

## REVIEW ARTICLE

## Sphingosine 1-phosphate signalling in mammalian cells

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Sphingosine 1-phosphate is formed in cells in response to diverse stimuli, including growth factors, cytokines, G-protein-coupled receptor agonists, antigen, etc. Its production is catalysed by sphingosine kinase, while degradation is either via cleavage to produce palmitaldehyde and phosphoethanolamine or by dephosphorylation. In this review we discuss the most recent advances in our understanding of the role of the enzymes involved in metabolism of this lysolipid. Sphingosine 1-phosphate can also bind to members of the endothelial differentiation gene (EDG) G-protein-coupled receptor family [namely EDG1, EDG3, EDG5 (also known as H218 or AGR16), EDG6 and EDG8] to elicit biological responses. These receptors are coupled differentially via  $G_i$ ,  $G_q$ ,  $G_{12/13}$  and Rho to multiple effector systems, including adenylate cyclase, phospholipases C and D, extracellular-signal-regulated kinase, c-Jun N-terminal kinase, p38 mitogen-activated protein kinase and non-receptor tyrosine kinases. These signalling pathways are linked to transcription factor activation, cytoskeletal proteins, adhesion molecule expression, caspase activities, etc. Therefore sphingosine 1-phosphate can affect diverse biological responses, including mitogenesis, differentiation, migration and apoptosis, via receptor-dependent

mechanisms. Additionally, sphingosine 1-phosphate has been proposed to play an intracellular role, for example in  $Ca^{2+}$  mobilization, activation of non-receptor tyrosine kinases, inhibition of caspases, etc. We review the evidence for both intracellular and extracellular actions, and extensively discuss future approaches that will ultimately resolve the question of dual action. Certainly, sphingosine 1-phosphate will prove to be unique if it elicits both extra- and intra-cellular actions. Finally, we review the evidence that implicates sphingosine 1-phosphate in pathophysiological disease states, such as cancer, angiogenesis and inflammation. Thus there is a need for the development of new therapeutic compounds, such as receptor antagonists. However, identification of the most suitable targets for drug intervention requires a full understanding of the signalling and action profile of this lysosphingolipid. This article describes where the research field is in relation to achieving this aim.

**Key words:** endothelial differentiation gene (EDG), lipid phosphate phosphatase, lysosphingolipid, sphingosine kinase, sphingosine 1-phosphate lyase.

## INTRODUCTION

Sphingosine 1-phosphate (S1P) is a polar sphingolipid metabolite which has been proposed to act both as an extracellular mediator and as an intracellular second messenger. Extracellular effects are mediated via a recently identified family of plasma membrane G-protein-coupled receptors (GPCRs), whereas specific intracellular sites of action remain to be defined. S1P is stored and released from platelets upon their activation, but can also be synthesized in a wide variety of cell types in response to extracellular stimuli, such as growth factors and cytokines. S1P affects diverse biological processes, including cell growth, differentiation, migration and apoptosis, and may have an important role in pathophysiological disease states, such as atherosclerosis and cancer.

## SPHINGOLIPID METABOLISM

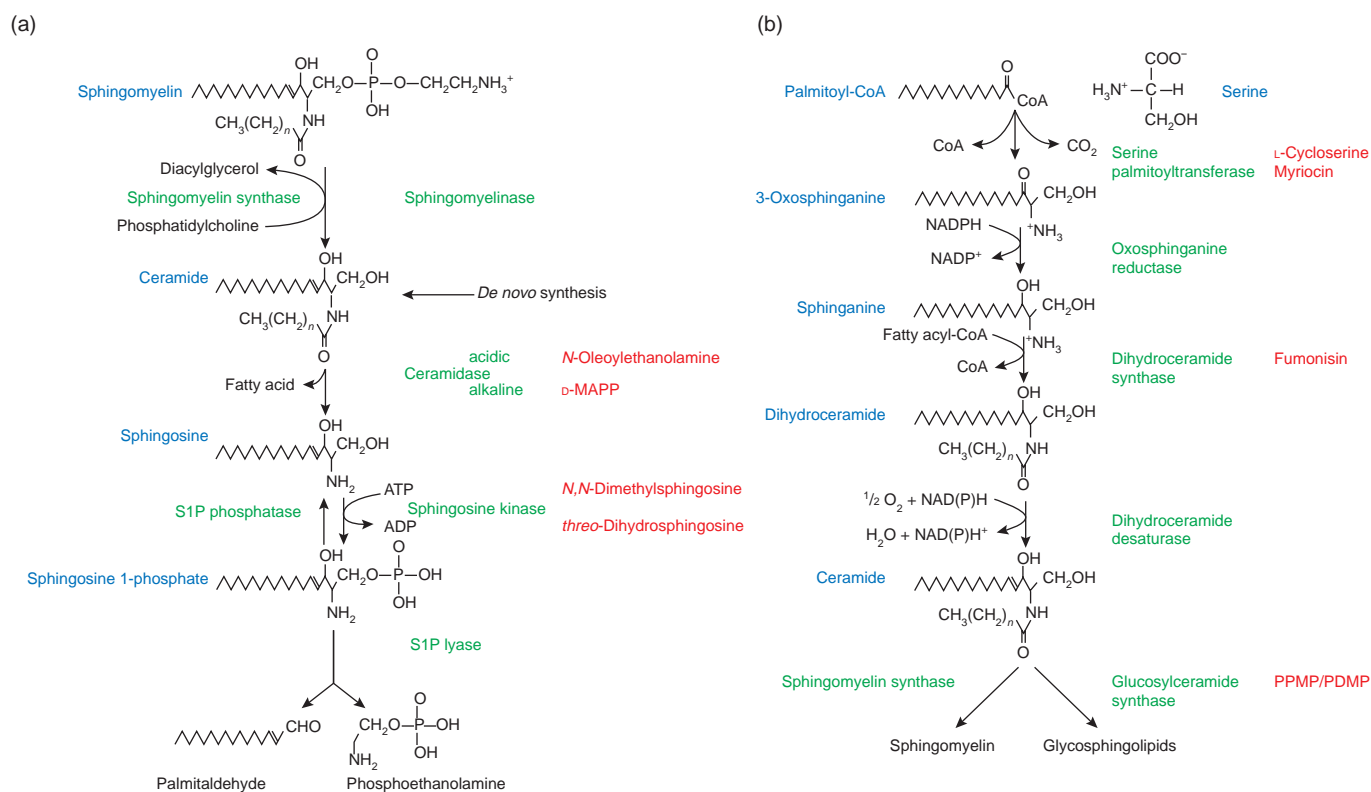
It is beyond the scope of this review to describe sphingolipid metabolism in detail, and the reader is referred to more specific articles on this subject [1,2]. However, a brief overview is provided, with details of several useful compounds for the selective inhibition of key enzymes, which may be used to

ultimately modulate S1P metabolism in cells (Schemes 1a and 1b).

Ceramide may be important in determining apoptotic responses to stress [1,2]. However, it may also serve as a precursor for the synthesis of S1P, which, in certain cell types, is implicated in cell survival. This suggests that the metabolic conversion of ceramide into S1P could switch cells from an apoptotic state to a proliferative one. Therefore inhibition of the conversion of ceramide into S1P might be usefully exploited to induce cell death in disease, such as cancer. Ceramide can be synthesized either in response to the agonist-dependent activation of sphingomyelinases or *de novo*. Agonist-dependent activation of sphingomyelinases has been observed in response to growth factors, cytokines and arachidonic acid [3]. Additionally, cellular stress and changes in the redox state of the cell can result in sphingomyelinase activation [4,5]. To date, five distinct sphingomyelinases have been identified, based on their pH optima, cellular localization and cation dependence [2,3]. Ceramidase catalyses the deacylation of ceramide to produce non-esterified fatty acid and sphingosine. Both an acidic ceramidase [6,7] (deficient in Farber's disease and inhibited by the ceramide analogue *N*-oleoylethanolamine) and an alkaline ceramidase [8]

Abbreviations used: BAPTA/AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester); EDG, endothelial differentiation gene; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; EST, expressed sequence tag; FAK, focal adhesion kinase; GPCR, G-protein-coupled receptor; IGF, insulin-like growth factor; JNK, c-Jun N-terminal kinase; LDL, low-density lipoprotein; LPA, lysophosphatidic acid; LPP, lipid phosphate phosphatase; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; PA, phosphatidic acid; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC/D, phospholipase C/D; SH2, Src homology 2; SPC, sphingosylphosphocholine; S1P, sphingosine 1-phosphate; SPHK, sphingosine kinase; SRE, serum response element; SRF, serum response factor; TNF $\alpha$ , tumour necrosis factor  $\alpha$ .

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### Scheme 1 Spingolipid metabolism

(a) S1P biosynthesis and degradation; (b) *de novo* ceramide biosynthesis. Structural formulae of the spingolipids are shown, with enzymes denoted in green and their inhibitors in red. Abbreviations: *D*-MAPP, *D*-*erythro*-2-(*N*-myristoylamino)-1-phenylpropanol; PPMP, *D*-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; PDMP, *D*-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol.

[inhibited by *D*-*erythro*-2-(*N*-myristoylamino)-1-phenylpropanol (*D*-MAPP)] exist. The resulting sphingosine may act as the substrate for sphingosine kinase (SPHK) to produce S1P. This is cleaved by S1P lyase (to produce palmitaldehyde and phosphoethanolamine) or dephosphorylated by S1P phosphatase. Alternatively, ceramide can be converted back into sphingomyelin by the addition of a phosphocholine headgroup (donated by phosphatidylcholine), catalysed by sphingomyelin synthase [9] (Scheme 1a)

*De novo* ceramide synthesis (which occurs in the endoplasmic reticulum) is initiated by the condensation of serine and palmitoyl-CoA to produce 3-oxosphinganine and CO<sub>2</sub>, catalysed by serine palmitoyltransferase. 3-Oxosphinganine is rapidly reduced to dihydrosphingosine (sphinganine) by an NAD(P)H-dependent reductase which is stereospecific for the *D*-isomer. Subsequently, dihydrosphingosine is *N*-acylated to dihydroceramide, catalysed by dihydroceramide synthase. Dihydroceramide desaturase catalyses the subsequent introduction of a *trans* double bond at C-4-C-5 to produce ceramide. *De novo* ceramide synthesis can be blocked at serine palmitoyltransferase by *L*-cycloserine [10] or by the fungal metabolite myriocin [11], whereas the mycotoxin fumonisin inhibits dihydroceramide synthase [12].

Ceramide produced *de novo* can be metabolized to glycosphingolipids, the first step of which is catalysed by a glucosylceramide synthase [13] (Scheme 1b). This enzyme is inhibited by the ceramide analogues *D*-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) and *D*-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), a potential anti-cancer agent [14]. Alternatively, ceramide can be

phosphorylated by a calcium-activated ceramide kinase to produce ceramide 1-phosphate [15,16].

### SPHK

SPHK was originally purified from rat kidney as a 49 kDa protein, with *K<sub>m</sub>* values of 5 μM and 93 μM for sphingosine and ATP respectively [17]. The enzyme binds calmodulin in the presence of calcium. Subsequently two SPHKs have been cloned [18]. Kohama and colleagues [18] trypsin-treated the purified kidney form and obtained amino acid sequences of eight peptides. Using tBLASTn algorithm searches they identified several expressed sequence tags (ESTs) which yielded predicted amino acid sequences similar to those of three of the peptides obtained by trypsin treatment of purified kidney SPHK. Subsequently, two clones from mouse were obtained with apparent open reading frames of 381 and 388 amino acids containing homologous sequence encoding seven of the eight peptides obtained from the rat kidney form. The enzymes were designated SPHK1a and SPHK1b. Their predicted molecular masses were 42.3 and 43.2 kDa. These isoforms differ in only a few amino acids at the N-terminus, suggesting that they may be derived by alternative splicing of the same mRNA transcript. However, since both cDNAs lacked Kozak sequences [19], the suggestion is that they may represent only partial cDNAs. Therefore, until upstream sequence at the 5' end is obtained, the exact relationship of the two forms is unknown.

SPHK mRNA expression has been detected as a 2.4 kb mRNA species in most tissues, with abundant expression in lung and spleen, and barely detectable levels in skeletal muscle and liver.

A SPHK gene family has been identified which includes LCB4 and LCB5 from *Saccharomyces cerevisiae* [20]. Other potential SPHKs were identified in the genomic sequences of *Caenorhabditis elegans* and *Schizosaccharomyces pombe*. Five critical regions of highly conserved amino acids (C1–C5) have been identified in all the forms, including the mammalian enzymes. Regions C1 and C3 of SPHK show 35% and 58% identity with residues 296–315 and 378–389 respectively of human diacylglycerol kinase  $\zeta$  [21], which are present in the conserved subdomain 1 of all diacylglycerol kinase isoforms. Subdomain 1 is believed to contain the ATP-binding site that is distinct from that present in protein-specific protein kinases [22]. Therefore, because of the close similarity with diacylglycerol kinase, Kohama et al. [18] have suggested that C1 and C3 in SPHK might also constitute an ATP-binding domain. More specifically, the invariant sequence, Gly-Gly-Lys-Gly-Lys, within the C1 domain of SPHK is suggested to form part of the ATP-binding site.

The cloned murine SPHK isoforms contain at least three calcium/calmodulin-binding consensus sequences and several potential protein kinase phosphorylation sites: one for protein kinase A, two for casein kinase II and eight for protein kinase C (PKC). The presence of the latter is of interest because SPHK in intact cells has been shown to be stimulated by PKC activators, such as PMA [23]. Furthermore, inhibition of ceramide-induced apoptosis by PKC activation is associated with the stimulation of SPHK and S1P formation [23]. The mammalian SPHKs also lack Src homology 2 (SH2), SH3 and pleckstrin homology domains, suggesting that they do not interact with phosphotyrosine-containing adaptor proteins, G-protein  $\beta\gamma$  subunits or phospholipids, such as phosphatidylinositol 3,4,5-trisphosphate. Earlier *in vitro* studies demonstrated that the enzyme could be activated by acidic phospholipids such as phosphatidate, phosphatidylinositol, phosphatidylinositol 4,5-bisphosphate and especially phosphatidylserine [24], suggesting a possible relationship between S1P levels and glycerophospholipid signalling.

SPHK1a activity, measured in lysates of transiently transfected HEK 293 cells, has specificity for the *trans* isomer of D-erythro-sphingosine ( $K_m$  2.15  $\mu$ M). The enzyme can also phosphorylate D-erythro-dihydrosphingosine, but to a lesser extent. Ceramide, diacylglycerol, phosphatidylinositol and phytosphingosine were not substrates. N,N-Dimethylsphingosine and D,L-threo-dihydrosphingosine were confirmed as inhibitors of SPHK, with  $K_i$  values of 2.64 and 2.15  $\mu$ M respectively.

Transient transfection of HEK 293, NIH 3T3 and Swiss 3T3 cells with SPHK1a resulted in a 400–800-fold increase in SPHK activity [18,25]. The transfected SPHK activity partitioned into both cytosolic and membrane fractions (approx. 70–80% cytosolic and 20–30% particulate). Indeed, the lack of hydrophobic-enriched domains and of a signal peptide in the protein are consistent with its predominantly cytosolic localization. Platelet-derived growth factor (PDGF) stimulated a 2-fold increase in the specific activity of cytosolic SPHK1a, but did not induce significant translocation to the membrane fraction [25]. Other studies support the presence of both membrane and cytosolic SPHK activities. Notably, in platelets, 70% of total activity was particulate and 30% cytosolic [26]. Chromatographic fractionation of the cytosol resolved two distinct SPHK activities. Therefore multiple isoforms of SPHK may exist, with different physiological roles and possibly derived from distinct genes.

Transfection of NIH 3T3 cells with SPHK1a resulted in a marked (21-fold) increase in S1P mass, with 67% and 29% decreases in sphingosine and ceramide respectively. However, the net increase in S1P (0.95 pmol/nmol of total phospholipid) did not equate with the observed decrease in ceramide and

sphingosine (which totalled 6.23 pmol/nmol of total phospholipid) [18]. This suggests the concomitant removal of S1P by, for instance, S1P lyase and/or S1P phosphatase and/or efflux.

### S1P lyase

S1P lyase catalyses the cleavage of S1P at the C-2–C-3 bond to produce hexadecanal (palmitaldehyde) and phosphoethanolamine [27,28]. The enzyme requires the coenzyme pyridoxal 5'-phosphate, has a pH optimum of 7.4–7.6 and is inhibited by heavy-metal ions. Activity is specific for the D-erythro isomers (in common with SPHK) of phosphorylated sphingoid bases, including S1P, dihydrosphingosine 1-phosphate and phytosphingosine 1-phosphate, although chain length can vary. The enzyme is ubiquitously expressed with regard to species and mammalian tissues, with the notable exception of platelets, where it is absent. S1P lyase activity was present in cultured human fibroblasts to which [ $^3$ H]dihydroS1P (1  $\mu$ M) was added [29]. [ $^3$ H]DihydroS1P was rapidly taken up by the fibroblasts and converted, in part, into [ $^3$ H]palmitaldehyde, which was further metabolized into glycerophospholipids and alk(en)yl-phospholipids.

S1P lyase was first cloned by D-erythro-sphingosine selection of *Saccharomyces cerevisiae* transformed with yeast genomic library carried on the pRS202 high-copy shuttle vector [30]. The most highly represented insert was subcloned and sequenced to identify a novel gene termed *BST1* (bestower of sphingosine tolerance). *BST1* (since renamed *DPL1*) is a 65.5 kDa protein comprising 589 amino acids, and is 23% identical with *gdaA* and *gadB* of *Escherichia coli*, which encode glutamate decarboxylase, another pyridoxal 5'-phosphate-dependent enzyme that catalyses the synthesis of  $\gamma$ -aminobutyric acid. Deletion of *BST1/DPL1* resulted in increased sensitivity to exogenous D-erythro-sphingosine and phytosphingosine. In addition, the *bst1/dpl1* deletion mutant accumulated S1P upon exposure to exogenous sphingosine.

A mammalian S1P lyase gene has also been identified [31] using a BLAST search of the Genbank EST database for sequence similarity with the S1P lyase gene of *Caenorhabditis elegans*. An EST with an open reading frame of 1707 nucleotides was found which encodes a protein of 568 amino acids and a predicted molecular mass of 63.7 kDa. Hydrophathy analysis indicates that the enzyme contains a single 20-amino-acid transmembrane domain close to the N-terminus. Indeed, the enzyme is located in membranes within mammalian cells. Comparison of the deduced amino acid sequence of the mouse S1P lyase with that of *Saccharomyces cerevisiae* *BST1/DPL1* and *C. elegans* revealed 59%/62% similarity and 40%/39% identity respectively. Furthermore, the mouse gene encodes S1P lyase activity, confirmed by its expression in the *Saccharomyces cerevisiae* *bst1/dpl1* deletion mutant. Under these conditions, the sphingosine-sensitive phenotype was reversed, demonstrating that the mouse gene can complement the yeast defect when expressed. Analysis of S1P lyase mRNA levels was consistent with previously reported S1P lyase activity levels, being most abundant in liver, followed by kidney, lung, heart and brain.

### S1P phosphatase

Several lipid phosphate phosphatases (LPP1/1a/2/3) have been cloned and exhibit a broad substrate specificity which includes S1P, lysophosphatidic acid (LPA), phosphatidic acid (PA) and ceramide 1-phosphate [32]. A mammalian S1P-specific phosphatase has yet to be cloned. However, a S1P phosphatase which

is distinct from the LPP activities has been detected in mammalian cells. Dephosphorylation of S1P to sphingosine has been observed in tissue homogenates [33] and whole cells [29]. For example, human fibroblasts incorporated exogenous [ $^3\text{H}$ ]dihydroS1P (1  $\mu\text{M}$ ) and, in part, dephosphorylated it to [ $^3\text{H}$ ]sphingosine, which was subsequently metabolized to [ $^3\text{H}$ ]ceramide and [ $^3\text{H}$ ]sphingomyelin. The rate of dephosphorylation of S1P by this phosphatase was calculated to be at least the same as that of its degradation by S1P lyase. A later study by the same group identified both high- and low- $K_m$  dihydroS1P phosphatase activities in liver homogenate [34]. The high-affinity enzyme was predominantly associated with the plasma membrane and exhibited a pH optimum of 7.5. Significantly, the activity was distinguished from that of LPP (and other phosphatases) by the differential effects of bivalent cations, chelators, water-soluble and amphiphilic phosphate esters and detergents.

Two specific genes (*YSR2/LBP2* and *YSR1/LBP1*, where *YSR* stands for yeast sphingosine resistance) have been identified in *Saccharomyces cerevisiae* which encode a dihydroS1P/phytoS1P phosphatase [35,36]. Thus a *ysr2* $\Delta$  deletion mutant accumulated dihydroS1P, whereas overexpression of *YSR2* conferred sphingosine resistance to a dihydroS1P lyase-defective mutant, which is hypersensitive to sphingosine [35]. Similarly, deletion of *LBP2* resulted in the accumulation of long-chain sphingoid bases and lowered ceramide levels [36]. Furthermore, *YSR2* and *LBP1* specifically dephosphorylated dihydroS1P/phytoS1P phosphatase and S1P, but not ceramide 1-phosphate or PA [35,36]. Deletion of *LBP1* and *LBP2* dramatically enhanced cell survival upon severe heat shock, suggesting that phosphorylated sphingoid bases modulate stress responses in yeast in a manner similar to that observed in mammalian cells.

## MEASUREMENT OF S1P

A number of methods are available to measure S1P levels in tissue and cell samples. S1P exhibits unusual solubility for a lipid due to its polar headgroup. S1P (and ceramide 1-phosphate) can be selectively separated from other phospholipids and sphingolipids when an alkaline lipid extraction is used. Under these conditions, S1P partitions into the aqueous phase. S1P can be quantified by its chemical modification to  $^3\text{H}$ -labelled ceramide 1-phosphate [37] or by dephosphorylation to sphingosine followed by rephosphorylation (in the presence of [ $\gamma$ - $^{32}\text{P}$ ]ATP) to [ $^{32}\text{P}$ ]S1P [38]. Alternatively, relative changes in the S1P content of cells can be measured by incorporating radiolabelled palmitate, serine or sphingosine into cellular sphingolipids.

Yatomi et al. [37] obtained quantitative measurements of S1P by its extraction from cells and chemical conversion into N- $^3\text{H}$ acetylated S1P (i.e. [ $^3\text{H}$ ]C $_2$ -ceramide 1-phosphate) by N-acylation with [ $^3\text{H}$ ]acetic anhydride. The assay detects between 30 pmol and 10 nmol. Using this technique, S1P was quantified in human platelets, plasma and serum and in all rat tissues examined, being most abundant in testis and intestine (see Table 1; [37–40]). The biological significance of the observed S1P tissue distribution is yet to be determined. However, a correlation exists between the S1P content in the plasma of platelet concentrates, poor platelet increments and the occurrence of transfusion reactions in patients [41].

Alternatively, SPHK preparations can be used to quantify S1P (in the range 0.25 pmol–2.5 nmol) in biological samples [38] (Table 1). S1P of cell extracts, prepared under alkaline conditions, is dephosphorylated with alkaline phosphatase to produce sphingosine, which is then incubated with a SPHK preparation, i.e. the cytosol from cells transfected with cloned mammalian SPHK

**Table 1 S1P and sphingosine levels**

Values are means, means  $\pm$  S.D. or ranges

Species	Plasma/serum	S1P ( $\mu\text{M}$ )	Sphingosine ( $\mu\text{M}$ )	Ref.
Human	Plasma	0.19 $\pm$ 0.08	–	[39]
Human	Serum	0.48 $\pm$ 0.08	–	[39]
Bovine (foetal)	Serum	0.34	0.05–0.1	[38]
Bovine (calf)	Serum	0.11	0.05–0.1	[38]
Bovine (calf)	Serum*	Negligible	0.05–0.1	[38]
Horse	Serum	0.68	0.05–0.1	[38]
Tissue (rat)	S1P (pmol/mg wet wt)	Sphingosine (pmol/mg wet wt)	Ref.	
Testis	98	–	[40]	
Testis	0.5	6.5	[38]	
Spleen	39	–	[40]	
Spleen	2.4	22	[38]	
Brain	37	–	[40]	
Brain	5.8	8	[38]	
Heart	8	–	[40]	
Heart	0.6	6	[38]	
Liver	1	–	[40]	
Liver	0.7	9	[38]	
Kidney	12	–	[40]	
Kidney	0.6	8	[38]	
Eye	2.1	1	[38]	
Intestine	90	–	[40]	
Lung	18	–	[40]	
Muscle	3	–	[40]	
Cell type	S1P (pmol/10 <sup>8</sup> cells)	Sphingosine (pmol/10 <sup>8</sup> cells)	Ref.	
Platelets	141 $\pm$ 4	37 $\pm$ 6	[37]	
Neutrophils	150 $\pm$ 28	–	[39]	
Erythrocytes	7.17 $\pm$ 1.66	–	[39]	
Swiss 3T3	1600 $\pm$ 200	–	[43]	
HL60	111 $\pm$ 10	2500 $\pm$ 280	[38]	
Jurkat	40 $\pm$ 10	610 $\pm$ 60	[38]	
U937	2030 $\pm$ 160	1380 $\pm$ 120	[38]	
C6 glioma	1670 $\pm$ 390	414 $\pm$ 860	[38]	
MCF7	1140 $\pm$ 140	11 000 $\pm$ 570	[38]	
PC12	860 $\pm$ 60	12 100 $\pm$ 710	[38]	

\* Denotes charcoal-stripped

[38]. The cytosol of Swiss 3T3 cells [24] or partially purified enzyme [17,42] can also be used. Chain length and degree of saturation also affect activity, which can be promoted by the inclusion of phospholipids [24].

A comparison between these two methods (Table 1) shows an approximate 10-fold difference in the chemical amounts of S1P detected. The reasons for this are not clear. However, SPHK selectively phosphorylates only *D-erythro*-sphingosine and to a lesser extent *D-erythro*-dihydroS1P, whereas the acylation method would detect other lysolipids containing an amino group that might be present in the alkaline lipid extract.

## AGONIST-STIMULATED S1P FORMATION

A wide variety of stimuli have been shown to increase SPHK activity and elevate S1P levels (Table 2). These include growth factors, GPCR agonists, cytokines, phorbol esters, vitamin D $_3$  and antigen.

PDGF and serum stimulated a rapid activation of SPHK (1.4–1.5-fold at 10–15 min) and transient production of S1P (maximum of 2.2–2.8-fold increase over basal at 3 min) in Swiss

**Table 2 Agonist-stimulated S1P production**

Abbreviations: FMLP, formyl-Met-Leu-Phe; HUVEC, human umbilical-vein endothelial cells.

(a) Acute and transient

Cell type	Stimulus	Ref.
Swiss 3T3	PDGF/foetal calf serum	[43]
Airway smooth muscle	PDGF	[44]
M <sub>2</sub> /M <sub>3</sub> -transfected HEK 293	Carbachol	[45]
RBL-2H3 mast cells	Antigen	[46]
U937	Antigen	[47]
HL60	FMLP	[48]
HUVEC	TNF $\alpha$	[49]
HEK 293	EGF	[51]

(b) Chronic and sustained

Cell type	Stimulus	Ref.
HEL	PMA	[53]
PC12	NGF	[54]
HL60	Vitamin D <sub>3</sub>	[55]
Vascular smooth muscle	Oxidized LDL	[56]
RP11 periosteal cells	Forskolin	[57]

3T3 fibroblasts [43]. PDGF also stimulated transient S1P formation in airway smooth-muscle cells [44]. Similar kinetics and magnitude of response were observed for carbachol stimulation of M<sub>2</sub> and M<sub>3</sub> muscarinic receptors in transfected HEK 293 cells [45], and antigen-stimulated clustering of high-affinity IgE receptors (Fc $\epsilon$ RI) in a rat mast cell line (RBL-2H3) [46] and of IgG receptors in cytokine-primed U937 cells [47], formyl peptide receptor-stimulated HL60 cells [48] and tumour necrosis factor  $\alpha$  (TNF $\alpha$ )-stimulated human umbilical vein endothelial cells [49]. These rapid and transient kinetics are consistent with a second messenger action and/or an autocrine/paracrine role. Epidermal growth factor (EGF) also stimulates SPHK, although this varies between different cell types. For example, EGF is without effect in Swiss 3T3 cells [50] and HEK2 clones, but is effective in a HEK3 clone [51].

The regulatory mechanisms governing SPHK activity have not been fully defined. The presence of calcium/calmodulin-binding domains and PKC phosphorylation sites in the protein suggest that receptor activation linked via phospholipase C (PLC) to inositol 1,4,5-trisphosphate and diacylglycerol formation may represent an acute mechanism for increasing activity of the kinase. Indeed, this appears to be the case for PDGF which has engaged PDGF receptor  $\beta$ . Using various mutants of PDGF receptor  $\beta$ , Olivera and colleagues [52] demonstrated that phenylalanine substitution of tyrosine-1021, which acts as a docking site for PLC $\gamma$ , blocked PDGF-induced activation of SPHK. In contrast, mutation of tyrosine-740/751 and -1009, which are responsible for binding of phosphoinositide 3-kinase (PI3K) and SHP-2 respectively, did not abrogate PDGF-stimulated SPHK activity. Calcium-dependent activation of SPHK was supported by the inhibitory effects of intracellular Ca<sup>2+</sup> chelators, such as BAPTA/AM [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis(acetoxymethyl ester)]. Whilst this supports a model in which Ca<sup>2+</sup> mobilization by PDGF may activate SPHK, other studies suggest that SPHK activation precedes Ca<sup>2+</sup> mobilization, e.g. in Fc $\epsilon$ RI-stimulated rat mast cells [46]. Other signalling pathways have also been implicated in the regulation of SPHK. For instance, in cytokine-primed U937 cells, SPHK activation was shown to be dependent upon the tyrosine kinase-

mediated activation of phospholipase D (PLD) [47]. Furthermore, in HL60 cells, SPHK was stimulated by direct G-protein activation [48]. The mechanism underlying TNF $\alpha$  activation of SPHK remains undefined, but does not require prior sphingomyelinase and ceramidase activities [49].

SPHK can also be regulated chronically (hours/days), e.g. by PMA [53], nerve growth factor (NGF) [54], vitamin D<sub>3</sub> [55] and oxidized low-density lipoprotein (LDL) [56]. PMA-induced increases in SPHK activity in HEL cells were dependent upon both transcription and translation [53] and may be involved in differentiation. For instance, the NGF-stimulated differentiation of PC12 neuronal cells correlates with a 4-fold increase in the  $V_{max}$  of SPHK, an effect which required protein kinase activity and protein synthesis [54]. In HL60 cells, SPHK activation by vitamin D<sub>3</sub> supports a cytoprotective role for S1P [55]. Similarly, cAMP suppression of apoptosis in RP-11 periosteal cells is mediated by activation of SPHK [57]. Stimulation of S1P production by oxidized LDL, which occurs downstream of sphingomyelinase and ceramidase, has also been implicated in smooth-muscle cell proliferation [56].

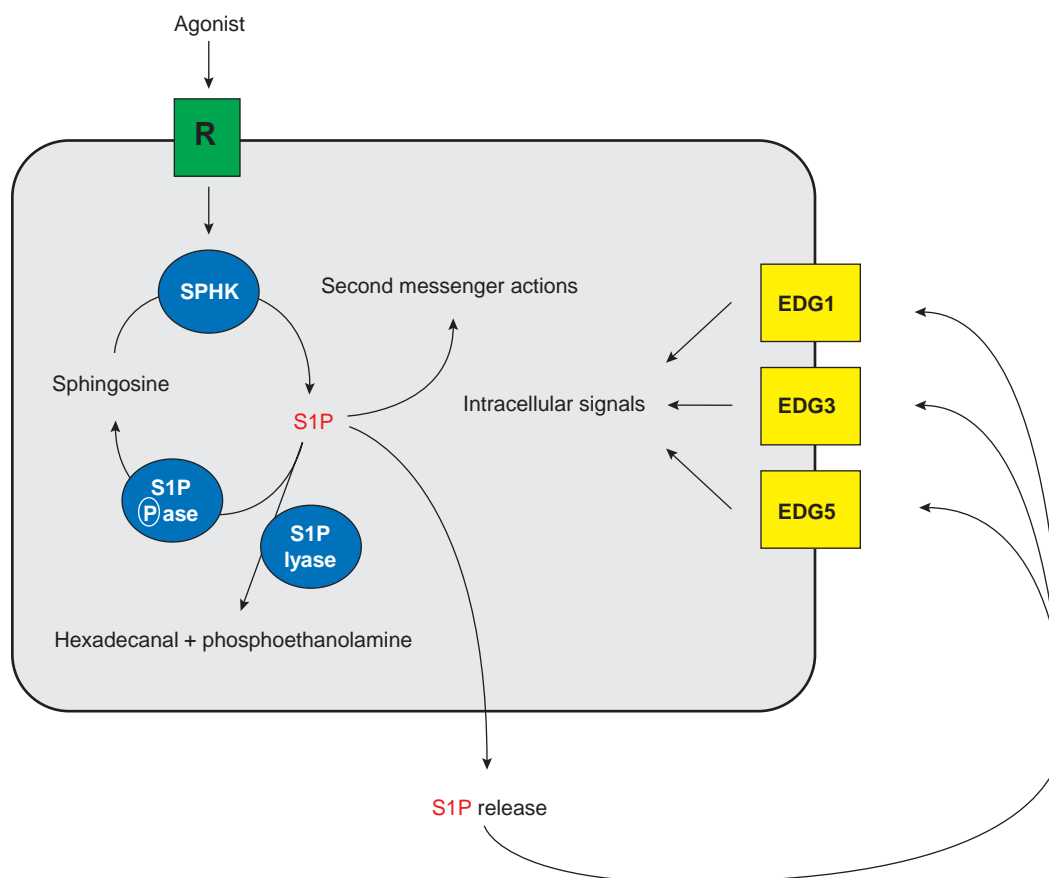
### EXTRACELLULAR VERSUS INTRACELLULAR ACTIONS

A key question is whether S1P formed intracellularly in response to extracellular agonists is released from cells to act on cell-surface GPCRs. In this manner, S1P would act as either an autocrine or a paracrine factor. Alternatively, S1P may function as a second messenger for growth factors, by altering the activity of specific intracellular target proteins (Figure 1). In the following, we have reviewed the evidence for both extracellular and intracellular actions of S1P.

### Identification of GPCRs for S1P

To date, five closely related GPCRs of the EDG (endothelial differentiation gene) family (EDG1, EDG3, EDG5/AGR16/H218, EDG6 and EDG8/*nrg-1*) have been identified as high-affinity S1P receptors [58–64]. They are integral membrane proteins that are probably glycosylated, predicted to have seven transmembrane domains and exhibit approximately 50% amino acid sequence identity.

EDG1 was identified as an immediate-early gene product in phorbol ester-differentiated human umbilical vein endothelial cells, and was suggested to play a role in the morphogenetic differentiation of vascular endothelial cells into capillary-like tubules and in angiogenesis [58]. Amino acid sequence analysis of EDG1 showed that it is a 380-amino-acid protein containing seven hydrophobic transmembrane-spanning domains and significant sequence similarity with the GPCR family [58]. Fusion of the third intracellular loop of EDG1 with glutathione S-transferase polypeptide was used to make an affinity matrix to which members of the G<sub>i</sub> family could bind [65]. Indeed, overexpression of EDG1 in HEK 293 cells led to association with G<sub>i1</sub> and G<sub>i3</sub> polypeptides [65]. Furthermore, EDG1-induced activation of extracellular-signal-regulated kinase (ERK) was blocked by pertussis toxin (which ADP-ribosylates and inactivates G<sub>i</sub>/G<sub>o</sub>) [65]. A high similarity (25%) between EDG1 and the cannabinoid receptor suggested that the EDG1 ligand may be a lipid. This was confirmed in assays of morphogenetic differentiation, which was dependent on EDG1 expression and the presence of serum-borne lipid, S1P. Further characterization studies confirmed EDG1 to be a GPCR with high affinity for S1P (measured  $K_d$  values of 8.1 nM [66] and 13.2 nM [67]). A variety of sphingolipids and lysolipids failed to significantly displace [<sup>32</sup>P]S1P in EDG1-transfected cells [66,67].



**Figure 1** Extracellular versus intracellular role of S1P

S1P produced in response to agonist stimulation may elicit second messenger actions before its removal by S1P phosphatase and/or S1P lyase. In addition, S1P may be released from cells to act in a paracrine and/or autocrine manner at EDG receptors. R, receptor.

Other studies using transfected CHO, HEL, Cos-7 and Sf9 cells have all confirmed that EDG1 functions as a high-affinity S1P receptor which couples to a number of signalling pathways via  $G_i/G_o$  [68,69]. More recently, EDG1-transfected Sf9 cells were used to establish that S1P binding to this receptor in fact activates several members of the G-protein family, including  $G_{i1}$ ,  $G_{i2}$ ,  $G_{i3}$ ,  $G_o$  and  $G_z$ , but not  $G_s$ ,  $G_q$ ,  $G_{12}$  or  $G_{13}$  [70]. Others have also shown that EDG1 receptors can utilize Rho-dependent signalling. For instance, S1P-stimulated morphogenetic differentiation of EDG1-transfected HEK 293 cells was shown by Lee et al. [66] to be  $G_i$ -independent and blocked by the C3 exoenzyme, a specific inhibitor of the small GTPase Rho, which regulates stress fibre formation and focal adhesions [71]. Recent studies have confirmed these findings in T lymphoma cells, where Rho and Rac appear to be involved in S1P-induced migration [72].

EDG1 has also been reported to act as a low-affinity receptor for LPA ( $K_d$  2.3  $\mu$ M), the binding of which induces EDG1 receptor phosphorylation [73]. However, no effects of LPA were evident in membranes of Sf9 cells co-expressing EDG1 and  $G_{i2}$ , whereas S1P was effective [70].

EDG3 and EDG5 also have high affinity for S1P (measured  $K_d$  values of 23 nM and 27 nM respectively [74] and 26.2 nM and 20.4 nM respectively [67]), which is poorly displaced by other sphingolipids and lysolipids, including LPA, sphingosylphosphocholine (SPC), cyclic S1P,  $C_8$ -ceramide 1-phosphate, ceramide and sphingosine [74]. DihydroS1P ( $K_d$  15 nM [75]) and SPC are most effective. The  $K_d$  for S1P binding at EDG6 is 12–19 nM

[62], with SPC exhibiting an approx. 50-fold lower binding affinity, whereas LPA was ineffective. The recently cloned EDG8 has a  $K_d$  of 2 nM for S1P; dihydroS1P was equipotent, whereas SPC was relatively ineffective [63].

EDG3 and EDG5 communicate not only via  $G_i$ , but also through  $G_q$ ,  $G_{12}$  and  $G_{13}$  [76–78]. Interestingly, in transfected Sf9 cells, the expression of EDG3 and EDG5 also produced constitutive activation of  $G_{12}$  and  $G_{13}$  respectively [70]. Several studies are consistent with the use of  $G_q$ ,  $G_{12}$  and  $G_{13}$  by these EDG receptors. Co-expression of a  $G_q/G_i$   $\alpha$  subunit chimaera ( $G\alpha_{qi}$ ) with EDG3 or EDG5 potentiated S1P-stimulated rises in intracellular  $Ca^{2+}$ . Furthermore, co-expression of  $G\alpha_{qi}$  with EDG1 allowed S1P-stimulated coupling to the  $G_q$  pathway [78]. In addition, it was also shown that suramin acts as a subtype-specific functional antagonist of EDG3 ( $IC_{50}$  22  $\mu$ M) [78]. EDG6 couples via  $G_{i/o}$ , whereas a role for  $G_q$ ,  $G_{12/13}$  and/or Rho has yet to be addressed [62]. EDG8 couples via  $G_{i/o}$ , but not through  $G_q$  [63].

As a general paradigm, the role of Rho in EDG receptor signalling has still to be fully defined. For EDG3 and EDG5 receptors, coupling to  $G_{12/13}$  may be the predominant route of Rho activation [79,80]. Indeed,  $G\alpha_{12/13}$  has been shown to initiate RhoGTP regulation of serum response element (SRE)-mediated transcription, cytoskeletal rearrangement, PLD and PI3K [81]. Moreover, RhoGEF, which promotes guanine nucleotide exchange in Rho, is stimulated by  $G_{13}$  [82,83]. The mechanism for this remains to be determined. One possibility is that the

$G\beta\gamma_{13}$  dimer may act as an adaptor for Rho. As mentioned above, EDG1 receptor signalling has also been shown to be Rho-dependent [66], e.g. S1P stimulates morphogenetic changes in EDG1-transfected HEK 293 cells. However, studies by Windh et al. [70] demonstrated that EDG1 does not functionally couple to  $G_{12}$  or  $G_{13}$ . Therefore EDG1 must activate Rho by a different mechanism.

These observations support the conclusion that S1P-specific EDG GPCRs couple productively to different types of G-protein  $\alpha$  subunits,  $\beta\gamma$  subunits and Rho (Figure 2).

A second group of EDG receptors (EDG2, EDG4 and EDG7) have high affinity for LPA (35% identity with EDG1, 3, 5 and 8). EDG2 (or Vzg-1) was the first EDG family member to have its ligand identified. EDG2 overexpression in a neuronal-like cell (TSM) exaggerated LPA-stimulated cytoskeletal changes (Rho-mediated) and inhibition of adenylate cyclase ( $G_i$ -mediated) [84]. A sequence-based search subsequently identified EDG4 [85]. EDG2- and EDG4-transfected HTC4 hepatoma cells (which have a LPA-null background) exhibit LPA-stimulated calcium transients [86], and EDG2 expression in yeast confers LPA responsiveness [87]. EDG7-transfected Sf9 cells exhibit LPA-stimulated calcium mobilization and increased cAMP levels [88]. These effects are similar to those elicited by stimulation of S1P-specific EDG receptors, and suggest that the two lysolipids may signal via common pathways.

### Extracellular GPCR-mediated actions of S1P

Many signalling pathways are activated in response to stimulation of cells by S1P. An S1P-dependent decrease in forskolin- and cholera toxin-stimulated intracellular cAMP levels has been observed in several cell types (see Figure 2). This has been correlated with EDG1 expression [67–69,75,89]. The effect is blocked by pertussis toxin pretreatment and is probably mediated by a typical  $G_i$ /adenylate cyclase interaction. More recently, S1P has been shown to inhibit forskolin-stimulated cAMP formation in EDG3-transfected CHO cells [90] and EDG8-transfected Rh7777 hepatoma cells [63] via a pertussis toxin-sensitive mechanism. In contrast, there are a number of cell types in which stimulation by S1P produces an increase in intracellular cAMP [67,91,92]. For example, S1P-stimulated cAMP formation is pertussis toxin-insensitive in EDG3- and EDG5-transfected CHO cells [67,92]. Since EDG receptors appear not to couple to  $G_s$ , it is probable that increased cAMP synthesis is mediated by cross-talk via the  $G_q$ /PLC signalling pathways. In this case the activation of calcium/calmodulin-sensitive or PKC-stimulated adenylate cyclase isoforms might be involved.

There are a number of studies in which S1P has been shown to activate PLC-catalysed inositol phosphate formation [67,68,91–93]. The EDG1-mediated response was blocked by pertussis toxin [68], whereas that mediated by EDG5 was only reduced by 30% [92]. For EDG1, this effect may be mediated by  $G\beta\gamma_1$  subunit regulation of PLC $\beta$ . More recently, EDG1-, EDG3- and EDG5-transfected CHO cells have been compared [67]. S1P-stimulated inositol phosphate production was observed in EDG3- and EDG5-transfected cells, which in both cases was moderately inhibited but not abolished by pertussis toxin. In contrast, S1P binding to EDG1 did not produce a response [67]. EDG6-transfected cells also exhibit a S1P-stimulated PLC activity that is blocked by pertussis toxin [62]. Consistent with the production of diacylglycerol and  $\text{Ins}(1,4,5)P_3$ -sensitive  $\text{Ca}^{2+}$  mobilization, conventional isoforms of PKC ( $\alpha$  and  $\beta$ ) translocate to the membrane in response to S1P, as does the atypical PKC $\epsilon$  [93]. Regulation of other glycerophospholipid signalling pathways by S1P also appears to be mediated by both  $G_i$ -independent

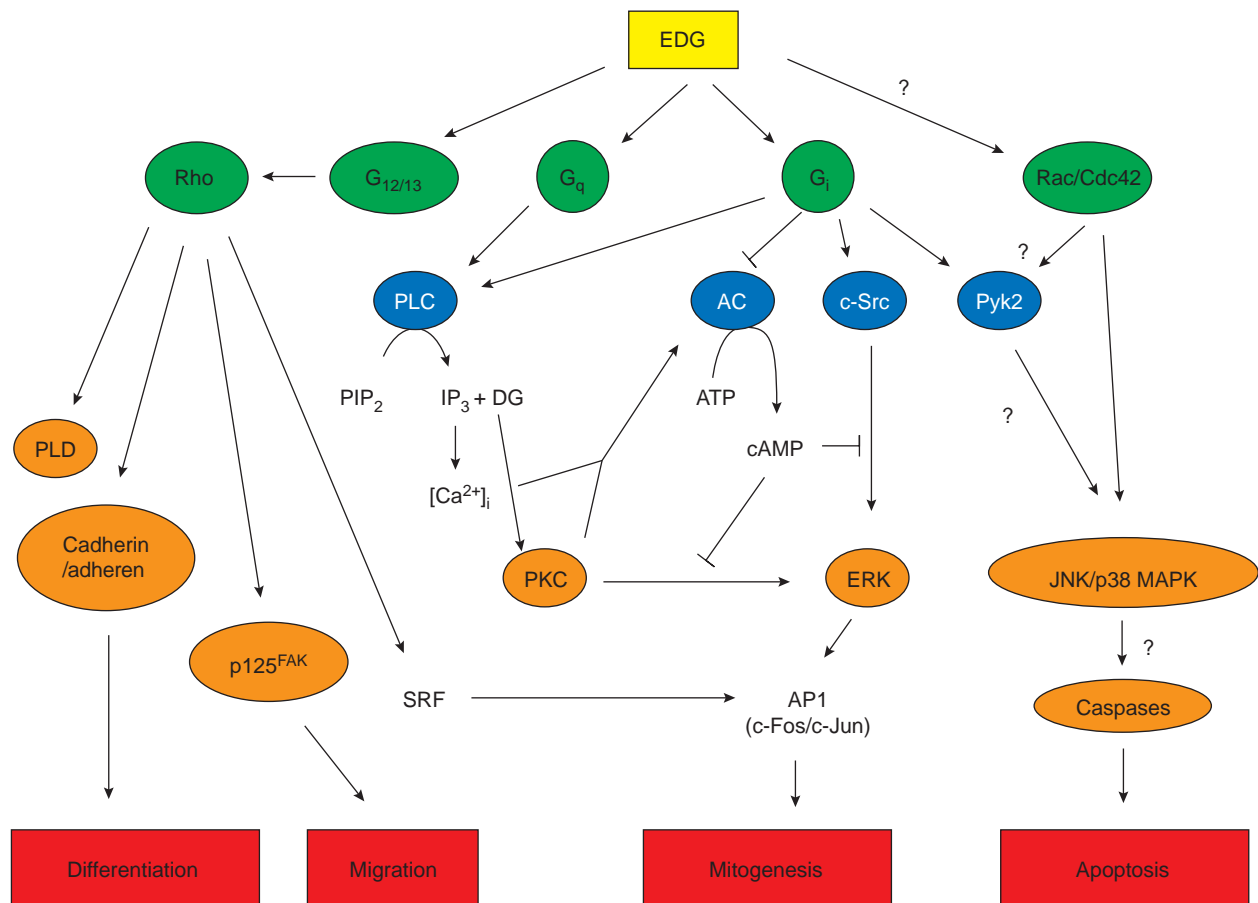
and  $G_i$ -dependent routes. S1P-dependent activation of PLD was reported to be independent of EDG1 expression [75] and insensitive to pertussis toxin [75,93].

Stimulation of  $\text{Ca}^{2+}$  mobilization by S1P ( $\text{EC}_{50}$  1 nM) has also been correlated with EDG1 expression [68]. High concentrations of S1P (10  $\mu\text{M}$ ) were required for  $\text{Ca}^{2+}$  mobilization in EDG1-transfected HEK 293 cells [75] and in the rat mast cell line RBL-2H3 [46]. EDG3- and EDG5-transfected cells also exhibit pertussis toxin-sensitive S1P-stimulated  $\text{Ca}^{2+}$  mobilization (reported  $\text{EC}_{50}$  values of  $\approx$  2 nM and 20 nM respectively [67], and of  $\approx$  3 nM (EDG5) [92]). However, others have shown the  $\text{Ca}^{2+}$  response in EDG3- and EDG5-transfected cells to be largely pertussis toxin-insensitive [90]. In oocytes microinjected with EDG3 and EDG5 mRNA, S1P stimulated  $\text{Ca}^{2+}$  mobilization via a  $G_q$ -dependent mechanism, although both receptors were also shown to couple to  $G_i$  [78]. S1P mobilizes intracellular  $\text{Ca}^{2+}$  in EDG6-transfected CHO cells in a pertussis toxin-sensitive manner [62], whereas S1P stimulation of EDG8-transfected Rh7777 cells failed to mobilize intracellular  $\text{Ca}^{2+}$  [63]. Therefore EDG-receptor-dependent  $\text{Ca}^{2+}$  mobilization appears to be differentially regulated by  $G_i$ - and  $G_q$ -linked pathways.

The S1P-stimulated activation of ERK1/ERK2 has been widely observed. Using cultured airway smooth muscle cells, which express EDG1 but not EDG3, we were one of the first to show that activation of ERK by S1P is pertussis toxin-sensitive [94,95], clearly implicating the involvement of a GPCR acting via  $G_i$  (airway smooth muscle cells do not express  $G_o$ ). Several others have shown that the S1P-stimulated activation of ERK is pertussis toxin-sensitive in EDG1-transfected cells [66,68,69]. Studies on GPCR signalling to ERK have identified the involvement of  $G\beta\gamma_1$  dimers [96,97], which regulate non-receptor tyrosine kinase-catalysed phosphorylation of Shc and the sequential recruitment of Grb-2 and mSos. In airway smooth muscle cells, S1P activates c-Src tyrosine kinase via a pertussis toxin-sensitive mechanism. Furthermore, c-Src inhibitors abrogated the S1P-dependent activation of ERK [89]. Thus S1P signalling via EDG1 may involve the generation of free  $G\beta\gamma_1$  subunits, which may stimulate c-Src to initiate activation of the ERK cascade.

$G\beta\gamma_1$  subunits have also been shown to regulate PI3K, which has been identified as playing a critical role in linking GPCRs to the stimulation of ERK [98,99]. We have shown that the S1P-dependent activation of ERK via EDG1 was inhibited by two structurally dissimilar PI3K inhibitors (LY294002 and wortmannin). Moreover, PI3K activity was present in immunoprecipitates of Grb-2 (an intermediate in the ERK pathway) prepared from S1P-stimulated cells, an effect which was pertussis toxin-sensitive. These findings support a model whereby the EDG1 receptor is coupled via  $G_i$  to Grb-2/PI3K complex-formation [89]. This is similar to observations made in Rat-1 and Cos-7 cells with LPA, which also acts via PI3K [100]. Kranenberg et al. [100] also showed that LPA stimulated the tyrosine phosphorylation of dynamin II, which binds to the C-terminal SH3 domain of Grb-2 in an agonist- and  $G_i$ -dependent manner. PI3K inhibitors prevented LPA-stimulated dynamin II tyrosine phosphorylation and ERK activation. The latter suggests that PI3K regulates an unidentified novel tyrosine kinase which phosphorylates dynamin II. These studies, together with those on S1P, suggest that LPA and S1P might utilize similar signalling pathways to regulate ERK.

The interaction between Grb-2 and PI3K is entirely consistent with studies demonstrating that insulin and insulin-like growth factor-I (IGF-I) receptors stimulate ERK via a PI3K-dependent pathway involving the tyrosine phosphorylation of Gab1 (Grb-2-associated binder-1 protein), which has been shown to activate



**Figure 2 S1P-stimulated signal transduction**

Diagram showing the known signalling pathways activated by S1P binding to EDG receptors regulating mitogenesis, chemotaxis, differentiation and apoptosis. It should be noted that this encompasses all EDG receptors. However, differential coupling to G-proteins and effectors may be apparent for EDG1, EDG3 and EDG5. Levels of cAMP can be increased indirectly via  $G_q$  or reduced via  $G_i$  to limit the mitogenic pathway. However, in some cell types (e.g. Swiss 3T3 cells), cAMP increases mitogenesis. Abbreviations: AC, adenylate cyclase;  $PIP_2$ , PtdIns(4,5) $P_2$ ;  $IP_3$ , Ins(1,4,5) $P_3$ ; DG, diacylglycerol; AP1, activator protein 1.

p85/p110 PI3K1a [101,102]. It remains to be determined whether S1P can induce stimulation of Gab1 tyrosine phosphorylation (c-Src mediated) as a possible mechanism for Grb-2-associated PI3K activation. The stimulation of certain GPCRs (e.g.  $\beta$ -adrenergic) induces binding of  $\beta$ -arrestin I and II to receptors and sequential phosphorylation of the Grb-2 adaptor protein, dynamin II (molecular mass 100 kDa), which mediates clathrin-dependent GPCR internalization by endocytosis [103]. Recent studies have shown that  $\beta$ -arrestin I/II- and dynamin II-mediated internalization of MEK-1 [mitogen-activated protein kinase (MAPK)/ERK kinase] is a mandatory stimulatory signal required for the GPCR-dependent activation of ERK. Thus inhibitors of clathrin-mediated endocytosis or the expression of dominant-negative mutants of  $\beta$ -arrestin I/II or dynamin II abrogate GPCR-stimulated activation of this kinase [104,105]. Significantly, using HEK 293 cells, Lee et al. [66] showed that expression of EDG1 fused with a C-terminal green fluorescent protein was localized primarily on the plasma membrane, but that this construct was internalized upon stimulation with 100 nM S1P for 2 h.

In general terms, the activation of the ERK pathway by S1P shows differential sensitivity to pertussis toxin [106,107]. In C6 cells, the pertussis toxin-insensitive component was abolished by

inhibition of PKC, and was attributed to EDG5 linked to  $G_q$ /PLC. In contrast, S1P-induced ERK activation occurs entirely through a  $G_{i/o}$ -dependent pathway in EDG5-transfected CHO cells [92] and in HTC4 cells transfected with either EDG3 or EDG5 [108]. Interestingly, in the HTC4 cells, the concentration-dependence for S1P activation of ERK differs for EDG3 and EDG5, being maximal at 10 nM and 100 nM respectively [108], suggesting that the efficiency of coupling to  $G_i$  differs.

S1P can activate a number of non-receptor tyrosine kinases in order to transmit signals to well defined effector systems. For example, S1P activates Pyk2, which has been identified as a  $Ca^{2+}$ - and PKC-regulated effector of GPCRs, resulting in the activation of ERKs [106,109]. However, in rat aortic smooth muscle cells, S1P-activated Pyk2 and ERK were not linked, since Pyk2 activation was pertussis toxin-sensitive, while that of ERK was not [106]. Other non-receptor tyrosine kinases which are activated by S1P include Syk [110], Crk [111] and p125<sup>FAK</sup> (where FAK is focal adhesion kinase) [75,112]. Syk requires PKC and transmits signals to Ras, PI3K and PLC $\gamma$ . Crk activation correlates with increased mitogenesis, while p125<sup>FAK</sup> phosphorylation is associated with cytoskeletal changes and formation of focal adhesions. S1P-stimulated p125<sup>FAK</sup> phosphorylation is EDG1-



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LPP1   : 118-KYSIGRLRP--37--SFYSGHS--38--IYVGLSRISDYKHHWSD-227
LPP1a  : 121-KYSIGRLRP--37--SFYSGHS--38--IYVGLSRVSDYKHHWSD-228
LPP2   : 117-KYMIGRLRP--37--SFYSGHA--38--LYVGYTRVSDYKHHWSD-224
LPP3   : 148-KVSIIGRLRP--37--SFFSGHA--38--FYTGLSRVSDHKHHPD-255

LBP-1  : 128-KDYWCLPRP--18--GAPSSHT--36--MTLVFGRIYCGMHGILD-214
LBP-2  : 129-KDYWCLPRP--18--GAPSSHS--36--LTLVFGRVYCGMHGMLD-215

Consensus  KXXhXXXRP--X12-54--XXXSnHn-X31-54--XXXXXnFhXXXXHXXXD

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**Figure 3** Conserved phosphatase motif of lipid phosphate phosphatases and S1P phosphatase

Amino acid sequence alignment of mammalian LPP isoforms (LPP1, LPP1a, LPP2 and LPP3) and *Saccharomyces cerevisiae* LBP1/LBP2 (S1P phosphatases). Fully conserved residues are indicated in red in the consensus sequence. X, any amino acid; h, hydrophobic amino acid; n, neutral amino acid (Ala, Cys, Gly, Ser).

independent [75,112] and is not inhibited by suramin [75] (an EDG3 antagonist [78]), thereby implicating EDG5 (or EDG6) as the probable GPCR involved. Furthermore, S1P-stimulated p125<sup>FAK</sup> phosphorylation was inhibited by C3 exoenzyme, suggesting the involvement of Rho [75,112].

The regulation of PLD by Rho family members, such as Rho, Rac and Cdc42, is well established [113]. Therefore the S1P-dependent activation of PLD might be mediated by these low-molecular-mass G-proteins. Other effector systems which involve Rac and Cdc42 and which are activated by S1P include the stress-activated protein kinases c-Jun N-terminal kinase (JNK) and p38 MAPK, as demonstrated in EDG5-transfected CHO cells [92]. Both p38 MAPK and JNK have been implicated in the regulation of apoptosis.

Gene transcription is also activated in response to stimulation by S1P. For example, S1P stimulates the induction of the AP1 transcription factors c-Jun and c-Fos [114]. This has recently been shown to be mediated by both EDG3 and EDG5 [108]. The induction of other immediate-early genes is mediated by the promoter SRE in a Rho-dependent manner. SRE is activated cooperatively by the transcription factors serum response factor (SRF) and Elk-1 [115,116]. Both of these transcription factors can be activated by S1P via EDG3 or EDG5 [108].

Bunemann et al. [117] have shown activation of the muscarinic K<sup>+</sup> current by S1P in isolated cultured guinea-pig atrial myocytes. Desensitization of the muscarinic receptor did not affect the response to S1P, which was blocked by pertussis toxin. These results were interpreted as reflecting S1P binding to a GPCR that could activate a K<sup>+</sup> channel normally linked to other GPCRs, such as muscarinic receptors. Others have shown that S1P increases the minimum stimulus current needed to induce an action potential in isolated myocytes. S1P reversibly inhibited the peak Na<sup>+</sup> current by 50 pA, while the inward rectifier K<sup>+</sup> current was unaffected [118].

### S1P signal termination at EDG receptors

The termination of EDG receptor signal transduction has not been established. We are focusing on a mechanism that involves the dephosphorylation of S1P to sphingosine. This could be catalysed by S1P phosphatase (an integral membrane protein with seven predicted transmembrane domains) [35] and/or a lipid phosphate phosphatase [LPP1, or type 2 PA phosphatase (PAP2), an integral membrane protein with six transmembrane domains] [32]. S1P phosphatase and members of the LPP

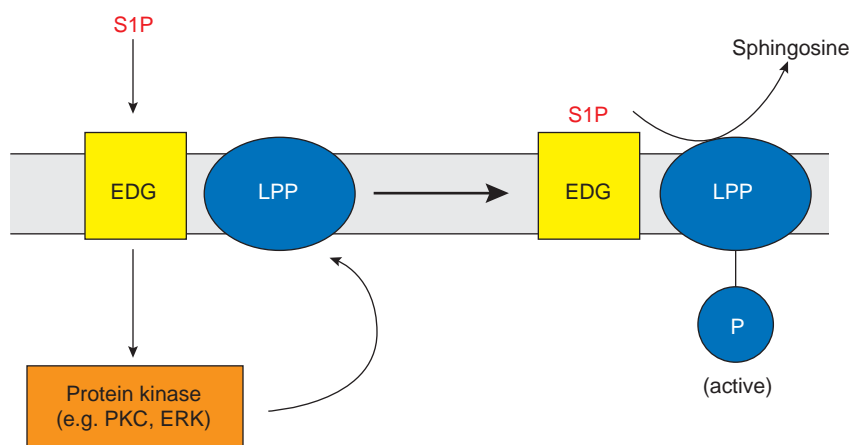
family, including LPP1, contain a novel phosphatase motif [KXXXXXXXXRP(X<sub>12-54</sub>)PSGH(X<sub>31-54</sub>)SRXXXXXXXXHXXXD] which is found in a superfamily of phosphatases [119] (Figure 3). Recently, site-directed mutagenesis has been used to establish that the conserved residues Lys, Arg, Pro, Ser, Gly, His, Arg and His are obligatory for the catalytic activity of LPP1 [120]. Significantly, the LPP1 isoform is located at the plasma membrane, with an externally oriented catalytic site [121]. Thus LPP1 may decrease extracellular S1P levels to limit its bioavailability at EDG receptors (Figure 4). This might require a co-ordinated mechanism that enables S1P to induce 'downstream' signalling responses before LPP1 is activated to reduce the extracellular concentration of S1P. This could involve an intracellular phosphorylation/dephosphorylation mechanism. In this regard LPP1 has putative cytoplasmic-facing phosphorylation sites for ERK, casein kinase, PKC and protein kinase A [122]. Over-expression of LPP1 has been shown to block LPA-stimulated ERK activation and DNA synthesis in Rat-1 cells [121].

### Evidence for an intracellular role for S1P

A second messenger role for S1P was first proposed when a kinase-dependent sphingosine-stimulated release of Ca<sup>2+</sup> from intracellular stores was observed [123,124]. In addition, mitogenesis of Swiss 3T3 cells in response to sphingosine was shown to be mimicked by S1P. The effect of sphingosine was believed to occur through its rapid conversion into S1P [125]. Furthermore, DNA synthesis in Swiss 3T3 cells was increased by microinjection of S1P [75]. Moreover, stimulation of cells with various mitogens elicits the production of S1P (see Table 2). However, many of the functions of S1P previously attributed to a second-messenger role may be due to its action at EDG receptors, especially since a large number of studies invoking intracellular actions involve the application of exogenous S1P to cells (see below; Figure 5).

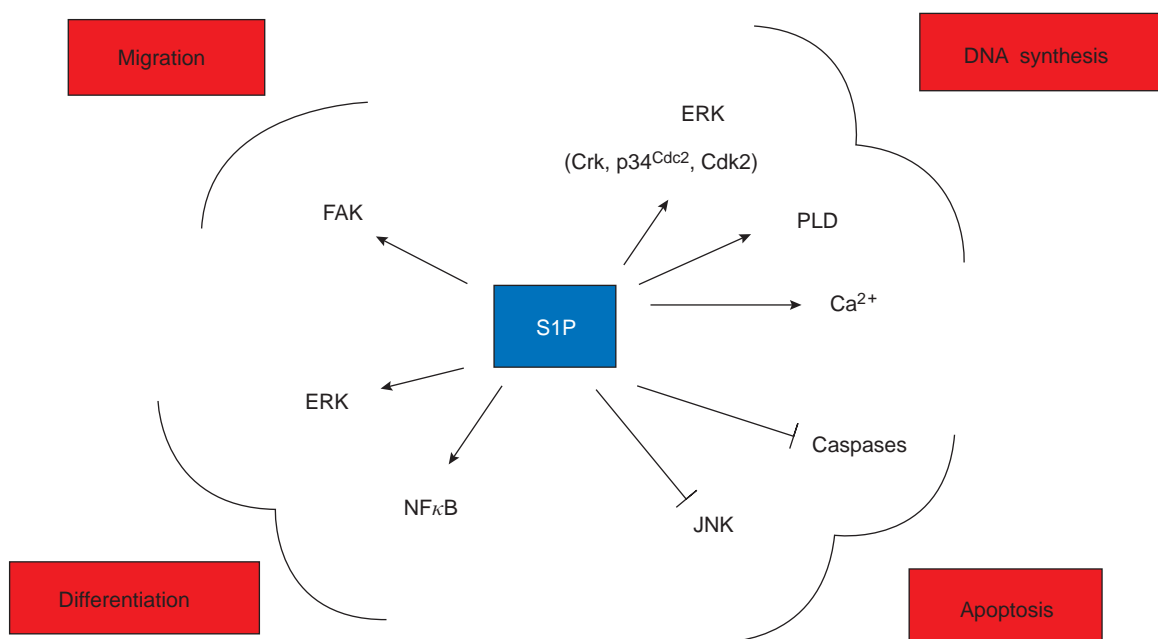
Evidence to support an intracellular role for S1P has been based mainly on the stimulation of SPHK by agonists, the use of SPHK inhibitors and, more recently, transfection of SPHK.

Exogenous-S1P-stimulated activation of the ERK1/2 pathway in Swiss 3T3 cells was observed to be partially (70–80%) inhibited by pertussis toxin [95]. Thus it was proposed that S1P acts both extracellularly (pertussis toxin-sensitive) and intracellularly (pertussis toxin-insensitive) to regulate the ERK1/2 pathway [126]. In hindsight, one cannot exclude a role for G<sub>q</sub>-linked activation of ERK, which would be pertussis toxin-insensitive. In contrast with Swiss 3T3 cells, S1P-stimulated ERK1/2 activity in ASM



**Figure 4** Model for signal termination at EDG receptors

S1P binding to the EDG receptor activates protein kinases which phosphorylate the ecto-lipid phosphate phosphatase (LPP). Activated LPP dephosphorylates extracellular S1P and thereby terminates its signalling and/or limits its bioavailability.



**Figure 5** Possible intracellular actions of S1P

Diagram showing possible intracellular actions of S1P affecting DNA synthesis, apoptosis, differentiation and cell motility. Abbreviation: NF $\kappa$ B, nuclear factor  $\kappa$ B.

cells was completely abrogated by pertussis toxin [94]. In this case, an entirely extracellular action of S1P was corroborated by studies which showed that the addition of sphingosine to cells and its intracellular conversion into S1P failed to support ERK1/2 activation in C6 glioma cells [107]. Thus intracellular increases in S1P do not support activation of this kinase in this case.

More recently, Van Brocklyn and colleagues [75] examined the effects of exogenous S1P in HEK 293 cells transfected with EDG1. S1P-stimulated calcium mobilization, p125<sup>FAK</sup> phosphorylation and activation of PLD were shown to be independent of EDG1 expression. Furthermore, mitogenesis and the prevention of apoptosis were correlated with S1P uptake into

cells, but not with EDG1 expression. These observations were interpreted as evidence for an intracellular role for S1P. However, one cannot exclude the possibility that additional types of S1P receptor are expressed which were not detected in ligand binding assays. In this regard, An et al. [108] have correlated the expression of EDG3 and EDG5 in HTC4 hepatoma cells with S1P-induced proliferation and survival. A receptor-dependent action of S1P was supported by reporter gene assays in EDG3- and EDG5-transfected Jurkat cells. These effects were inhibited by abrogation of G<sub>i/o</sub>- and Rho-dependent pathways [108]. Therefore partial pertussis toxin sensitivity can no longer be presumed to reflect a receptor-independent, intracellular site of action of S1P. However, the intracellular action is supported by

experiments which showed that dihydroS1P (sphinganine 1-phosphate) competes with S1P for binding to EDG1 [75], EDG3 and EDG5 [74], but lacks a cytoprotective effect.

Using Swiss 3T3 fibroblasts, Rani et al. [50] proposed that S1P formed in response to PDGF can function as a second messenger. This was based upon experiments which showed that the SPHK inhibitor *D,L-threo*-dihydrosphingosine abrogated PDGF-stimulated S1P formation and ERK1/2, Crk, p34<sup>cdc2</sup> and Cdk2 kinase activation. We obtained identical results for inhibition of PDGF-stimulated ERK1/2 activity with *D,L-threo*-dihydrosphingosine in cultured ASM cells [127,128]. However, *D,L-threo*-dihydrosphingosine is also a PKC inhibitor [129], and this might represent an alternative route through which ERK1/2 activation is abrogated, especially as PKC is an intermediate in the ERK1/2 cascade activated by PDGF [130,131]. This was further supported by studies which showed that other sphingoid bases, such as *N,N*-dimethylsphingosine and *D,L-erythro*-sphingosine (the substrate of SPHK), which also inhibit PKC [129], blocked ERK1/2 activation with identical concentration-dependence [130]. Thus inhibition by dihydrosphingosine or *N,N*-dimethylsphingosine should not be presumed to be at the level of SPHK alone. In contrast, Edsall et al. [132] reported that, in PC12 cells, 10  $\mu$ M *N,N*-dimethylsphingosine had no effect on PMA- and NGF-stimulated PKC activity or on PMA-induced PKC $\alpha$  and PKC $\delta$  translocation to membranes, despite competitively inhibiting SPHK. However, PKC was assayed using a commercial PKC kit which is not optimized for individual PKC isoforms. Furthermore, the PKC isoforms translocated in response to NGF and the effects of *N,N*-dimethylsphingosine were not established.

*N,N*-Dimethylsphingosine has also been used to implicate S1P as an intracellular signal involved in the inhibition of apoptosis and the promotion of cell survival. For example, Cuvillier et al. [133] showed that activation of JNK and apoptosis of promyelocytic HL60 and U937 monoclonal leukaemia cells induced by TNF $\alpha$ , sphingomyelinase, cell-permeable ceramide or PKC inhibitors was prevented by the addition of exogenous S1P. Similarly, the cell survival effect of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> in HL60 cells is via a mechanism involving activation of SPHK and S1P formation, whereas cytoprotection against ceramide-induced cell death was blocked by *N,N*-dimethylsphingosine [55]. Additionally, S1P inhibits various caspase activities and protects against ceramide- and Fas-induced apoptosis [23,133]. However, the sensitivity of these effects to pertussis toxin and C3 exoenzyme and, therefore, the involvement of EDG receptors was not investigated.

Inhibition of SPHK by dihydrosphingosine ( $K_i$  5–18  $\mu$ M) has also been used to support a second-messenger role for S1P in antigen clustering of Fc $\epsilon$ RI and subsequent intracellular calcium mobilization in a rat mast cell line (RBL-2H3) [46]. In oligodendroglial cells, PDGF-BB stimulates SPHK and induces oscillatory and non-oscillatory calcium signals. The oscillatory signal is due to the action of sphingosine, while the non-oscillatory component is mediated by S1P. Thus dihydrosphingosine was shown to increase the percentage of cells producing Ca<sup>2+</sup> spikes in response to PDGF-BB. This effect was correlated with a decrease in PDGF-stimulated S1P formation and elevated levels of sphingosine [134,135]. Similarly, stimulation of muscarinic M<sub>2</sub> and M<sub>3</sub> receptors expressed in HEK 293 cells also induced calcium signals that were blocked by dihydrosphingosine and *N,N*-dimethylsphingosine [45]. A general role for S1P in Ca<sup>2+</sup> mobilization has been suggested for some, but not all, GPCRs [45]. Furthermore, microinjection of S1P induced rapid, transient Ca<sup>2+</sup> mobilization which was not antagonized by heparin, thereby excluding a role for inositol trisphosphate and its receptor [45]. Similarly, heparin did not block the S1P-induced mobilization of

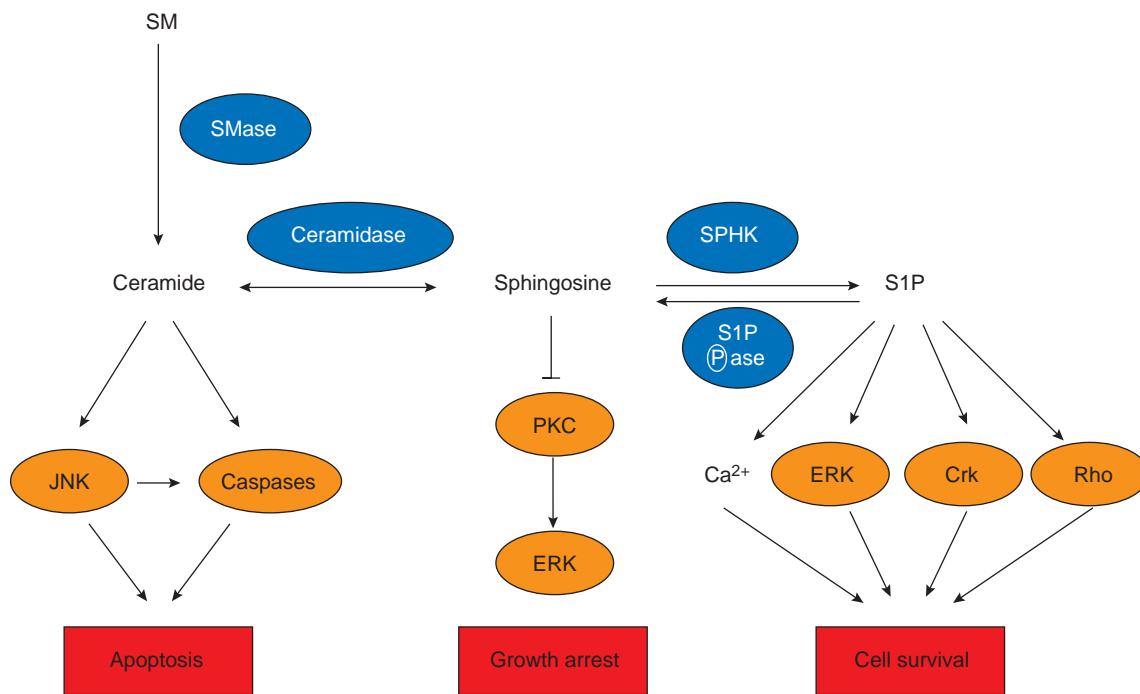
Ca<sup>2+</sup> in a microsomal preparation [124]. However, intracellular release of caged dihydrosphingosine has also been shown to mobilize Ca<sup>2+</sup> from ryanodine-sensitive intracellular stores in cultured neurons [136]. Moreover, SPC also releases microsomal Ca<sup>2+</sup> [124,137], thereby questioning the specificity of the S1P effect. In addition, others have failed to detect S1P-induced mobilization of intracellular calcium [138,139].

Sphingosine-stimulated increases in intracellular Ca<sup>2+</sup> appear to be mediated by PA. This may involve PA species derived from a PLC/diacylglycerol kinase pathway. Thus the diacylglycerol kinase inhibitor R59002 decreased both PA formation and Ca<sup>2+</sup> mobilization [139]. S1P does not appear to activate this pathway, but instead can stimulate the formation of distinct PA species via a PLD route. Thus the effect of sphingosine on calcium mobilization is not due to its conversion into S1P. Other effects of sphingosine include a PKC-dependent action on Ca<sup>2+</sup> dynamics [140] and blockade of ryanodine receptors [141]. Additionally, SPC releases Ca<sup>2+</sup> from isolated sarcoplasmic reticulum in a ryanodine-receptor-dependent manner [142] and activates GPCR-mediated Ca<sup>2+</sup> mobilization [143,144]. The advent of caged sphingolipids, including caged S1P [145,146], should progress this area of research, which remains controversial.

Cloning of SPHK [18] has led to an alternative approach to investigate the intracellular role of S1P. A 500-fold overexpression of SPHK in NIH 3T3 cells and HEK 293 cells resulted in a 4–8-fold increase in S1P levels, and promoted growth in low-serum medium, the G1/S transition, DNA synthesis and increased cell number. Overexpression of SPHK also protected against apoptosis induced by serum deprivation or ceramide in NIH 3T3 and HEK 293 cells, and reduced that in Jurkat T cells stimulated with anti-Fas. The protective effects of SPHK were blocked by *N,N*-dimethylsphingosine [25]. An autocrine role for S1P was not apparent, since S1P was not detected in the extracellular medium. Furthermore, the protective effect of S1P was not blocked by pertussis toxin, thereby eliminating a role for EDG1. However, signalling via pertussis toxin-insensitive pathways involving EDG3 and EDG5 receptors remains a possibility. Indeed, S1P-induced proliferation and survival have recently been demonstrated in EDG3- and EDG5-transfected HTC4 hepatoma cells [108]. Additionally, other effects of SPHK overexpression may play a role, e.g. a concomitant decrease in cellular ceramide levels.

## THE CERAMIDE/S1P RHEOSTAT

The balance between cellular concentrations of ceramide and S1P has been proposed to determine the physiological fate of the cell. Generally, S1P and ceramide elicit opposing cellular fates, e.g. proliferation and survival versus growth arrest and apoptosis. However, it should be noted that there is considerable controversy as to whether ceramide is a physiological mediator of apoptosis [147,148], as most studies have either correlated increased intracellular ceramide with apoptosis or have used exogenous short-chain ceramides (which may have non-specific membrane-perturbing effects). The rheostat model is, therefore, currently based on the assumption that ceramide does indeed induce apoptosis. This is believed to be due to the differential regulation by ceramide and S1P of members of the ERK and stress-activated protein kinase families and caspase proteases [23,44,149]. Importantly, the rheostat model for controlling the ceramide/S1P balance and cell fate does not rely upon whether S1P acts intracellularly or as an EDG receptor agonist (Figure 6).



**Figure 6** The rheostat model

The conversion of ceramide into sphingosine and S1P determines cell fate, based upon data which show that, as a general rule, ceramide induces apoptosis (caspase activation), whereas S1P induces cell survival. Agonists differentially regulate the rheostat enzymes, ceramidase and SPHK. Abbreviation: SM, sphingomyelin.

Cell-permeant ceramides strongly activate JNK in cultured airway smooth muscle cells, but are weak or ineffective with regard to activation of ERK1/2 [44]. Sphingosine inhibits ERK1/2 activation by PDGF, S1P and bradykinin, and is anti-proliferative in airway smooth muscle cells [128]. In contrast, exogenously applied S1P activates the ERK1/2 cascade and is co-mitogenic. Furthermore, ceramide/sphingosine and S1P elicit opposing effects on cAMP levels which might also contribute to their differential outcomes on DNA synthesis [94]. Similar observations have been made in glomerular mesangial cells [149], where cell-permeable ceramides activate JNK and inhibit PDGF-stimulated DNA synthesis. However, sphingosine stimulates ERK1/2 and proliferation. Thus the effect of sphingosine varies between cell types. This may depend on the metabolism of sphingosine, e.g. to S1P in glomerular mesangial cells.

A key piece of evidence to support the rheostat hypothesis is that ceramide-mediated programmed cell death is suppressed by the addition of S1P [23]. Thus ceramide-induced JNK activation and apoptosis were prevented by the addition of S1P. Furthermore, the PKC-mediated activation of SPHK and the concomitant increase in S1P levels were shown to have a similar inhibitory effect on ceramide-mediated apoptosis. Subsequently, Fas ligation or C<sub>2</sub>-ceramide-mediated caspase-3/ CPP32 and caspase-7/ Mch3 activation and resulting poly(ADP-ribose) polymerase cleavage were shown to be blocked by PKC activation and attenuated by the addition of exogenous S1P (0.5–5 μM) [133]. Similarly, either S1P or PKC activation inhibited C<sub>2</sub>-ceramide-mediated caspase-6/ Mch2 activation and subsequent lamin B cleavage. However, neither S1P nor PKC stimulation were effective at blocking Fas-induced caspase-8/ FLICE (Fas-associated death domain-like interleukin-1β converting enzyme) activation. Thus S1P/ PKC activation probably acts downstream of caspase-8/ FLICE activation and ceramide formation, and

upstream of caspases-3 (CPP32), -6 (Mch2) and -7 (Mch3) [133]. Similarly, S1P promotes survival of neuronal cells [150].

Further support for the rheostat model comes from studies showing that growth factors and cytokines tend to differentially regulate sphingomyelinase/ ceramidase and SPHK activities and, consequently, ceramide and S1P levels. For example, alkaline membrane-associated ceramidase is up-regulated (within 1 h) by growth factors, including PDGF, fibroblast growth factor, IGF and EGF, in a tyrosine kinase-dependent manner, with a concomitant increase in sphingomyelinase activity. In contrast, cytokines such as TNFα and interleukin-1 activated sphingomyelinase but not ceramidase activity, thereby resulting in the accumulation of ceramide within cells. Inhibition of ceramidase activity by *N*-oleylethanolamine reduced PDGF-stimulated DNA synthesis, but was without effect upon the proliferative response to endothelin, which failed to modulate ceramidase and sphingomyelinase activities [151].

Furthermore, manipulation of intracellular S1P levels can also affect the levels of ceramide and sphingosine. For example, NIH 3T3 cells transfected with SPHK1a exhibit a substantial decrease in their ceramide content [18]. This correlates with increased survival upon serum depletion, which usually induces apoptosis [25]. Therefore the concomitant removal of ceramide upon SPHK overexpression may prevent ceramide-induced apoptosis. Additionally, the subsequently formed S1P may promote cell survival by inducing mitogenic signalling pathways that counteract the effects of ceramide.

#### CELL PROLIFERATION, DIFFERENTIATION, MIGRATION AND APOPTOSIS

S1P appears to be both a full mitogen and a co-mitogen in different cell systems. For instance, in Swiss 3T3 cells it elicits a

marked increase in DNA synthesis [95], whereas in airway smooth muscle cells S1P is co-mitogenic with PDGF and non-mitogenic alone [89,94]. In Swiss 3T3 cells, S1P-stimulated PLD-derived PA [152] and  $\text{Ca}^{2+}$  mobilization [125] were proposed to be key events in the regulation of DNA synthesis. In contrast, PA failed to mimic the effects of S1P in thyroid FRTL5 cells, thereby excluding a role for PLD. In that study, S1P activated the  $\text{Na}^+/\text{H}^+$  exchanger, which promotes a subsequent increase in intracellular calcium, an important commitment step in the stimulation of DNA synthesis [153].

The mitogenic efficacy of growth factors has been correlated with the magnitude and kinetics of ERK activation [154]. Sustained ERK activation is thought to govern the amount of kinase translocating to the nucleus, the extent of subsequent transcription factor activation and, therefore, the magnitude of DNA synthesis. We have demonstrated that a greater activation of ERK occurs over 30 min in airway smooth muscle cells when S1P and PDGF are applied together than is observed with either agonist alone [89]. These results suggest that the amount of DNA synthesis might be governed by various 'threshold' levels of ERK activation throughout the 30 min stimulation. The extent to which these levels are exceeded at each time point might explain why combined stimulation with PDGF/S1P produced more DNA synthesis than stimulation with PDGF alone. S1P alone produces much weaker ERK activation via EDG1, which is probably below the 'threshold' levels and is therefore non-mitogenic.

Another possible mechanism to explain the co-mitogenic action of S1P is its synergism with choline phosphate. This was observed in NIH 3T3 fibroblasts, where a combination of the two lipids elicited a synergistic increase in DNA synthesis [155]. This was reported to involve amplification at the level of  $\text{p70}^{\text{S6K}}$  and to a lesser extent PI3K, based on the use of rapamycin (inhibitor of  $\text{p70}^{\text{S6K}}$ ) and wortmannin (PI3K inhibitor) and dominant-negative  $\text{p85}$  PI3K, which block synergism. Choline phosphate and S1P also elicited synergistic stimulation of DNA synthesis in Swiss 3T3 fibroblasts, an effect mediated by amplification of ERK activation. In these experiments, high concentrations of ethanolamine also potentiated the synergism on DNA synthesis, via an unidentified mechanism [156]. In contrast, low concentrations of ethanolamine actually inhibited choline phosphate/S1P-induced DNA synthesis [157]. Therefore the action of PLD on phosphatidylethanolamine might result in a bimodal regulation of S1P stimulation of DNA synthesis. In this case, the specificity of action may be dependent upon the extent of PLD activation. S1P can also mobilize intracellular calcium and promote activation of the transcription factor nuclear factor  $\kappa\text{B}$ , which was blocked by BAPTA/AM [158]. Therefore this action of S1P may involve calcium-dependent phosphatases and redox potential changes in the cell, which could affect mitogenesis.

S1P can both stimulate and inhibit chemotactic responses in different cell types. For instance, high concentrations (micromolar) of S1P inhibited, whereas low concentrations (10–100 nM) stimulated, the migration of EDG1-transfected HEK 293 cells [159]. S1P also stimulated the migration of endothelial cells [159] and CHO cells overexpressing EDG1 or EDG3 [67]. In contrast, EDG5-transfected CHO cells were unaffected [67]. S1P also inhibited PDGF-stimulated chemotaxis of aortic smooth muscle cells, via a calcium- and cAMP-dependent mechanism [91]. Furthermore, S1P stimulation of  $\text{p125}^{\text{FAK}}$  may be involved in the inhibition of breast cancer cell motility [160]. Indeed, S1P inhibits integrin-dependent cell motility, by affecting actin filament reorganization. In contrast, formyl-Met-Leu-Phe stimulates SPHK in HL60 cells, and may play a role in promoting chemotaxis of these cells [48].

S1P is also implicated in differentiation processes. For instance, NGF-induced neural differentiation correlates with increased SPHK activity [54]. Both were blocked by the inhibitor K252a. In endothelial cells,  $\text{TNF}\alpha$ -stimulated ERK, nuclear factor  $\kappa\text{B}$  activation and VCAM-1 (vascular cell-adhesion molecule-1)/E-selectin expression were mediated by SPHK activation and S1P production. This was established using *N,N*-dimethylsphingosine, which blocked the effects of  $\text{TNF}\alpha$ . Furthermore, S1P, but not ceramide or sphingosine, mimicked the effect of  $\text{TNF}\alpha$  [161]. Expression of E-selectin and, to a lesser extent, VCAM were also increased by S1P in human aortic endothelial cells [162]. S1P may also affect differentiation processes indirectly by inducing the release of cytokines. For instance, in osteoblast-like MC3T3-E1 cells, S1P appears to be involved in  $\text{TNF}\alpha$ -induced interleukin-6 synthesis [163].

S1P can both promote and protect against apoptosis in mammalian cells. For instance, in human hepatoma cells, S1P up-regulates Bax expression, which is a pro-apoptotic protein belonging to the Bcl-2 family. This was shown to be independent of  $\text{p53}$ -mediated transcription and growth arrest [164]. In neuronal cells, neurite retraction is one of the first steps in the apoptotic process. S1P induced neurite retraction in N1E-115 cells via a GPCR- and Rho-dependent pathway that involves contraction of the actin cytoskeleton [165]. Others have confirmed that the action of S1P is confined to an extracellular receptor, by using S1P immobilized on glass beads that fail to penetrate the cells [166]. Recent studies have shown that S1P-induced neurite retraction involves EDG5 and, possibly, EDG3 [74]. In contrast, agonist stimulation of SPHK protects against apoptosis, as observed in  $\text{TNF}\alpha$ -stimulated endothelial cells [49] and vitamin  $\text{D}_3$ -stimulated HL60 cells [55]. In osteoblastic cells, prostaglandin  $\text{E}_2$  stimulates cAMP formation and inhibits apoptosis. This appears to be mediated by a cAMP-dependent modulation of SPHK and S1P production [57]. Furthermore, protection of T lymphocytes from apoptosis by S1P was associated with suppression of Bax expression via an EDG5- and EDG3-dependent mechanism [167].

## S1P AND DISEASE

S1P may have an important role in a number of disease states. A particular focus is to determine whether EDG receptor levels and signalling via heterotrimeric G-proteins and Rho are altered in disease. A recent study by Goetzl et al. [168] identified EDG4 as a marker for ovarian cancer cells. A similar situation may exist for S1P-specific EDG receptors in other cancers. S1P has also been implicated in breast cancer. For example, S1P induces the secretion of type H IGF (IGF-II), which increases proliferation [169]. In contrast, S1P inhibits chemo-invasiveness of an oestrogen-independent breast cancer cell line, MDA-MB-231, through a  $\text{p125}^{\text{FAK}}$ -dependent pathway [160].

The role of S1P in angiogenesis (the formation of new blood vessels) is another key area. This involves endothelial-cell differentiation and increased motility/mitogenesis of smooth muscle cells, which may be influenced by lipoproteins, such as oxidized LDL [56]. Several reports of the angiogenic role of S1P have been published [159,170,171]. Leucocyte migration is also affected by S1P, which may play a role in inflammation. For example, S1P inhibits neutrophil motility [172] and modulates adhesion molecule expression [162]. Additionally, the relative levels of sphingosine and S1P in mast cells may determine, in part, their allergic responsiveness [173]. Thus changes in the phenotypic expression of EDG receptors, SPHK, S1P lyase and LPPs may have a critical role in governing the intracellular/extracellular concentration of S1P and its efficacy at EDG

receptors, and this may impact upon a number of disease processes.

Essential studies are required to produce gene knockouts, to establish the effect of abolishing the expression of EDG receptors, SPHK and other metabolizing enzymes. Deficiency in enzymes involved in S1P production and action may be associated with defects in important cellular processes, such as neuronal differentiation and apoptosis. It will be interesting to see whether these knockout animals exhibit neurological disorders, such as Alzheimer's disease and Parkinson's disease, which are associated with de-regulated apoptosis.

There are a number of potential approaches in the area of therapeutics. These include the development of selective EDG receptor antagonists, discrete inhibitors of enzymes involved in S1P metabolism and gene therapy. It may be possible to produce drugs or antisense oligonucleotides that can be delivered specifically to tumours to prevent growth and cell motility. Alternatively, it may be possible to overexpress enzymes which destroy S1P, such as LPP and S1P lyase, in order to prevent proliferation.

### FUTURE STUDIES

The biology of S1P has advanced considerably over the past 3–5 years. However, there are many questions still to be answered, promising further exciting and interesting developments in this area of lipid biology.

There are likely to be novel EDG receptors expressed in cells that remain to be identified. Whether these all conform to classical GPCR signalling will be of interest. Certain ligands can bind to both GPCRs and receptor ion channels, e.g. the 5-hydroxytryptamine receptors 5-HT<sub>1</sub>, 5-HT<sub>2</sub>, 5-HT<sub>4</sub> and 5-HT<sub>5</sub> are GPCRs, while 5-HT<sub>3</sub> is a receptor ion channel, more closely related to the nicotinic acetylcholine receptor. It will also be of interest to determine whether there is an intracellular S1P receptor, analogous to that for inositol 1,4,5-trisphosphate. Once the entire complement of receptors has been established, the evolutionary processes that governed their biology can be defined. Identification of members of this family in lower organisms should provide details regarding their divergence and functional heterogeneity. Moreover, the structure/functional properties of identified EDG receptors need to be fully characterized. This will be achieved over the next few years and will involve site-directed mutagenesis studies to define the amino acids involved in binding S1P and the molecular basis for divergent signalling from a single receptor subtype via G<sub>i</sub>, G<sub>q</sub> and G<sub>12</sub>/G<sub>13</sub>. Hopefully, characterization of the functional groups in each receptor will allow rational drug design for the production of receptor antagonists.

### Receptor signalling mechanisms

Although a large number of effector systems that are regulated by the binding of S1P to EDG receptors have been identified, the molecular organization of signalling events downstream of these receptors has not been fully defined. In this regard, a large amount of information can be borrowed from general paradigms of GPCR signalling, and future research will proceed down these lines. There are many questions to be answered. For instance, how do the different EDG receptors activate Rho-dependent signal transduction? Is this via the intermediacy of G<sub>12</sub>/G<sub>13</sub> and RhoGEF? Do EDG receptors communicate with JNK via a Rac/Cdc42-dependent mechanism? Do the G<sub>i</sub>-linked EDG receptors utilize  $\beta\gamma$  subunits to regulate non-receptor tyrosine kinases, and how does this initiate activation of the ERK pathway? What is the role of PI3K in this pathway, and does this

involve endocytic signalling mediated by  $\beta$ -arrestins and dynamin II? Is there potential cross-talk with other growth factor receptors, such as the PDGF receptor? In certain cases, growth factor receptors (e.g. those for insulin, IGF-I and PDGF) appear to utilize G<sub>i</sub> to signal downstream to ERK [101,131]. It would be very interesting to know whether G<sub>i</sub> activated by EDG receptors can be used by growth factor receptor tyrosine kinases. This may allow the PDGF receptor, for example, to signal more efficiently, and could provide the basis for co-mitogenicity. Certain GPCR agonists have also been shown to transactivate growth factor receptors, by stimulating their tyrosine phosphorylation. These tyrosine-phosphorylated receptors can then scaffold with signalling proteins. The best example of this is LPA, which stimulates tyrosine phosphorylation and transactivation of the EGF receptor [174]. It remains to be determined whether S1P stimulates tyrosine phosphorylation and transactivation of growth factor receptors.

Another important question is: what are the factors that enable S1P binding to EDG5 receptors to elicit opposite cellular outcomes, e.g. proliferation versus apoptosis? This may be dependent upon specific localization of EDG5 with the appropriate signalling machinery. Certain, but not all, JNK isoforms have been purported to be upstream of caspases, which play a key role in cell death. Therefore the specificity of signalling from EDG5 might be achieved by co-expression of the relevant JNK isoform in these cells. It is also important to determine whether the EDG5 receptor has functional determinants, distinct from those of other EDG receptors, that allow selective activation of JNK and cell death.

Finally, Rho-dependent kinase activation plays a key role in calcium sensitization of smooth muscle contractility. It remains to be determined whether S1P binding to EDG receptors can elicit contraction of smooth muscle via this Rho-dependent mechanism. This is important because S1P is released by platelets at atherosclerotic plaques, and this might contribute to increased vasoconstriction leading to hypertension in patients with atherosclerosis.

### Intracellular versus autocrine role of S1P

Perhaps the key question is whether S1P can also function as an intracellular second messenger. This is still controversial, because of the uncertainty as to whether S1P is released from cells to act at EDG receptors. Although there are many studies which support a second messenger role, these are based upon the use of inhibitors of SPHK, which at best are only selective for this enzyme. In our opinion, the question of an intracellular role for S1P has to be redefined, especially as unequivocal proof would place S1P in a rather unique position as a signalling molecule with both intracellular and extracellular actions. In this regard, it will be necessary to develop more sensitive assays to measure extracellular S1P (e.g. in the presence of LPP inhibitors), in order to establish whether certain growth factors and cytokines can stimulate its release. The molecular cloning of SPHK offers another approach to addressing this question. Increased S1P levels have been correlated with enhanced cell growth and survival in cells transfected with SPHK. In cells with a null background for EDG receptors, this might be indicative of an intracellular action of S1P. PDGF stimulates SPHK and S1P formation. Therefore, if S1P is released, it can be predicted that PDGF-stimulated signalling might be modified by altered EDG receptor expression. These experiments would distinguish between intracellular and autocrine models. Further information on the role of SPHK in cells might also be obtained by using antisense deoxyoligonucleotides to ablate expression of the protein.

Further studies are required to clone and characterize mammalian forms of SPHK and SIP lyase. These enzymes are likely to have a very significant role in regulating the kinetics of SIP formation. Both intra- and ecto-sphingosine 1-phosphate lyase activities may be expressed in cells, and these might serve as markers for extracellularly and intracellularly acting SIP. A similar paradigm has already been established for the LPPs, which exist as a family of isoforms. Some of these enzymes have ecto-activity against SIP, LPA, PA and ceramide 1-phosphate. As explained above, these ecto-enzymes may limit SIP bio-availability at EDG receptors. The best approach here would be to evaluate the mechanisms regulating ecto-LPP and to establish a structure/function relationship. Such regulatory mechanisms could serve as a feedback route for lowering the extracellular concentration of SIP after the cellular response has occurred.

## CONCLUSION

SIP is an important bioactive lysolipid that appears to have duality in its function, since it may act as both an extracellular and an intracellular mediator. It has profound biological activity, and aberrant regulation is implicated in certain disease states. Recent advances in this area have identified many potential targets for therapeutic intervention, such as EDG receptors and SIP-metabolizing enzymes. This suggests that further research may yield compounds that have clinical benefit.

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