mutated tumor suppressor genes in many tumors [170]. Switching it off results in defects of motility associated with directionality, such as disorientation of the leading pseudopods and increased frequency of the lateral pseudopod formation [166]. As a consequence, the random motility can be altogether increased. Fibroblasts lacking PTEN migrate with increased speed, which returns back to normal upon re-expression of the wild-type PTEN [168]. In contrast, SHIP1 but not PTEN is involved in neutrophil chemotaxis [169]. Knocking out SHIP1 reduced speed of neutrophil movement 5-fold, but did not affect the directionality [169]. Both localization of PTEN and SHIP1 and their redistribution upon stimulation have not been assessed in fibroblasts and neutrophils.

In unstimulated *Dictyostelium* cells PI3K is cytosolic and rapidly translocates to the leading membrane upon stimulation. In contrast, PTEN uniformly associates with the plasma membrane of the resting cells via the N-terminal, 15-residue PIP2-binding motif. By converting PIP2 into PIP3, PI3K reduces the number of PTEN binding sites at the leading membrane, causing PTEN to move into cytosol and accumulate at the rear in stimulated cells [130, 166]. There it lowers PIP3 levels, progressively increases PIP2, and thereby creates its own binding sites on the rear membrane [167]. Thus redistributed, PI3K and PTEN markedly amplify the internal PIP3 gradient. This gradient is further maintained by a positive feedback loop that involves Ras and actin polymerization [8, 171].

There is no doubt that PI3K and PIP3 play a crucial role in signal transduction and directed migration of many cells. However, recent studies indicate that they do not make up the sole guidance device. Full inhibition of PI3K exerts strong, but still partial effects on chemotaxis of *Dictyostelium* cells, detected only in shallow but not in steep gradients [172], it partially inhibits directed migration of macrophages in animal models [161] and has little if any effect on T-lymphocyte migration [133]. Even multiple deletions of all five isoforms of PI3K and PTEN phosphatase in *Dictyostelium* cells did not fully block chemotaxis [173]. This indicates that there are parallel receptor-dependent pathways that recognize external gradients and mediate chemotactic signaling.

Phospholipase A2. Phospholipase A2 (PLA2) acts in parallel to PI3K/PIP3 in chemotaxis of amoeboid cells [135, 172, 174] (Fig. 5a). This pathway is still barely studied and it is unclear whether it is activated directly by chemotactic receptors, or downstream of Ras [139].

PLA2 removes the 2′-acyl from glycerophospholipids, which is often arachidonic acid, and leaves behind a lysophospholipid. Most cells, including leukocytes, contain three major classes of PLA2: the secretory (sPLA2), cytosolic Ca^{2+}-dependent (cPLA2), and cytosolic Ca^{2+}-independent (iPLA2) enzymes. Among them only the latter two are thought of as signaling molecules. Thus, cPLA2 is activated by protein kinase C and low Ca^{2+} concentrations and selectively hydrolyzes arachidonic acid [174]. However, it is unlikely to control directionality of cell migration, more likely it is involved in regulation of pseudopodial activity and movement velocity. A uniform treatment of cPLA2-deficient *Dictyostelium* cells with an exogenous arachidonic acid restored their chemotaxis [135], suggesting that cPLA2 function is due to the general effects of arachidonic acid rather than lysophospholipids. cPLA2 is cytoplasmic in leukocytes and translocates to the endoplasmic reticulum upon stimulation, but not to the leading membrane [175]. cPLA2 deficient mutants of *Dictyostelium* fail to branch leading pseudopodia and randomly form new lateral ones [129]. As a result, the cells often turn and do not keep the direction of movement.

Arachidonic acid is further converted to active eicosanoids by cyclooxygenase, lipoxygenase, or cytochrome P450-dependent epoxygenase; the latter is thought to mediate chemotactic signaling by cPLA2 [174]. Arachidonic acid and its products stimulate Ca^{2+} efflux from the internal stores and elevate Ca^{2+} level in cytosol [139, 176]. Ca^{2+} is required for migration of leukocytes [100]. Although it contributes to chemotaxis of *Dictyostelium* cells, it is not a critical regulator in these cells [135, 172]. Ca^{2+} activates myosin II and contractility in the cell posterior [7, 100], but other mechanisms are more important for activation of myosin II.

In contrast to cPLA2, iPLA2 is not specific to the 2′-acyl group, but uses mainly phosphatidic acid as a substrate. Chemotaxis of iPLA2-deficient cells is restored by lysophosphatidic acid (LPA), which acts as an intermediate [174]. It has long been thought that iPLA2 is constitutively active and not regulated; however, it is now considered as a likely regulator of directionality and speed of migration [135, 172, 174, 175]. MCP-1, the major chemoattractant for monocytes, stimulates iPLA2 translocation from cytosol to the plasma membrane, its accumulation in the leading pseudopods, and colocalization with Cdc42 small GTPase, coincident with cell polarization [175].

LPA and structurally similar sphingosine-1-phosphate (S1P) activate chemotactic signaling and migration in many cells, including chemokine-induced leukocyte chemotaxis and PDGF-stimulated migration of fibroblasts [174, 176]. Cells contain several LPA receptors, which are GPCRs coupled to the $G_{q/11}$, $G_{i/0}$, and $G_{12/13}$ proteins [176]. They are thought to support cell migration in an auto- or paracrine manner, perhaps via receptor-dependent activation of PLA2. Recent studies suggest that LPA and its receptors signal to the cell rear leading to phosphorylation and activation of myosin II [177] via the Rho-family GTPases [176].

Finally, a still unexplored and potentially important mechanism of PLA2 function may involve local changing

of membrane fluidity due to formation of lysophospholipids that have detergent-like properties. Similar activity is also known for polyunsaturated fatty acids such as arachidonate. They all lower viscosity and cause local bending of membranes, normally participating in maturation and scission of intracellular vesicles for their transport to and from the Golgi complex, or in the budding of virus-containing vesicles during infection [178]. It is therefore possible that local increase in fluidity of the plasma membrane following PLA2 activation may facilitate or promote formation of membrane protrusions during directed migration. Localization of iPLA2 in the areas of vigorous pseudopodial activity [175] is consistent with this possibility. In addition, the major cPLA2 product is lysophosphatidylcholine, which most strongly increases the membrane fluidity. Although largely located in cytosol, cPLA2 partially translocates to the plasma membrane where it binds specifically to the $p47^{phox}$ subunit of NAD(P)H oxidase and regulates generation of reactive oxygen species (ROS) [179]. ROS and their more stable metabolite, hydrogen peroxide, are now considered as potential regulators of directed migration (see the last section).

Thus, PLA2 plays a critical role in chemotaxis along with the PI3K/PIP3 module. Nonetheless, when PI3K and PLA2 are switched off, chemotaxis is not completely blocked: a triple mutant of *Dictyostelium* lacking both PLA2 and two major PI3K isoforms is still capable of detecting cAMP gradients [135, 180]. This indicates that redundant chemotactic pathways exist that take over function of the first two. They appear at the cytosolic cascade level.

Submembrane Level

A hallmark of chemotaxis is translocation to the plasma membrane of cytosolic proteins that contain PH domains and their binding to and activation by the lipid signaling molecules. These proteins can be conventionally divided into two groups: (1) proteins that locally activate actin dynamics and polymerization, and (2) proteins that initiate cytoplasmic cascades. The former specify sites of protrusion formation, and their effectors are actin nucleation and polymerization factors. The second is less studied; it branches out the signal in cytosol and transmits it into the cell body and rear.

Pleckstrin homology (PH) domain consists of approximately 120 amino acids. It is a structural element of many signaling and cytoskeletal molecules that function in association with membranes [181]. Various PH domains are found in guanine nucleotide exchange factors for small GTPases, phospholipase C, protein kinase PKB/Akt and β-adrenergic receptor kinases (βARK), Btk tyrosine phosphatase, as well as in their substrates and adaptor and scaffold proteins. PH domains may have different specificity and discriminate between phosphoinositides with a phosphate group attached to different positions of the inositol ring, i.e. PI(3,4)P2, or PI(4,5)P2, or PI(3,4,5)P2.

Actin dynamics. Small GTPases Rac and Cdc42 control activity of actin nucleation and polymerization factors at the leading edge of migrating cells. These proteins contain PH domains but are activated by different phospholipids. Cdc42 and WASP are activated by binding of PIP2 and a protein partner. Cdc42 interacts with a nucleotide exchange factor, whereas WASP binds activated Cdc42 [63, 77]. The WASP activity is much higher than that of all other NPFs [63]. Because Cdc42 and WASP do not require accumulation of PIP3 for activation, they may provide for the first rapid, but transient phase of actin polymerization when they dissociate from the membrane upon conversion of PIP2 into PIP3.

Cdc42 is the primary regulator of filopodia formation and cell polarity [73, 138, 140]. Gβγ-subunits and a signaling complex composed of Cdc42, the guanine nucleotide exchange factor PIXα, and p21-activated kinase (PAK) 1 mediate activation of Cdc42 by chemoattractants in leukocytes [182]. PAK1 serves as a scaffold protein to recruit the activated PIXα into the complex by binding its specific domain. Within the complex PIXα accelerates guanine nucleotide exchange on Cdc42. In addition to turning on the actin-polymerized machine, Cdc42 activates PAK1, thus creating a positive feedback loop (see Fig. 6).

Rac activation by chemotactic receptors takes longer because it requires PI3K-mediated accumulation of PIP3. It is PIP3 that recruits to the membrane nucleotide exchange factors for Rac as most of them contain PH domains [181, 183]. Then Rac locally initiates actin polymerization at the leading edge by activating all major NPFs including formins [77], Scar/WAVE [77, 80], and Spire [78, 79]. In addition to Rac, PIP3 directly controls membrane localization and activation of Scar/WAVE and Spire [63, 79]. Based on the adaptor protein–protein interactions, this mechanism of signal transduction is highly specific at the price of effective amplification, which is needed to activate numerous actin regulators. The amplification is achieved through a positive feedback loop and by enzymes possessing high catalytic power and turnover rate. These mechanisms are switched on by the Rac activation and sustain their activity allowing for massive polymerization and growth of actin filaments (see Fig. 6). The Arp2/3-complex targeted by WASP and Scar/WAVE mediates further branching of these filaments and formation of the dendritic actin network [80, 85].

Various adaptor proteins also contribute importantly to activation of actin dynamics. The receptor-dependent adaptors Nck and Grb2 directly activate WASP [85], and in cooperation with Erk1/2 and Src kinases they activate the regulator of Arp2/3 complex cortactin [63]. The

insulin receptor substrate protein IRSp53 is critical for activation and/or localization of Rac and lamellipodium/filopodium formation [85]. Acting together, these proteins may provide for a second phase of WASP activation and maintain activity of WASP and Arp2/3 for a period needed for protrusion formation.

There are at least three PAK isoforms, which are promiscuously activated by various Rac and Cdc42 homologs [74]. This feature underlies another signaling mechanism that leads to increased actin dynamics through inactivation of cofilin. While unphosphorylated cofilin promotes severing and depolymerization of actin filaments, phosphorylation by LIM-kinase lowers this cofilin activity resulting in actin polymerization [65]. PAKs phosphorylate and activate LIM-kinase. This mechanism is clearly distinct from the function of PAK1 in activation of Cdc42 mediated by αPIX.

Similarly, Rac and Cdc42 play an important role in fibroblast migration in response to RPTK stimulation [145, 184, 185]. A fluorescent biosensor detecting Rac activation in living cells showed that active Rac forms a gradient toward the leading edge and concentrates in lamellipodia of moving fibroblasts [186]. Gene knockout [187] or silencing Rac expression [184] prevented lamellipodia formation and reduced speed of migration, but no severe orientation defects were observed. Fibroblasts retained ability to detect PDGF gradient and to move along it using thin filopodia-like protrusions [187]. Controlled expression of Rac in fibroblasts demonstrated that decreasing the Rac activity reduces formation of lateral protrusions and switches motility from random to directionally persistent, PI3K-dependent chemotaxis [185]. Taken together, these results substantiate the role of Rac in regulation of local actin dynamics and lamellipodial growth. They show that moderate activation of Rac leads to its accumulation at the leading edge where Rac cooperates with PI3K to create sites of increased protrusion activity, but at high activity levels Rac disorients migration by inducing lateral pseudopodia. Consistent with the expected function of Cdc42 in establishing primary actin polymerization sites and cell polarity axis (see Fig. 6), blocking expression of Cdc42 caused severe defects in directionality of fibroblast migration, but it had no effect on random motility in the absence of chemoattractant [184, 185]. Intriguingly, such a mechanism as it shown in Fig. 6 has not been found in fibroblasts [13, 15], suggesting it has different organization in these cells.

Interestingly, chemoattractant also causes two waves of actin polymerization at the periphery of *Dictyostelium* cells that match with two phases of translocation to the plasma membrane of proteins containing PH domains. Similarly, only the second wave depends on activity of PI3K [188]. Unlike mammalian mesenchymal cells and fibroblasts, *Dictyostelium* lacks genes of Cdc42 and Rho [30]. Therefore, it is likely that some of the multiple Rac isoforms act as functional homologs of Cdc42 and Rho in *Dictyostelium* [138]. While different in details, they seem to also provide for biphasic actin polymerization response in these cells.

PH-domain proteins. PI3K is a second after Ras signal distributor at the leading edge of moving cells. Initiating accumulation of PIP3, this enzyme amplifies signal delivered by chemotactic receptors and converts it into steep gradients of PIP3 target molecules inside cells. Besides nucleotide exchange factors for Rac, there are other proteins that contain PH domains, which recognize and bind specifically to 3′-phosphorylated PIP3 generated by PI3K [153]. They include protein kinases PKB/Akt and PDK1, soluble tyrosine kinase Btk (component of the B-lymphocyte receptor signaling complex), guanine nucleotide exchange factors for Arf proteins of the Ras superfamily (regulators of cytoskeletal dynamics and vesicular transport), and a variety of adaptor and scaffold proteins (components of the MAP kinase and other signaling pathways, e.g. Grb2) [153, 170, 181].

The isolated PH domains retain PIP3-binding activity and when genetically fused to fluorescent proteins, they are widely used for detection and quantification of chemotactic gradients generated in cells. The most popular are the chimeric constructs PHAkt–GFP, which contains the PH-domain of PKB/Akt [13, 160, 163, 165, 169, 185, 187, 189], and PHPhd–GFP or PHCrac–GFP that contain the PH domains of specific *Dictyostelium* proteins [130, 164, 166, 171, 188, 190]. These sensors report protein translocation and require the use of time-lapse fluorescent confocal or evanescent wave microscopy, also known as TIRF-microscopy [13]. When expressed in *Dictyostelium* or neutrophils cells, these sensors show that PIP3 forms gradients that are approximately an order of magnitude steeper than the external chemoattractant gradients [165].

There are more than 300 human genes encoding proteins with at least one PH-domain [181]. Not all of them recognize only the 3′-phosphorylated PIP3, but some can bind both PI(3,4)P2 and PI(4,5)P2 with different affinity and specificity [170, 181]. For instance, PH-domain of PTEN binds its own product, PI(4,5)P2; this domain plays a critical role in intracellular localization of PTEN during chemotaxis [167]. Similarly, PH-domain of phospholipase C-γ favors its substrate, the same PI(4,5)P2, and almost does not bind the 3′-products of PI3K. In contrast, PH domain of PKB/Akt recognizes only the products of PI3K, with a preference to PI(3,4)P2 as compared to PI(3,4,5)P3. The similar PH-like domain of Btk is highly specific to PI(3,4,5)P3 [170, 181]. Thus caution should be exercised when selecting an adequate PH-domain reporter for studying a particular signaling pathway, including the PIP3-activated cascades.

PKB/Akt is regarded as the primary target of the PI3K pathway [191]. Its activation is closely linked to the plasma membrane and involves binding of the PH-domain of PKB/Akt to PIP3. Full activation of PKB/Akt

requires phosphorylation of two sites, which is mediated by two additional enzymes, the phosphoinositide-dependent kinase 1 (PDK1) and 2 (PDK2) [192]. PDK1 translocates to the plasma membrane owing to binding of its PH domain to PIP3; it phosphorylates Thr308 in the activation loop of PKB/Akt. The nature of PDK2 has long been unknown, probably because few enzymes with such an activity have been reported. Only recently it became clear that the principal PDK2 is the TorC2 complex, which phosphorylates Ser473 residue in the hydrophobic motif of PKB/Akt [193]. Other protein kinases can also act like PDK2, including the integrin-linked kinase (ILK), p38 MAP kinase activated protein kinase 2 (MAPKAPK2), and DNA-dependent kinase (DNA-PK) [192]. Thus, PKB/Akt is not only targeted by PI3K cascade, but also by other pathways.

The functions of PKB/Akt in cells are diverse. Over a hundred nonredundant substrates of PKB/Akt have been reported [191, 194]. PKB/Akt is linked to regulation of metabolism, growth, survival, and proliferation, as well as of angiogenesis [191, 192]. The control of endothelial NO synthase activity, glucose transport, and glycogen metabolism make PKB/Akt one of the candidates for intracellular mediators of type 2 diabetes and metabolic syndrome [194].

Until recently, involvement of PKB/Akt in cell migration has been uncertain [191]. However, several important observations have been made that pinpointed its role in *Dictyostelium* [160] and macrophage [195] chemotaxis, migration of fibroblasts [189] and endothelial cells [196]. Several PKB/Akt mechanisms have been identified that are coupled to activation of actin dynamics in cell anterior and myosin II in the cell rear.

The detailed analysis showed that mechanism of PKB/Akt in the cell front is associated with an actin-binding protein girdin [197]. In mammalian cells, PKB/Akt phosphorylates a residue within a PIP2-binding site of girdin. As a consequence, girdin dissociates from the membrane but retains the ability to bind actin and mediate the lamellipodial network formation. Silencing of girdin results in decreased velocity of cell movement and its re-expression restores the migration [197]. PKB/Akt may transmit signal to cell posterior via activation of PAK-mediated regulation of myosin II. Other as yet unknown pathways activated by PKB/Akt are likely to also exist.

Studies in *Dictyostelium* have shown that TorC2 activates PKB/Akt bypassing the PI3K/PIP3 module [198, 199]. Identification of TorC2, PLA2 [135, 172], sGC, and cGMP [200] as independent signaling modules in *Dictyostelium* cells made critical contributions to the conceptual paradigm that several mechanisms function in parallel to regulate chemotaxis [2, 125, 129, 180].

Rac and Rho antagonism. Antagonism between Rac/Cdc42 and Rho is one of the most intriguing mysteries of cell signaling. The fact it exists has long been accepted [201], but the question as to how it works is still not completely resolved. While intracellular localization of this mechanism is not precisely clear, it tentatively might be attributed to the submembrane level (Fig. 5b).

Inhibition of Rho causes responses characteristic of Rac activation, and conversely, inhibition of Rac induces changes typical for Rho activation [201]. Among multiple manifestations, the Rac and Rho antagonism is clearly seen in adhesion contact dynamics [93], suggesting it likely couples chemotactic signals to regulation of cell adhesion. It is Rac rather than Rho that is responsible for creation of nascent adhesions and focal complexes in the lamellipodium, whereas a switching to Rho-mediated control in the lamellum is required for their subsequent maturation to focal adhesions [93]. These observations led up to the idea that cells have two spatially and functionally separated Rac- and Rho-domains [93]. Yet, their function is not limited to regulation of cell adhesion. They provide critical inputs into formation and regulation of the protrusive and contractile compartments of cells (see [201] and references in this review). The chemotactic signals act via Rac and Rho to couple these domains and align them in the direction of movement, thus providing for growth and consolidation of the front protrusions, traction of the cell body, and the rear retraction, respectively.

Cascade Level

Cascade level is localized in cytoplasm and provides for transmission of signal to the remote receptor targets. One path branches out at the cell front and involves TorC2 and PKB/Akt (Fig. 5a). The second branch sends signal to the cell rear and lateral compartments, amplifying it by soluble second messengers and protein kinases. It is aimed at assembly of myosin II molecules to form the contractile elements that move the cell body (Fig. 5b). Finally, the third group of signals diffusely distributes in cytosol, coordinating events at the leading and trailing edges.

TorC2. In *Dictyostelium* cells Ras triggers one more pathway important for chemotaxis, which involves protein complex TorC2 and acts in parallel to the PIP3-based cascades [134, 199]. The target of rapamycin (Tor) protein kinase is the central component of this complex; it receives signals from the insulin and other growth factor receptors, as well as from the nutrient extracellular amino acids [193, 202, 203]. Tor forms two major complexes in eukaryotic cells; their multicomponent structure is determined by the constituent proteins raptor (complex TorC1) or rictor (complex TorC2) [203, 203]. Unlike TorC1, the rictor-containing TorC2 is insensitive to rapamycin.

TorC2 regulates actin polarization and growth in yeasts [202]. It phosphorylates Ser473 in PKB/Akt and

regulates actin cytoskeleton in mammalian cells [193, 203, 204]. In *Dictyostelium* cells, it phosphorylates PKB/Akt-related kinase PKBR1, which in contrast to classical PKB/Akt (PKBA in *Dictyostelium*) does not require PIP3 for the binding and anchors to membrane through an N-terminal myristoyl moiety. Consequently, activation of PKBR1 by TorC2 does not require the secondary phosphorylation of Thr308 and therefore is independent of PIP3/PI3K [134, 199].

The mammalian TorC2 contains at least five proteins: TOR, LST8, AVO1, AVO2, and AVO3/rictor [203, 204]. AVO1 and AVO3/rictor are the orthologs of two *Dictyostelium* proteins, Rip3 (Ras interacting protein 3) and Pia (Pianissimo). Rip3/AVO1 has a Ras-binding domain (RBD), which likely mediates activation of TorC2 by Ras. Genetic knockout of LST8, Rip3/AVO1, or Pia (AVO3/rictor) results in profound defects in *Dictyostelium* chemotaxis, including loss of polarity, velocity, and direction of movement [198]. Moreover, it also impairs myosin II polymerization and synthesis of cAMP needed for cell-to-cell signal relay. Because both these processes are localized in the rear of the cell, it has been concluded that TorC2 functions both at the leading edge and in the rear. The major target of TorC2, PKB/Akt, is thought to transmit the rearward signal [2, 199].

Adenylyl cyclase and cAMP. *Dictyostelium* cells use the adenylyl cyclase mechanism to relay information on the movement direction from one cell to another [25]. Adenylyl cyclase is located on the rear membrane and emits cAMP outside of the migrating cell. For some reason, cAMP does not serve in *Dictyostelium* as an internal signaling molecule, but merely acts as a ligand for chemotactic receptors for neighbor cells to navigate their movement. Perhaps the intracellular adenylyl cyclase mechanism was acquired later in evolution, because some *Dictyostelium* species that do not use cAMP as chemoattractant still do not use it for intracellular signaling [200].

In contrast, adenylyl cyclase mediates another important mechanism in mammalian leukocytes, which is not shown in Fig. 5, and results in axial distribution of chemotactic signal and cell polarization [205]. This mechanism implements the principle of functional bifurcation of signal downstream of Gs-coupled GPCRs and uses the intracellular cAMP as second messenger [205]. Importantly, it also uses the guanine nucleotide exchange factor directly activated by cAMP (Epac), and a small G-protein Rap1, in addition to the classic cAMP-dependent protein kinase A pathway. These cascades are located in cytosol. One of them leads from cAMP to activation of integrins and front adhesions through sequential activation of Epac and Rap1. The other is mediated by protein kinase A; it weakens adhesions and activates myosin II in the cell rear [205, 206]. The function of this mechanism has not yet been studied in fibroblasts, but it might be expected as the Epac protein mediates smooth muscle cell migration and formation of neointima in injured vessels [207].

Interestingly, a similar but little characterized pathway appears to operate in *Dictyostelium* cells. It involves small GTPases Ras and Rap1 and is activated by cAMP receptors [206, 208]. It is assumed that it acts via myosin heavy chain kinase (see below) and mediates translocation of myosin II to the cell rear [2, 138]. Remarkably, it appears to use cGMP instead of cAMP, and cGMP-binding protein GbpC as the functional analog of Epac. Unlike in mammalian cells, cGMP mediates regulation of myosin II structure and activity in *Dictyostelium* cells [113, 209].

Guanylyl cyclase and cGMP. Among the cell models studied so far only *Dictyostelium* appears to use the intracellular guanylyl cyclase mechanism in chemotactic signaling for regulation of myosin II. Several important notes can be outlined from its analysis. (1) This mechanism is activated by chemotactic receptors and involves Ras-like GTPase. (2) It is not a mainstream pathway and works in combination with other cascades. (3) It comprises two functionally different branches, one of which localizes the soluble guanylate cyclase to the leading edge and the other mediates cGMP regulation of myosin II in the cell body. (4) This mechanism is clearly seen only in migrating cells; it supports leading pseudopods and suppresses formation of the rear and lateral pseudopods, that is, it unlikely governs polarization and only supports it.

Acting through the $G\alpha 2\beta\gamma$ complex, the chemoattractant cAMP stimulates rapid increase in cGMP in the cytoplasm and chemotaxis [200]. Both the membrane and soluble (sGC) guanylate cyclases produce cGMP, but only the latter mediates the majority of chemotactic responses associated with polymerization and activation of myosin II [180]. cGMP binds to one of the four unique cGMP-binding proteins (GbpC) in *Dictyostelium*, which has a leucine-rich motif, Ras-domain, an effector MAP-KKK domain, and two cGMP-binding sites capable of regulating nucleotide exchange in the Ras-domain [209]. GbpC binds cGMP with high affinity and activates its own Ras-domain, which in turn activates its own MAP-KKK domain. The latter is homologous to the first kinase of MAP kinase module and likely launches a similar cascade that leads to polymerization of myosin II in the cell rear [209]. Interestingly, the cGMP-binding proteins GbpA and GbpB are phosphodiesterases that hydrolyze cGMP, and cGMP is probably their low-affinity allosteric activator.

Inhibition of the PI3K/PIP3 module along with PLA2 blocks chemotaxis only of the initially unpolarized *Dictyostelium* cells. The cells that have been polarized remain resistant to simultaneous switching off the PI3K/PIP3, PLA2, and even TorC2 genes; they migrate effectively toward cAMP [180]. Additional knockout of the sGC gene completely blocks chemotaxis of polarized cells, and re-expression of sGC restores their chemotaxis

[180]. This indicates that a mechanism involving sGC/cGMP is important, but it does not operate on its own and requires the other pathways.

The guanylyl cyclase mechanism causes two relatively independent events: the transition of sGC to the cell front where it colocalizes with filamentous actin, and generation of cGMP [210] (Fig. 5b). Although the sGC mechanism is still not clear, it somehow increases the probability of generating subsequent pseudopods at the base of preceding pseudopods. In contrast, cGMP mediates myosin II localization and polymerization in the posterior region, suppressing formation of the lateral and rear protrusions [180]. Thus, sGC and cGMP act differently and do not establish intrinsic polarity in the *Dictyostelium* cell, but orient them during migration in shallow gradients [129].

Myosin II. Activation of myosin II critically contributes to cell polarization. It is differently tackled in such evolutionary distant organisms as *Dictyostelium* and mammals, thus illustrating a clear and elegant example of using newly evolved alternatives to optimally deal with a conserved task. All myosins II have a similar structure and consist of two large (heavy chains) and two pairs of small (light chains) polypeptides. The heavy chains form two globular motor domains (heads) at the N-terminus that interact with actin and mediate ATP-dependent contraction [104, 105], while the long C-terminal parts associate into coiled-coil tail, which is responsible for polymerization of individual myosin molecules into filament [114]. Activation of myosin II involves two events, i.e. an increase in enzymatic activity of individual monomers, and their assembly into regular filaments [113]. In the inactive state non-muscle myosin II monomers assume characteristic conformation inhibited by the intramolecular interactions. The superhelical tail forms a few kinks and bends up such that its C-terminal part is brought towards the N-terminal motor domains [112]. In this folded state the lateral association of tails and filament formation become impossible, and the binding of myosin heads to actin is hindered resulting in low actin-activated ATPase and motor activity of myosin II.

The folded conformation of *Dictyostelium* myosin II is maintained by phosphorylation of three threonine residues in the distal part of the superhelical tail [211]. These sites are phosphorylated by three myosin heavy chain kinases: MHCK-A, MHCK-B, and MHCK-PKC. The catalytic domain of the first two differs completely from any of the traditional serine/threonine or tyrosine kinases. The mechanism of MHCK-A and MHCK-B activation is unknown; it may involve autophosphorylation. The situation with MHC-PKC is even more confusing. It has been thought to be a cGMP-activated fusion product of the diacylglycerol kinase gene and that of protein kinase C, whose domains have been found in MHC-PKC [211]. However, recent studies indicated that this is erroneous because of the incorrect reading of the MHC-PKC gene. In fact, the *Dictyostelium* genome encodes an independent diacylglycerol kinase, which is somehow involved in chemotaxis, possibly via MHCK-A activation (see [113]). A myosin II heavy chain phosphatase has been identified and found not to be regulated [113]. Thus it appears that MHCK-A, MHCK-B, and relevant phosphatase(s) are constitutively active in *Dictyostelium* cells. They provide for background level of myosin II heavy chain phosphorylation and for about 50% of myosin molecules being in monomeric state.

It is likely that chemotactic receptors are coupled to myosin II mainly by PAK in *Dictyostelium* cells [211]. PAK has a typical p21-binding domain by which it binds one of the Rac proteins and specific motifs that enable its translocation to the sites of myosin II polymerization (Fig. 5b). PAK does not directly phosphorylate myosin II in *Dictyostelium*; instead it phosphorylates and inhibits all the MHCKs [211]. Cells with disrupted PAK gene display low level of myosin filaments, which does not alter upon activation of chemotactic receptors. The behavior of these cells is similar to those with knocked out myosin II.

In addition, *Dictyostelium* myosin II is phosphorylated by myosin light chain kinase (MLCK-A) on Ser13 of the regulatory light chains, which corresponds to Ser19 of the mammalian myosin regulatory light chain [211]. MLCK-A activity indirectly depends on cGMP, which apparently acts through cGMP-dependent protein kinase. MLCK-A activity does not require Ca^{2+}. Phosphorylation of Ser13 neither affects polymerization, nor actin-activated ATPase of myosin II, it only slightly increases activity of filamentous myosin II. Site-directed mutagenesis confirms that Ser13 phosphorylation does not affect the function of myosin II in *Dictyostelium* (see [211]).

In contrast, in mammalian cells phosphorylation is a key event in activation of myosin II, but it is regulated by different means and occurs exclusively at Ser19 of the regulatory light chains [112]. This phosphorylation destabilizes the "folded" myosin II conformation, and simultaneously causes assembly of myosin molecules into filaments and enzymatic activation. Two major kinases phosphorylate the myosin II regulatory light chains [98]. One is classical MLCK, which is activated by Ca^{2+}-calmodulin and uses only myosin II as a substrate [96]. The other phosphorylates additional substrates, but it predominantly mediates myosin II activation in non-muscle cells. It is directly activated by Rho and is known as Rho-kinase or ROCK [94]. Two ROCK isoforms are known that are differently localized in cells. They perform similar but independent functions [95] and may regulate different isoforms of myosin II [110]. Importantly, ROCK activation is largely determined by localization of Rho inside cells, which acts in cell rear and adhesion contacts and thereby locally activates myosin II in these cell compartments where contractile activity is most needed (Fig. 5b). In addition, ROCKs phosphorylate and inhibit the phos-

phatase that dephosphorylates Ser19, thus upregulating myosin II activation.

Thus, regardless of when and how the regulatory switch from the cGMP/PAK-mediated to the Rho-dependent control of myosin II has occurred in evolution, it became a cornerstone to dramatically increase the efficiency of myosin II activation. Natural selection apparently followed a few lines to improve the efficacy of signal transduction (antagonism of Rho and Rac, Rho localization in the cell rear) and myosin II phosphorylation (activation of kinases and inhibition of phosphatase), and myosin II activation (the dual effect of phosphorylation at the level of a single molecule).

Other pathways. *Erk1/2* MAP-kinase is a classic target of Ras [7, 14, 145]. This pathway consists of three protein kinase modules, MEKK, MEK, and their effector Erk1/2 (Fig. 5a). In the case of RPTK, this MAP kinase pathway is initiated by binding of the adaptor proteins Grb2 and/or Shc to RPTK and by subsequent activation of Ras. In the case of GPCR, Ras is recruited from cytosol and activated by the Gβγ complex. The Erk1/2 substrates mostly mediate the proliferation responses in cells; the role of Erk1/2 in migration is probably linked to regulation of adhesion contacts. The active Erk1/2 is translocated to the early focal adhesions upon activation of integrins, which involves cytosolic tyrosine kinase Src, myosin II, and MLCK [212]. The function of Src in regulation of adhesion and migration of fibroblasts is well known; in these cells Src is activated directly by chemotactic RPTKs. Because of the general mode of action, the role of MAP-kinases and Src in detection of gradients and establishing direction of movement is unlikely, but they may considerably contribute to basic motility. The other MAP kinase pathways such as p38 and JNK cascades are arranged in a similar manner, and their role in chemotaxis is still contradictory [7].

Ca^{2+} does not play an essential role in chemotaxis, but differently contributes to motility and signaling components depending on the cell type. It is important for signaling in the nerve growth cone [9] but plays a minor role in detection of gradients in other cells. Ca^{2+} is a general secondary messenger and like MAP kinases acts fairly nonspecifically. Chemoattractants stimulate an increase in intracellular Ca^{2+}, which however forms very subtle and shallow internal gradients. Ca^{2+} is thought important for neutrophil motility on adhesive substrates as it modulates the function of integrins [100]. In these amoeboid cells it mainly regulates myosin II activity in the cell rear [7]. Fibroblasts might be closer to the growth cone in the importance of Ca^{2+} signaling [9]. The effects of Ca^{2+} are in most cases mediated by calmodulin and MLCK activation. MLCK phosphorylates and activates myosin II in the lamellum, thus providing contractile forces for translocation of the cell body [98]. In addition, Ca^{2+} may activate calpain and contribute to the rear detachment [121].

Phospholipase C-γ is consistently reported to play an important role in cell migration. It is most evident in fibroblasts [14, 142, 145], but not in amoeboid cells [139, 180]. The mechanism largely involves generation of membrane diacylglycerols and soluble inositol trisphosphate, which triggers Ca^{2+} efflux from intracellular stores (Fig. 5b). An important function of diacylglycerol in signaling from phospholipase C-γ may be due to binding of proteins that possess a lipid-binding C1 domain [213]. Classic examples of such proteins are protein kinase C and chimerins, the latter being the only proteins known so far that have a Rac-GAP activity and thus mediate inactivation of Rac [213]. Whether chimerins are involved in chemotaxis is not known, but they are potential Rac inhibitors in the posterior region of cells and regulators of Rac and Rho antagonism, which would be consistent with a reported function of phospholipase C-γ in establishing asymmetric cell morphology [142].

Microtubules also regulate Rac and Rho antagonism and polarization of cells [38, 39, 86, 214]. Although tubulin cytoskeleton has not been explicitly demonstrated to be required for detection, processing, or transmission of chemotactic signals [138], it is certainly involved in regulation of adhesion dynamics and basic motility (see [38, 86, 87] for details). The growing microtubules are captured and stabilized by the adhesion contacts, slow down their following maturation, and induce disassembly. The action of microtubules is often linked to regulation of actin cytoskeleton, so that depolymerization of microtubules causes activation of Rho and stabilization of actin–myosin cytoskeleton, whereas re-polymerization results in Rac activation. Perhaps feedback mechanisms are involved, as myosin II regulates the dynamic balance of actin and tubulin cytoskeleton [109].

FEEDBACK REGULATION

Ideas that feedback mechanisms are needed for cytoskeleton regulation, polarization, and persistent motility have long been suggested [54, 59]. There is no doubt that a relationship, that integrates the protrusive, adhesive, and contractile activities [6, 40], involves feedback regulation. Although these mechanisms are much less studied with respect to chemotaxis [8], it is clear that signal localization and amplification at the leading edge, as well as maintenance and adaptation of cell responses to changes of external stimuli, are essential for chemotactic signaling.

Feedback Loops

Dictyostelium cells provide a clear example of the biphasic changes in actin dynamics at the leading edge that reflect signal delivered by chemotactic receptors (see

[26, 188, 190] for details). The first phase is marked by a rapid appearance of the proteins containing PH-domains at the cell periphery. It is shortly followed by a prolonged phase of their accumulation in limited areas of the plasma membrane. During the first phase, which is insensitive to inhibitors of PI3K, cells shrink and lose polarity, and in the second phase they begin to protrude pseudopodia, which are blocked by PI3K inhibition. These changes clearly coincide with two phases of actin polymerization that take place in these areas.

It would be expected that activation of a feedback mechanism underlies the two-phase behavior of the actin dynamics (see Fig. 6). The first phase is likely immediately initiated by the receptors by switching on the small GTPases Cdc42 and Rac [138, 182]. As mostly protein–protein interactions are used to transmit the signal, no significant amplification occurs at this point, but PAK1 may provide some gain in as yet not entirely clear manner [182]. As a result of this first rapid wave, a local actin platform is created (Fig. 6) that is required for organization of a second positive feedback loop using polymerized actin and PI3K/PIP3.

In neutrophils Cdc42 acts as an organizer of short-lived actin filaments [215], which are the likely candidates for such a platform. These structures are also the likely precursors of filopodia and microspikes. Their further development requires additional regulators, such as Ena/VASP proteins, IRSp53, fascin, cofilin, and formins [53]. None of these proteins are able to create by themselves the branched actin network characteristic for lamellipodia. This becomes possible only in the presence of Rac [215] and a large number of regulatory molecules that promote actin nucleation, elongation, and branching [52, 62, 73, 81]. PIP3 is needed to activate Rac because it recruits to the membrane a number of guanyl nucleotide exchange factors containing PH domains [183].

Massive formation of PIP3 is achieved by a feedback mechanism and by the second wave of activation of actin dynamics. The feedback loop involving PI3K and polymerized actin has been described in *Dictyostelium* cells [159, 171, 190] and neutrophils [146, 163, 215, 216]. Amplification of the PIP3 signal is achieved by the kinase activity of PI3K, and actin localizes this loop to the sites of lamellipodia formation (Fig. 6). The second signaling wave to actin polymerization is mediated by the receptor-dependent activation of the PI3K/PIP3 cascade and recruitment from cytosol of Rac guanyl nucleotide exchange factors containing PH-domains.

Such a model explains the two activation waves of actin polymerization triggered by attractant, while only the second depends, albeit partially, on the integrity of actin cytoskeleton [8, 188]. The first wave does not depend on PI3K activation and therefore is resistant to PI3K inhibitors [188], whereas the second wave does depend on PI3K. The second phase of actin polymerization and formation of lamellipodia/pseudopodia (but not filopodia) depend entirely on Rac activation [215]. The uniform (no gradient) addition of chemoattractant or exogenous PIP3 causes appearance of PIP3 patches and subsequent self-enhanced accumulation of PIP3, leading to activation of Rac and increased actin dynamics [163, 190]. Such a mechanism may explain why *Dictyostelium* cells with knocked out Gβγ-subunits migrate in the absence of gradients [171].

A similar mechanism may also account for the intrinsic polarity and the ability of certain cells to move in the absence of chemoattractants. The most striking example is fibroblasts. Both in the absence of external stimuli [217] and in gradients [218] their motility is associated with local hot spots of PIP3 that are somehow dynamically and stochastically coupled such that their lifetime becomes comparable with that of protrusion dynamics [219]. Lack of the actin-dependent positive feedback loop in these cells suggests an existence of other means that locally maintain chemotactic signaling. For instance, the activated cell surface RPTKs are internalized by endocytosis, but they maintain signaling or even organize new signaling platforms on endosomes [143, 220]. In addition, receptor-dependent activation of the membrane NAD(P)H oxidase by PI3K and Rac leads to ROS generation [221], which is thought to maintain activity of signaling cascades.

Endocytosis

Endocytosis plays a critical role in PDGF-dependent migration of fibroblasts [47]. It involves association into a complex of PDGF receptors, DOCK4, an adaptor protein Grb2, and dynamin-2. DOCK4 is a Rac1 guanine nucleotide exchange factor that is involved in migration of lymphocytes, phagocytosis, and tumor growth. DOCK4 has a PH-domain, localizes to the leading edge of migrating fibroblasts by binding to PIP3, and interacts there with Grb2. It specifically activates Rac1, but not Rho, Cdc42, or Rap, and does not affect the activation profile of the ERK1/2 MAP-kinases or PKB/Akt in response to PDGF. Dynamin-2 is the molecular motor that mediates fission of the clathrin-coated vesicles from the membrane. It binds to active PDGF receptors and mediates endocytosis of the whole complex. Intracellular traffic and sorting of the receptors that is regulated by Rab GTPases critically contributes to cell migration [222]. In the migrating cells DOCK4 [47], clathrin [223], and endosome transport [222] are polarized in the direction of movement, and receptors remain longer in the phosphorylated state while in the endosomal compartment.

Similarly, activation and endocytosis of the receptors of EGF and PVR (an analog of PDGF receptor) are required for boundary cell migration during oogenesis in *Drosophila* [19, 222]. The absence of proteins that mediate early stages of their endocytosis (*Cbl*, *Sprint*, and *Hrs*)

impairs localization of tyrosine phosphorylation to the leading membrane and directed migration [19].

Thus, the role of endocytosis in cell migration is not apparently limited to endosomal transport of integrins and membrane lipids to the leading edge, as previously thought [61]. Increasing evidence for signaling function of endocytosis implies a potentially novel regulatory mechanism for chemotaxis. Internalized chemotactic receptors are routed to a separate cellular compartment and become resistant to inactivation. They maintain signaling activity for 30-40 min, within the time scale sufficient for pseudopodia formation in fibroblasts [47]. Whether a similar mechanism operates in amoeboid cells, which have fast pseudopodial dynamics, as yet remains unexplored.

NAD(P)H Oxidase

Together with NAD(P)H oxidase, endocytosis may prolong duration of the receptor-dependent signaling. In fibroblasts such a mechanism may involve Rac-mediated activation of the membrane NAD(P)H oxidase and local accumulation of intracellular hydrogen peroxide [224, 225]. Currently, H_2O_2 is considered as a signaling molecule complying with all the characteristics of a classic second messenger. The major H_2O_2 mechanism is linked to inactivation of signaling tyrosine phosphatases, but other targets of H_2O_2 have been identified as well.

The membrane NAD(P)H oxidase complex consists of two lipid-binding components, $gp91^{phox}$ (Nox2 or its homologs) and $p22^{phox}$, as well as several regulatory proteins recruited from cytosol upon activation by cell surface receptors. They are $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac GTPase in the case of the classic plasma membrane NAD(P)H oxidase. They bind to $gp91^{phox}/p22^{phox}$ and activate the electron transfer from NAD(P)H to molecular oxygen [226]. The resulting superoxide radical is a precursor for multiple ROS, whose regulatory role in cell physiology has been reviewed in detail elsewhere [226-228]. Stimulation of cells by different factors (i.e. PDGF, EGF, tumor necrosis factor-α, or IL-1β) results in rapid activation of NAD(P)H oxidase and increased intracellular free radicals. This response involves Ras, PI3K, Rac-GEFs, and Rac [221, 229]. The superoxide radical is then rapidly dismutated, either spontaneously or by dismutase into longer lasting H_2O_2. Hydrogen peroxide is not a radical and therefore it is much more stable and less active, properties that considerably narrow the range of its potential targets.

Numerous effects of ROS and H_2O_2 on intracellular signaling have been described (see [230] for a review). ROS enhance activation of the ERK1/2 cascade and other stress-activated MAP-kinases, as well as phosphorylation of PKB/Akt and Src triggered by RPTK stimulation. It is often paralleled by increased levels and/or duration of receptor phosphorylation. Exogenous H_2O_2 increases tyrosine phosphorylation in different cell types, whereas catalase that inactivates H_2O_2 eliminates this effect.

H_2O_2 reversibly oxidizes the cysteine residues in target proteins that are predominantly ionized in the intracellular environment [224, 228]. The tyrosine phosphatases are the best candidates for this modification because they contain a conserved $Cys\text{-}(Xxx)_5\text{-}Arg$ motif in their active center. In this microenvironment, SH-groups of these cysteines are often dissociated and serve as H_2O_2 targets [224]. These proteins include PTP-1B (the major RPTK phosphatase) [231] and PTEN (PIP3 phosphatase) [232, 233]. In addition to these phosphatases directly related to chemotaxis, H_2O_2 has been shown to activate cofilin, the critical regulator of actin dynamics, by targeting adaptor protein 14-3-3 and, indirectly, the cofilin phosphatase *Slingshot* [234]. There is ample evidence, perhaps best reviewed by Sroka and Madeja [235], which implicates free radicals and H_2O_2 in cell migration. However, the relevant intracellular targets and H_2O_2 mechanisms in chemotaxis remain largely unexplored.

The oxidized cysteines are reduced back by the highly active peroxiredoxins, whereas H_2O_2 is destroyed by catalase, which however is not freely around because it localizes in peroxisomes. The topology of the plasma membrane NAD(P)H oxidase predicts that superoxide anion is produced outside of the cell, and several studies confirm this (see [226, 228]). Therefore, superoxide anion or H_2O_2 have to diffuse across the cell membrane to reach intracellular targets. However, H_2O_2 is destroyed inside cells rapidly unless it immediately hits a nearby reactive target. This requires that a steep transmembrane gradient to be maintained for H_2O_2 to exert prolonged effects. A similar situation has been recently reported in zebrafish when excess H_2O_2 produced in acute wounds served as chemoattractant to recruit leukocytes [326]. While proving a principle that H_2O_2 has a role in chemotaxis, it does not however suggest that such H_2O_2 gradients can be formed and maintained in physiological milieu.

The first clues that H_2O_2 signaling is relevant to chemotaxis began to emerge after discovery of NAD(P)H oxidase components at the leading edge of migrating cells (see [224, 225] for reviews). In addition, NAD(P)H oxidase has been found in caveolae and lipid rafts that polarize in migrating cells. Of particular interest are observations of the receptor- and NAD(P)H-oxidase-dependent H_2O_2 generation in endosomes [237, 238]. Stimulation of cells with IL-1β, EGF, or PDGF caused endocytosis of the cognate receptors and endosome-associated generation of H_2O_2. When H_2O_2 dynamics were assessed in live cells, it appeared to be highly localized and sustained over 30 min in the endosome fraction [238]. This indicates that NAD(P)H oxidase remains active in endosomes, likely due to maintained activity of internalized receptors and associated Rac.

Altogether, the data accumulated so far highlight the importance of feedback regulation in chemotaxis and shape up emerging mechanisms that involve polarized endocytosis and receptor-dependent production of intracellular hydrogen peroxide, a novel second messenger capable of enhancing and extending receptor-dependent signaling.

PERSPECTIVES

Delving into the basic and regulatory mechanisms of guided motility has been vastly progressing in this millenium uncovering a myriad of participants intertwined into two elaborate networks, which sense the external clues and perform the mechanical responses. Despite their complexity, common principles of composition and molecular communication appear and give a hope that understanding of how these intricate machines work will be achieved. The picture emerges that cells are the flexible beings that use common tools to bias behavior in accord with surroundings. They perceive outer signals and process them using divergent, but still shared mechanisms, and move by choosing appropriate means. To manipulate and exploit this intrinsic behavior in helping our needs, we must learn fundamental aspects and specifics. While *Dictyostelium* allows for easier access and provides a significant insight into basics of chemotaxis, other regulatory strategies might have evolved in mammalian cells and some have been lost, as we see with cGMP regulation. Still, using valuable knowledge gained in these model organisms should facilitate studying more sophisticated but relevant systems such as neural, immune, inflammatory, stem, and progenitor cells.

The modern techniques provide novel tools to visualize and research how cells sense the natural cues and behave in the physiological 3-D context, what mechanisms underlie cell–cell dialogs and their collective behavior, and how cells deal with and make use of their environment. Long-distance contacts by means of small molecules are well expected for cells in the body, yet we know little of how their stable gradients are formed and maintained. This is especially important in regenerative medicine and immunology. It is also not clear what mechanisms the cells use to relay information in the form of a gradient over long distances. What is the nature of chemotactic signals and how are multiple signals being dealt with? A fairly unexplored area is how particular cells are navigated within tissues, whether the cells of different types give a hand to each other? That some cells such as leukocytes express rather low proteolytic activity may require their mesenchymal neighbors to provide it or physical guidance. Hematopoietic and mesenchymal cells, which most of the time live in a niche as a couple, may provide an insight into the natural case. These answers are clearly on demand in stem and progenitor cell biology.

Going inside, the questions remain as to how the cells sort out and process the external information, how they adapt to the spatio-temporal changes of the external gradient? Along these lines, what are the mechanisms that maintain the internal signaling gradients? It seems to be likely that slow mechanics of mesenchymal cells would require feedback mechanisms, perhaps different from those in fast-moving amoeba and leukocytes. New concepts are coming around suggesting that, in addition to classic transduction design, the signaling architecture may involve new partners and whereabouts we have not thought much of. They may seem to be novel to us and hardly characterized chemical second messengers, such as hydrogen peroxide, or the well-known acquaintances such as endocytosis and vesicle traffic. Further research will reveal how these mechanisms are built and whether they are essential, evolutionary conserved, and abundant in cells.

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