Sphingosine 1-phosphate signaling: providing cells with a sense of direction

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Sphingosine 1-phosphate (S1P) is a sphingolipid metabolite that regulates diverse biological functions. S1P has been identified as a high-affinity ligand for a family of five G-protein-coupled receptors, known as the S1P receptors. The physiological role of the S1P receptor $S1P_1$ in vascular maturation was recently revealed by gene disruption in mice. In addition to other cellular processes, the binding of S1P to its receptors regulates motility and directional migration of a variety of cell types, including endothelial cells and vascular smooth muscle cells. This review focuses on the important role of S1P and its receptors in cell migration and describes a new paradigm for receptor cross-communication in which transactivation of $S1P_1$ by a receptor tyrosine kinase (PDGFR) is crucial for cell motility.

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Formed by activation of sphingosine kinase in response to diverse stimuli, the bioactive sphingolipid metabolite sphingosine 1-phosphate (S1P) has been implicated in many biological processes, including cell growth, suppression of apoptosis, stress responses, calcium homeostasis, cell migration, angiogenesis and vascular maturation. S1P is found in many types of organisms, from plants, yeast, worms and flies, to mammals. While the observation that S1P can induce cell proliferation was made over 10 years ago [1], the identification of S1P receptors (reviewed in Refs [2–5]) has only recently led to major advances in our understanding of the role of this lipid in homeostatic and pathologic processes and the signaling pathways that its receptors activate.

The level of S1P in cells is low and is regulated by the balance between its synthesis, catalyzed by sphingosine kinase, and its degradation, catalyzed by an endoplasmic reticulum S1P lyase and a newly discovered S1P phosphohydrolase (see Fig. I in Box 1). Sphingosine kinase is activated by numerous external stimuli, among which growth and survival factors are prominent [6,7]. Intracellularly generated S1P can then mobilize calcium from internal stores independent of inositol trisphosphate [8] and regulate signaling pathways that stimulate cell growth [9–11] and suppress apoptosis [7,12–14]. Released from cells, S1P can ligate specific receptors, leading to increased chemokinetic or random motility, regulation of directional migration (chemotaxis) and dynamic changes in the actin cytoskeleton. As the intracellular actions of S1P have been extensively reviewed recently [2,15], this review will examine the role of S1P and its receptors, especially $S1P_1$, in cell migration and discuss several new hypotheses pertaining to the mechanisms involved. Our focus will be on the model of crosstalk between different classes of receptors, such as the tyrosine kinase platelet-derived growth factor (PDGF) receptor and the G-protein-coupled S1PRs, and the importance of such cross communication for cell motility.

Pleiotropic functions of sphingosine 1-phosphate

Recent studies have begun to shed light on the physiological functions of S1P, beginning with the discovery that it is a specific ligand for a family of G-protein-coupled receptors (GPCRs), recently named S1PR [2–5]. To date, five members of this receptor family have been identified, including EDG-1/$S1P_1$, EDG-5/$S1P_2$, EDG-3/$S1P_3$, EDG-6/$S1P_4$, and EDG-8/$S1P_5$. These receptors are expressed ubiquitously and couple to diverse G proteins, except for $S1P_1$, which is coupled mainly to G_i. As a result, S1PRs regulate different biological processes depending on their pattern of expression and the various G proteins present. For more extensive discussions of G protein signaling downstream of these receptors, the reader is referred to several recent reviews [2–5].

Although S1PRs are differentially expressed, several members have been implicated in both positive and negative regulation of cell migration [16–20]. Activation of $S1P_1$ or $S1P_3$ by S1P or sphinganine 1-phosphate (dihydroS1P), which has the same structure as S1P (Box 1, Fig. I) except for lack of the 4,5-*trans* double bond, in many cell types both increased random migration and induced directional or chemotactic migration [16,21,22], whereas binding to $S1P_2$ abolished chemotaxis and membrane ruffling [23]. In agreement, the S1PRs differentially regulate the small GTPases of the Rho family, particularly Rho and Rac, which are downstream of heterotrimeric G proteins and are important for cytoskeletal rearrangements [24]. Binding of S1P to $S1P_1$ mediated cortical actin assembly and Rac activation [25,26], whereas binding to $S1P_3$ and $S1P_2$ induced stress fiber formation and activation of Rho [23]. Interestingly, $S1P_2$ negatively regulates Rac activity [23], thereby inhibiting cell migration.

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Box 1. Regulation of S1P levels

Two distinct isoforms of sphingosine kinase, which phosphorylate sphingosine to form sphingosine 1-phosphate (S1P), have been cloned [a,b]. Overexpression of sphingosine kinase type 1 in fibroblasts results in enhanced proliferation [c], growth in soft agar and the ability of cells to form tumors in nude mice [d]. With the use of a sphingosine kinase inhibitor and a dominant-negative mutant of this enzyme, it was elegantly demonstrated that sphingosine kinase contributes to cell transformation mediated by oncogenic Ha-Ras, suggesting a novel signaling pathway for Ras activation [e]. Overexpression of sphingosine kinase 1 also protected cells against apoptosis, particularly cell death induced by elevation of ceramide (*N*-acyl sphingosine) [c,e]. The cytoprotective effect has been attributed to inhibition of activation of caspases-2, -3 and -7 and of the stress-activated protein kinase JNK [e].

Recently, a specific hydrophobic phosphohydrolase that degrades only S1P, and not the related phospholipids, lysophosphatidic acid, phosphatidic acid or ceramide 1-phosphate, has been described [f]. This unique S1P phosphatase, which converts S1P to sphingosine (Fig. I), contributes to regulation of the dynamic balance between S1P and sphingosine/ceramide in mammalian cells and, consequently, influences cell fate [f].

S1P lyase is a pyridoxal-dependent enzyme that cleaves S1P at the C2–C3 bond to yield ethanolamine phosphate and hexedecenal (Fig. I). S1P lyase, like S1P phosphatase, appears to be localized to the endoplasmic reticulum of cells. Although murine and human S1P lyases were cloned recently [g,h], their functions in mammalian cells have not yet been thoroughly examined. However, disruption of the gene encoding S1P lyase in *Dictyostelium discoideum* results in aberrant morphogenesis, as well as increased viability during stationary phase. The absence of the lyase affects multiple stages throughout development, including the cytoskeletal architecture of aggregating cells, the ability to form migrating slugs and terminal spore differentiation, suggesting that S1P lyase has a central role in the development of multicellular organisms [i].

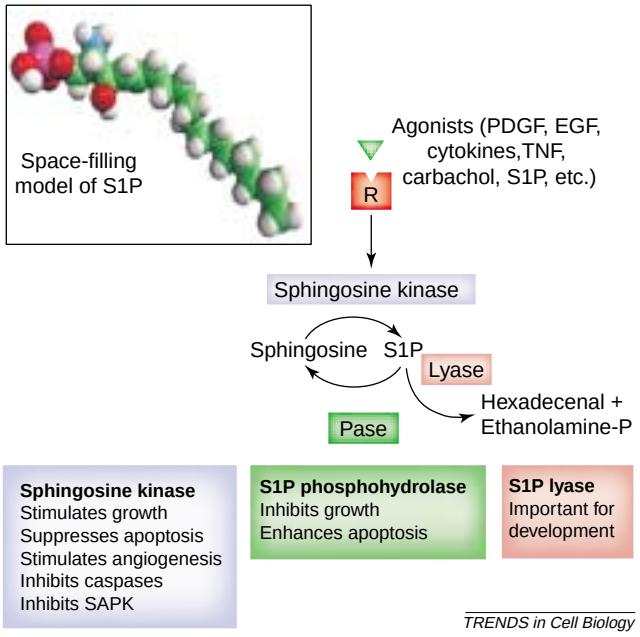

Fig. I. Metabolism of sphingosine 1-phosphate (S1P). Agonists, including growth factors, cytokines, carbachol and even S1P itself, activate sphingosine kinase in cells, which phosphorylates sphingosine, generating S1P. S1P can be degraded by specific phosphatases (Pase) to sphingosine or by cellular lyases to form hexadecenal and phosphoethanolamine. Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; R, receptor; SAPK, stress-activated protein kinase; TNF, tumor necrosis factor.

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Sphingosine 1-phosphate in cell migration and angiogenesis

Cell migration is an essential process involved in embryonic differentiation as well as in many physiological and pathological processes, including inflammation, wound healing, tumor growth, metastasis and the formation of new blood vessels, known as angiogenesis. Recently, a mutation in the zebrafish homolog of the *edg-5/s1p2* gene, 'miles apart' or *mil*, was shown to cause defective migration of myocardial cells during vertebrate heart development [20]. The function of $S1P_2$ in the development of the mammalian cardiovascular system has not yet been described.

The best studied of the S1P receptors is $S1P_1$, which has been implicated in migration of many types of cells, including endothelial [16,22,25] and smooth muscle [27,28] cells, an important event in angiogenesis. Indeed, S1P has recently been demonstrated to induce angiogenic responses *in vitro* and *in vivo* [16,18,22,25]. Both $S1P_1$- and $S1P_3$-regulated signaling pathways were required for endothelial cell morphogenesis into capillary-like networks [25]. Moreover, S1P also promotes endothelial cell barrier integrity by $S1P_1$ and $S1P_3$ linked to Rac- and Rho-dependent cytoskeletal rearrangement and might act late in angiogenesis to stabilize newly formed vessels [29].

Lesson from the $S1P_1$ knockout

Further understanding of the physiological function of S1P emerged recently from the knockout of genes encoding S1PRs in mice [26,30,31]. The remainder of this review focuses on $S1P_1$ because much less still is known of the functions of the other S1P receptors. Disruption of the gene for this receptor by homologous

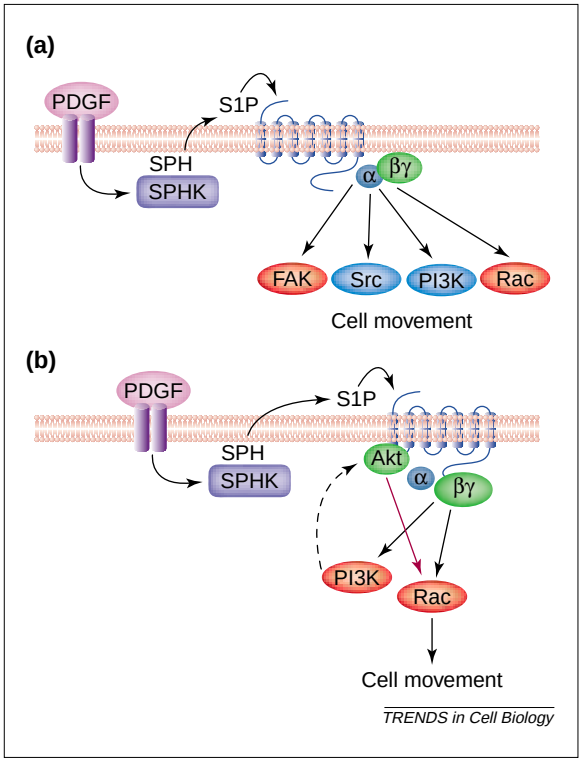

Fig. 1. The role of $S1P_1$ in cell motility directed by platelet-derived growth factor (PDGF). (a) Transactivation of EDG-1/$S1P_1$ by PDGF regulates cell motility. This scheme depicts a proposed paradigm for intracellular communication between a tyrosine kinase growth factor receptor (the PDGF receptor PDGFR) and G-protein-coupled seven transmembrane span receptor ($S1P_1$) signaling pathways crucial for cell motility. Binding of PDGF to PDGFR results in activation and translocation of sphingosine kinase (SPHK) to the leading edge and restricted phosphorylation of sphingosine (SPH), resulting in the local generation of sphingosine 1-phosphate (S1P). Spatial and temporal formation of S1P in turn activates the S1P receptor $S1P_1$, leading to dissociation of heterotrimeric G proteins into $G\alpha_i$ and $G\beta\gamma$ subunits. This in turn can lead to stimulation of focal adhesion kinase (FAK), Src, phosphoinositide 3-kinase (PI3K) and Rac. See text for more details of recruitment and/or activation of downstream signaling molecules, including Src, FAK and Rac. Note that the more conventional pathway by which the PDGFR can directly activate numerous signaling pathways activating the same molecules [46,59] is not shown. Because the PDGFR can activate Rac on its own, the S1P–$S1P_1$ signaling loop might provide an amplification step that could enhance the chemotactic response. (b) Potential involvement of Akt-mediated phosphorylation of $S1P_1$ in Rac activation. A new pathway for Rac activation by $S1P_1$ has been proposed recently [58]. $S1P_1$ can lead to activation and recruitment of Akt to the plasma membrane, where it phosphorylates $S1P_1$ on Thr236, which is required for Rac activation and cell migration. PDGF can also stimulate Akt, which then can phosphorylate and activate $S1P_1$ in an S1P-independent manner.

recombination in mice resulted in massive intra-embryonic hemorrhaging and intrauterine death between E12.5 and E14.5 owing to incomplete vascular maturation [26]. This defect resulted from a failure of vascular smooth muscle cells and pericytes to migrate to arteries and capillaries and properly reinforce them, resulting in blood vessels comprising mainly naked endothelial tubes, a finding consistent with the notion that $S1P_1$ plays an essential role in the regulation of vascular maturation.

Cell locomotion requires communication of individual cells with their environment and is dependent on integration of diverse signals derived from growth factors that promote cell motility such as PDGF, extracellular matrix adhesion receptors (integrins) and associated molecules. Interestingly, disruption of the genes encoding PDGF-β or PDGFR-β in mice resulted in a lethal phenotype somewhat similar to that caused by $S1P_1$ disruption [26,32,33]. Furthermore, in many different cell types, PDGF stimulates sphingosine kinase, leading to an increase in S1P levels [34]. Thus, we proposed that interaction between PDGFR and $S1P_1$ signals might be required for cell migratory responses essential for the development of functional neovessels [27].

Interaction between a receptor tyrosine kinase and $S1P_1$

Several approaches have been used to examine the role of $S1P_1$ in cell migration, including $S1P_1$ antisense, treatment with pertussis toxin, which ADP-ribosylates and inactivates Gi, and studies of cells from genetic knockout mice [27,35]. Although these approaches can suffer from specific drawbacks, they all support the conclusion that, in the absence of $S1P_1$, migration towards S1P is markedly reduced. Importantly, deletion of $S1P_1$ by antisense or knockout not only blocked cell migration towards S1P but also reduced migration of human aortic smooth muscle cells and fibroblasts towards PDGF [27], without affecting PDGFR functions important for cell-cycle progression, including tyrosine phosphorylation of the receptor itself and activation of the mitogen-activated protein (MAP) kinases ERK 1/ERK 2 [36]. These results suggest that migration of cells towards PDGF is likewise dependent on expression of $S1P_1$. However, $S1P_1$ deletion did not markedly affect migration towards fibronectin [27,36], indicating that $S1P_1$ deletion does not disrupt all essential mechanisms of cell migration. The proposition that $S1P_1$ signaling acts downstream of PDGFR is further bolstered by the observations that pertussis toxin and *N*,*N*-dimethylsphingosine (inhibitors of Gi and sphingosine kinase, respectively) blocked PDGF-directed migration in wild-type fibroblasts expressing $S1P_1$ [27]. Hence, these results demonstrated that $S1P_1$ functions in an unprecedented manner as an integrator linking the PDGFR to the cellular machinery of cell migration and suggest a new mechanistic concept for cross communication, or transactivation, between tyrosine kinase receptors and GPCRs. According to this paradigm, stimulation of PDGFR by PDGF activates sphingosine kinase, resulting in increased formation of S1P (Fig. 1a). S1P in turn, in an autocrine or paracrine fashion, stimulates $S1P_1$, leading to activation of downstream signals crucial for cell locomotion [27,36]. Further support for this concept recently emerged from the demonstration that PDGFR is tethered to $S1P_1$, providing a platform for integrative signaling by these receptors [37]. Although the physiological relevance of this new type

of intracellular signaling crosstalk is not yet clear, it might be important for normal microvasculature maturation during vasculogenesis and angiogenesis. In this process, mural support cells (pericytes and smooth muscle cells) are normally recruited to the vessel walls in response to PDGF secreted from endothelial cells. As discussed above, embryos lacking either $S1P_1$ or PDGFR-β, as well as embryos deficient in PDGF-BB, display similar defects in the pericyte (mesenchymally derived mural cell precursors) coating of their capillary walls. These defects cause the vessels to rupture at late gestation, leading to massive hemorrhaging [26,32,33,38]. Dysfunctional migration of $S1P_1^{-/-}$ cells towards PDGF [27] provides an attractive hypothesis that links these two phenotypes at the final steps of vascular development. Based on the demonstration that $S1P_1$ plays a crucial role in PDGF-directed motility of smooth muscle cells [27], it is tempting to speculate that this might also provide the underlying mechanism of the newly discovered role of S1P in maturation of blood vessels [26] and *in vivo* angiogenesis [18,22,25]. Further work is needed to critically evaluate the role of S1P in vascular maturation *in vivo* and tumor angiogenesis.

How does $S1P_1$ signaling regulate PDGF-induced cell motility?

During cell movements, there is dynamic reorganization of the actin cytoskeleton and focal adhesion formation, which directs protrusion at the leading edge of the cell and retraction at the rear. Given the complexity of the process of cell motility, it is not surprising that this is regulated by multiple signals. Indeed, the Rho family of small GTPases – Rac, Cdc42 and Rho – effect complex changes in the actin dynamics required for the migratory response [39]. In addition, focal adhesion complexes modulated by tyrosine kinases such as focal adhesion kinase (FAK) [40] as well as upstream effectors, including Src [41,42], phosphoinositide 3-kinase (PI3K), different MAP kinase members (ERK and p38) and phospholipase C, have all been shown to play a role in migration of various cell types (reviewed in Refs [43,44]). The role of certain mediators in responses of endothelial cells to S1P, including PI3K, phospholipase C, MAP kinases and Src-family members might depend on the cells' origin and conditions used to assess migration. This topic has recently been reviewed in great detail elsewhere [45]. Deletion of $S1P_1$ revealed a link to several of the key elements of cellular migration, including Rac, FAK and Src, and stress-activated protein kinase 2 (p38), and will be discussed in more detail below (Fig. 1a).

Role of the tyrosine kinases FAK and Src

It is well established that the tyrosine kinases FAK and Src are necessary for formation and turnover of focal complexes [40–42,46]. Active recruitment and activation of Src-family protein tyrosine kinases, Src, Yes and Fyn (hereafter collectively referred to as 'Src') to FAK at its phosphorylated Y397 site might be the first of several signaling events necessary to promote migration [41,46]. Recent evidence indicates that PDGF promotes phosphorylation of FAK at Y397, thereby creating a Src-homology 2 (SH2)-binding site to recruit Src to focal adhesion complexes [46]. In order for migration to proceed, these focal adhesions must continuously be disrupted and reformed in a dynamic manner as the cell moves forward. FAK phosphorylation at this indispensable Src-binding site dynamically functions as part of the cytoskeleton-associated network of signaling molecules downstream not only of PDGFR and EGFR, but also of integrins and GPCRs, to regulate cell motility [46]. Interestingly, autophosphorylation of FAK on Y397 was found to be essential for regulation of cell motility by S1P [47].

Migratory deficits have been noted in cells lacking Src [48] or FAK, and reintroduction of FAK, but not the unphosphorylatable mutant Y397F FAK, in FAK-null cells restored their ability to migrate [46]. Because the tyrosine kinase activity of Src has been shown to promote turnover of focal contact structures during cell migration [42], the migratory defects reflect defects in focal adhesion turnover. Notably, in $S1P_1$-null fibroblasts, PDGF had no effect on tyrosine phosphorylation of FAK, which appeared to be constitutively hyperphosphorylated, and activation of cytoskeleton-associated Src and FAK, as well as FAK–Src association, were inhibited [36]. This indicates that recruitment and activation of Src by PDGF is dependent on activation of $S1P_1$. In agreement, pertussis toxin, which inactivates Gi signaling, significantly inhibits activation of Src by PDGF in airway smooth muscle cells [49], suggesting that this may be a general phenomenon. However, it is unlikely that Src is solely responsible for $S1P_1$ migratory defects as triple-null mutations of Src, Yes and Fyn had little effect on PDGF-directed motility [48].

Role of the Rac GTPase

Rac is required for chemotaxis of fibroblasts towards PDGF and is essential for producing the leading edge lamellipodial protrusions required for forward movement [39,50]. In $S1P_1$-null cells, not only was Rac activation induced by S1P and PDGF drastically reduced [26,27] but lamellipodia formation was also deficient [36]. Moreover, assembly of actin into a functional myosin motor unit capable of generating contractility forces and membrane ruffling are regulated by distinct signaling pathways in migratory cells, where the former is regulated by ERK activation and the latter by assembly of Crk-associated substrate (Cas)–Crk complexes and Rac activation [51]. This is consistent with the novel scheme proposed by Ohmori *et al.* in which Fyn-dependent Cas tyrosine phosphorylation and

membrane ruffling resulting from signals delivered from activated $S1P_1$ cooperate with the Rho-dependent responses originating from ligation of $S1P_3$ [21] to regulate endothelial cell migration. Yet another downstream target of Rac that might be also be involved in PDGF-induced cell motility is p38 [52]. Indeed EDG-1 deletion, which had no significant effect on activation of ERK induced by PDGF, completely eliminated p38 activation [36]. Similarly, binding of S1P to $S1P_1$ in several cell types has been shown to activate p38 [18,53], and inhibitors of this MAP kinase, but not of ERK, inhibit motility responses to S1P [54].

How does $S1P_1$ activate Rac?

Although it seems clear that Rac is involved in migratory responses induced by S1P, the mechanism whereby $S1P_1$ regulates Rac activation is not well understood. Rac is known to be activated by G protein βγ complexes, and a comprehensive review of the connections between GPCRs and Rac activation has recently been published [43]. Briefly, tyrosine kinases of the Src family and PI3K potentially link Gi and βγ to activation of Rac through regulation of GDP–GTP exchange factors (GEFs). One of these, T-lymphoma invasion and metastasis gene 1 (Tiam1), a specific GEF for Rac, might be involved in Rac-dependent migration as it is activated by both PDGF and $S1P_1$ [25,55]. Alternatively, Src, when activated by Gβγ or by recruitment to the membrane mediated by β-arrestin [56] following $S1P_1$ activation [27], can directly phosphorylate Ras-GRF1, thereby inducing GEF activity towards Rac [57].

It is well known that Akt is activated after it interacts with the lipids generated as a consequence of PI3K activation in stimulated cells. Lee *et al.* suggested a different link between $S1P_1$ and Rac and showed that activated Akt binds to $S1P_1$ and phosphorylates Thr236. This phosphorylation was indispensable for Rac activation, cortical actin assembly and chemotaxis induced by S1P [58]. Because many chemoattractants, including PDGF, also stimulate Akt, the results of Lee *et al.* infer that perhaps Akt could phosphorylate and activate $S1P_1$ to specifically couple to the chemotaxis signaling pathway, without generation of its ligand S1P (Fig. 1b). If so, sphingosine kinase, the enzyme that forms S1P, should not be involved in directed cell movement. Further studies are needed to clarify the possibility of ligand-independent activation of $S1P_1$. Moreover, it should also be emphasized that there are several reports of a lack of inhibition of S1P-induced migration of endothelial cells by PI3K inhibitors [17,18], leaving the role of Akt in $S1P_1$-mediated Rac activation unsettled.

However, activation of PI3K is crucial for initiation of PDGF-induced migration, and, in addition to Akt, Rac also has a major role downstream of PI3K [59]. Thus, an intriguing possibility is that, while PDGF can activate Rac on its own, the S1P–$S1P_1$ signaling loop might provide an amplification step that could enhance the chemotactic response (Fig. 1b).

$S1P_1$ signaling at the leading Edg?

The acquisition of spatial and functional asymmetry between the front and the rear of the cell is a necessary step for directional migration. It has been suggested that components of G-protein receptor systems might accumulate at the front of polarized cells, accounting for increased responsiveness to chemoattractants at the anterior [60]. Nonetheless, chemoattractant receptors remain evenly distributed on the surface of *Dictyostelium* and polarized neutrophils [44,61], and intermediate intracellular signals that are important for directional migration might be produced in a spatial and temporal manner. An early event that marks the directional responses is the restricted translocation of proteins containing pleckstrin-homology (PH) domains [owing to local generation of phosphatidylinositol (3,4,5)-trisphosphate] in a manner that is similar to the polarity of distribution of Gβγ subunits along the leading edge [43,44,60]. However, the asymmetric redistribution of βγ subunits is not sufficiently localized to restrict events to the leading edge [60], and it has been suggested that chemoattractant-associated PH domain recruitment requires an intermediate pathway dependent on the activity of one or more of the small GTPases (for an extensive review on the role of PI3K in establishing polarity, see Ref. [43]). An additional mechanism that impinges on the signaling cascade to bring about a steep signaling gradient could be restricted activation of $S1P_1$. A tantalizing notion is that local generation of S1P might convert tyrosine kinase receptor signaling into directed migration. Hence, spatially and temporally restricted generation of S1P in response to PDGF could result in restricted activation of $S1P_1$ that in turn stimulates tyrosine kinases, such as Src and FAK, and the small GTPase Rac at the inner plasma membrane facing the stimulus. Rac might then amplify the initial receptor signals, thus creating a positive-feedback loop at the leading edge of the cell.

How could cells generate a steep gradient of sphingosine 1-phosphate?

Unlike many other chemoattractants, S1P is synthesized by cells that respond to it. It is also degraded by specific enzymes in these cells, some of which may reside on the outer surface of the plasma membrane. Although not well understood, it is reasonable to assume that synthesis and degradation of S1P are differentially regulated. We are thus proposing that enhanced formation of S1P by PDGF (and perhaps also by other chemoattractants) could be governed by local activation of sphingosine kinase, while global, rapid and efficient degradation is catalyzed by lipid phosphate phosphatases or by S1P lyase. The net result would be an asymmetric build-up of S1P at the site of its formation. Intracellularly

generated S1P might not be released in appreciable quantities – vanishingly small amounts could be secreted and only activate nearby receptors. This mechanism could account for the failure to detect S1P release by S1P-producing cells even though local activation of $S1P_1$ was observed [27]. In addition, directionality could be further governed by asymmetric translocation of sphingosine kinase from the cytosol of stimulated cells to specific areas of plasma membrane ruffles, as previously visualized in cells transfected with a green-fluorescent sphingosine kinase fusion protein following treatment with PDGF [36]. Furthermore, because sphingosine, the substrate of sphingosine kinase, is a membrane-associated lipid, this translocation of sphingosine kinase could increase the production of S1P as a result of increased substrate availability.

Thus, it is possible that PDGF might elicit a steep S1P gradient by recruiting sphingosine kinase to the leading edge, where local formation of S1P could result in restricted activation of its receptor. Spatial regulation of signal-transduction pathways in this manner could play a role in directional responses to chemoattractants. Interestingly, disruption of the gene encoding S1P lyase in *Dictyostelium discoideum* affects multiple stages throughout development, including the cytoskeletal architecture of aggregating cells and the ability to form migrating slugs, suggesting an important role for S1P in development of multicellular organisms [62].

Where does S1P take us now?

This review has focused mainly on only one of the S1P receptors in cell movements. Because, in one way or another, binding of S1P to each of the S1PRs regulates motility, much more needs to be learned about the other four S1PRs, especially $S1P_2$ and $S1P_5$, that are coupled to unique signaling pathways. In addition, it will be important to determine whether other growth factors and chemokines, which are known to stimulate sphingosine kinase, also transactivate the S1PR family and, if so, to discover the physiological consequences. Although much attention has been focused in recent years on the role of S1PRs, little is still known of the regulation of S1P levels in cells. We need to complete the identification, cloning and characterization of all of the enzymes involved in S1P metabolism. Moreover, we still do not understand how these enzymes are regulated in response to external stimuli and whether different isozymes play distinct roles in different cellular compartments. A specific antibody against S1P would be extremely useful to determine its distribution within the cell. Transport of S1P into and out of cells is also an important area of investigation that has received little attention. The cystic fibrosis transmembrane conductance regulator (CFTR) has recently been implicated in uptake of S1P into cells [63]. It is thus possible that exogenous S1P is transported into cells by a specific transporter that could serve to terminate S1PR signaling and/or to supply S1P for some intracellular function. An open question concerns where in the cell S1P is produced. If it is formed in the inner leaflet of the bilayer, there must be a specific transporter or a flippase to bring it to the outer leaflet where it can bind to its receptors. Such a transporter has been identified for other sphingolipids. Finally, the development of specific agonists and antagonists for the S1PRs will help to dissect their function. The answers to these questions could lead to development of new sphingolipid-directed therapeutics for treatment of the many human disorders in which S1P has been implicated.

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