Sphingosine-1-phosphate inhibits actin nucleation and pseudopodium formation to control cell motility of mouse melanoma cells

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Abstract Sphingosine-1-phosphate (Sph-1-P), the initial product of sphingosine (Sph) catabolism, has been reported to inhibit motility of mouse melanoma B16/F1 and other types of cells at very low concentrations (10-100 nM). Sph-1-P (100 nM-1 µM) inhibited pseudopodium formation by blocking polymerization and reorganization of actin filaments in newly formed pseudopodia, and reduced F-actin by ~25% in F1 cells. A pyrene-labeled actin nucleation assay revealed that Sph-1-P (100 nM) inhibits actin nucleation mediated by F1 cell plasma membranes. These results suggest that Sph-1-P interacts with molecules associated with actin nucleation to inhibit reorganization of pseudopodium formation and cell motility.

Key words: Sphingosine-1-phosphate; Actin nucleation; Pseudopodium; Cell motility

1. Introduction

Sphingosine-1-phosphate (Sph-1-P), the initial product of catabolism of Sph by Sph kinase [1], was found to be a strong inhibitor (in the nM range) of conditioned medium (CM) and extracellular matrix (ECM) induced motility [2,3]. The molecular basis of the inhibitory effect was unknown. Several biological functions of Sph-1-P have been reported. Sph-1-P has been shown to be a protein kinase C (PKC)-independent signaling molecule which mobilizes intracellular Ca^{2+} [4,5] and stimulates cellular proliferation [6-9]. Olivera and Spiegel claimed that Sph-1-P is a novel second messenger in platelet-derived growth factor- and serum-dependent cell growth [10]. Sph kinase activity is highly stimulated by phorbol esters in BALB/c 3T3 (A31) cells but not in Swiss 3T3 cells [11]. Here we show that the motility-inhibitory effect of Sph-1-P is based on inhibition of actin nucleation, polymerization, and reorganization of pseudopodium formation.

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Abbreviations: C2-Cer, C2-ceramide (N-acetyl sphingosine); CM, conditioned medium; DMEM, Dulbecco's modified Eagle's medium; DMS, N,N-dimethylsphingosine; DMS-1-P, N,N-dimethylsphingosine-1-phosphate; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; SPC, sphingosylphosphorylcholine; Sph, sphingosine; Sph-1-P, sphingosine-1-phosphate; TMS, N,N,N-trimethylsphingosine; TMS-1-P, N,N-trimethylsphingosine-1-phosphate; TPA, 12-O-tetradecanoylphorbol 13-acetate

2. Materials and methods

2.1. Cell culture

B16 melanoma cell variant F1 was obtained from Dr. I.J. Fidler (M.D. Anderson Cancer Center, University of Texas, Houston, TX). F1 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT), 2 mM L-glutamine, 2 mM pyruvic acid, 4.5 mg/ml D-glucose, 100 U/ml penicillin G and 100 mg/ml streptomycin.

2.2. Sphingolipids

Sph was synthesized from the protected serine aldehyde and lithium alkyl derivative [12,13]. N,N-dimethylsphingosine (DMS) was prepared by reductive amination with HCOOH and $NaBH_4$ [14]. N,N,N-trimethylsphingosine (TMS) [15] and Sph-1-P [16] were synthesized as described previously. N,N-dimethylsphingosine-1-phosphate (DMS-1-P) and N,N,N-trimethylsphingosine-1-phosphate (TMS-1-P) were synthesized [17]. Dihydro-Sph-1-P [15,18] and C2-Cer [19] were synthesized as previously described. Sphingosylphosphorylcholine (SPC) was purchased from Sigma Chemical Co. (St. Louis, MO) and purified by HPLC with $CHCl_3/CH_3OH/NH_4OH$ (3:3:1). These sphingolipids were dissolved in ethanol/water (1:1), and stored at $-20°C$ until required.

2.3. Morphological study

F-actin was stained by FITC-labeled phalloidin after cells were permeabilized by the method previously described [20], with slight modification. Briefly, Lab-Tek cover glass chambers (Nunc, Naperville, IL) were coated with Matrigel (20 mg/ml) at room temperature for 2 h. F1 cells were suspended in DMEM containing 2% FCS or CM at 5×10^4 cells/ml. Test compounds at various concentrations were added to the suspension. 0.5 ml of the suspension was seeded in chambers and allowed to adhere at 37°C. After periods of 2, 3, 4, and 18 h, the cells were observed with phase-contrast microscopy and fixed for 30 min by addition of 0.5 ml of 0.1 M phosphate buffer (pH 7.2) containing 4% paraformaldehyde. The cells were permeabilized with 0.2% Triton X-100 for 5 min and incubated with PBS containing 1% BSA for 30 min. For F-actin staining, the cells were incubated with 0.1 µg/ml FITC-labeled phalloidin (Sigma) for 45 min.

2.4. Quantification of F-actin content

Quantification of F-actin content was performed essentially as previously described [21]. Briefly, 24-well plates (Falcon 8111) were coated with Matrigel (20 µg/ml) in distilled water at room temperature for 2 h. F1 cells (5×10^4/ml) were suspended in DMEM containing 2% FCS or CM, with or without effector lipids. 0.5 ml of the suspension was seeded in the coated well and allowed to adhere at 37°C. After 3 h incubation, the cells were fixed with 2% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 5 min, and stained with BODIPY FL phallacidin (Molecular Probe, Eugene, OR) (5 U/ml) for 45 min. Cells were washed in PBS, and fluorescent dye was extracted from the cells in 0.5 ml of 100% methanol for 30 min. Fluorescence of the methanol extract was measured by fluorometer at 480 nm excitation and 515 nm emission.

2.5. Extraction of plasma membranes

F1 cells were homogenized in a Dounce homogenizer with 20 mM MOPS, 0.2 M sucrose, 10 mM EDTA, pH 7.0 at 4°C, and centrifuged at $500 \times g$ for 10 min at 4°C. The supernatant was centrifuged at
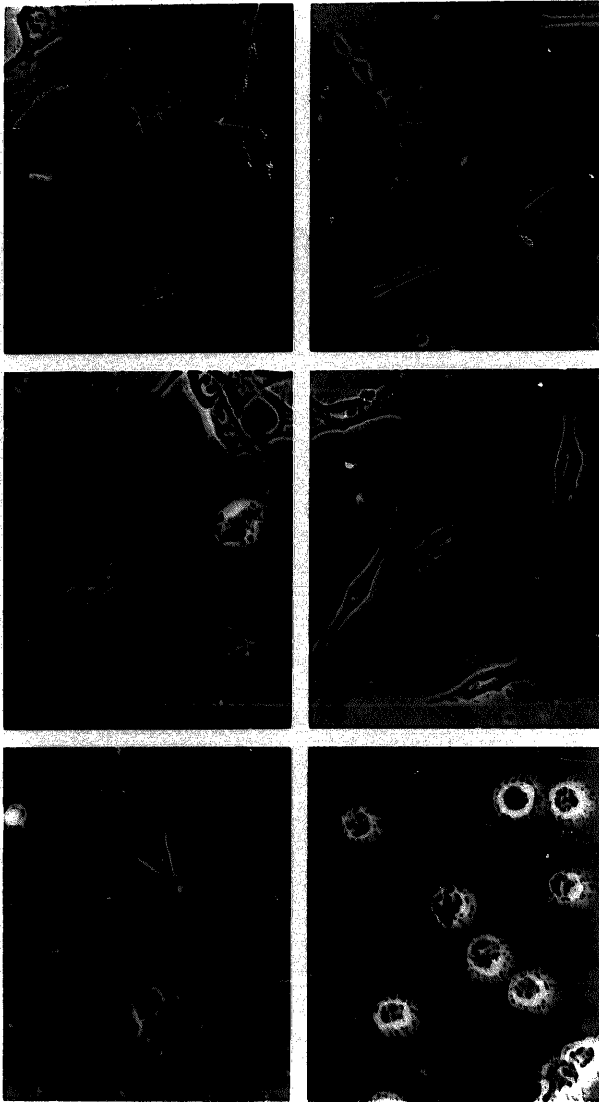
Fig. 1. Morphological changes of F1 cells induced by various test compounds, as observed by phase-contrast microscopy. Cells were incubated for 4 h in glass chambers coated with Matrigel, in the presence of test compounds. (a) CM. (b) CM and 10 nM TPA. (c) CM and 1 μM Sph-1-P. (d) CM, 10 nM TPA and 1 μM Sph-1-P. (e) CM and 1 μg/ml colcemid. (f) CM and 2 μg/ml cytochalasin D. Pseudopodia are indicated by arrows. In the presence of Sph-1-P, F1 cells spread and elongated but developed no pseudopodia. Bar, 40 μm.

$7000 \times g$ for 10 min, followed by at $10000 \times g$ for 1 h at 4°C. The pellet was homogenized in 0.05 M Tris-HCl (pH 7.0) at 4°C, and stored at −80°C [22]. 5'-Nucleotidase activity [23] as a marker for plasma membrane was 9-fold of the activity in the cell homogenate.

2.6. Actin labeling

Pyrene-labeled actin was prepared according to the method of M. Carson et al. [24] with slight modifications. Briefly, actin from rabbit muscle cell (Sigma, St. Louis, MO) in buffer (0.1 mM $CaCl_2$, 0.2 mM ATP, 1 mM $NaHCO_3$, 1 mM dithiothreitol, 0.02% NaN_3, pH 7.6) was polymerized by addition of $MgCl_2$ to 2 mM and KCl to 0.1 M. A 7.5 molar excess of N-(1-pyrenyl)-iodoacetamide (Molecular Probes Inc., Eugene, OR) in dimethylformamide was added immediately after addition of KCl. The reaction mixture was gently rotated in the dark for 24 h at room temperature. The F actin was pelleted by centrifugation at $180000 \times g$ for 2 h at 4°C. The pellet was homogenized in 0.2 mM $CaCl_2$, 0.4 mM ATP, 1 mM dithiothreitol, 0.02% NaN_3, 2 mM

Tris-HCl, pH 8.0, and dialyzed against the same buffer. The homogenate was centrifuged at $180000 \times g$ for 30 min at 4°C, and the labeled actin was obtained in the supernatant.

2.7. Actin nucleation assay

Various concentrations of Sph-1-P in ethanol-H_2O (1:1) were added to the homogenate of plasma membranes from F1 cells, and the sample was preincubated at room temperature for 10 min. The nucleation assay was performed by the addition of buffer (0.4 mM ATP, 25 mM imidazole, pH 7.0, final concentrations) and pyrene-labeled actin to the sample. Fluorescence was measured with an LS-2B filter fluorometer (Perkin-Elmer Corp., Pomona, CA) at wavelengths of 366/407 nm [24,25]. Final concentration of ethanol was <0.25%, which did not affect actin nucleation.
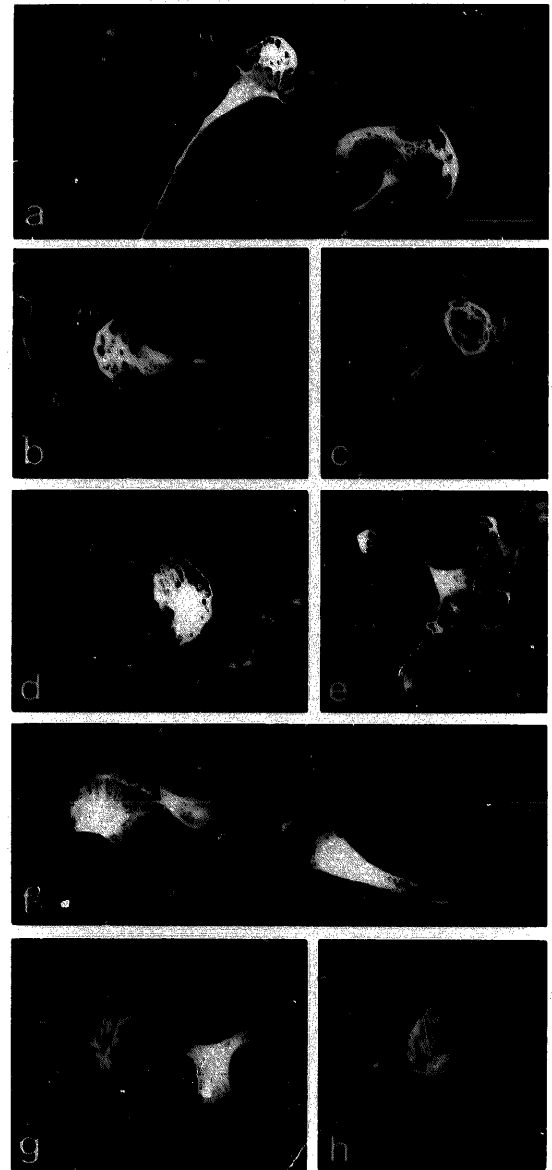

Fig. 2. FITC-phalloidin staining of F1 cells treated with CM, TPA, and/or Sph-1-P. F1 cells were incubated for 4 h, and stained with FITC-phalloidin. (a-c) CM. (d,e) CM and 10 nM TPA. (f,g) CM and 1 μM Sph-1-P. (h) CM, 10 nM TPA and 1 μM Sph-1-P. F-actin filaments are abundant in pseudopodia (lamellipodia), and sometimes form ring-like structures in CM-treated cells. TPA treatment leads to stronger peripheral staining, suggesting active membrane ruffling. Sph-1-P-treated cells show stress fibers but neither ring-like structures nor clear peripheral staining. Bar, 40 μm.
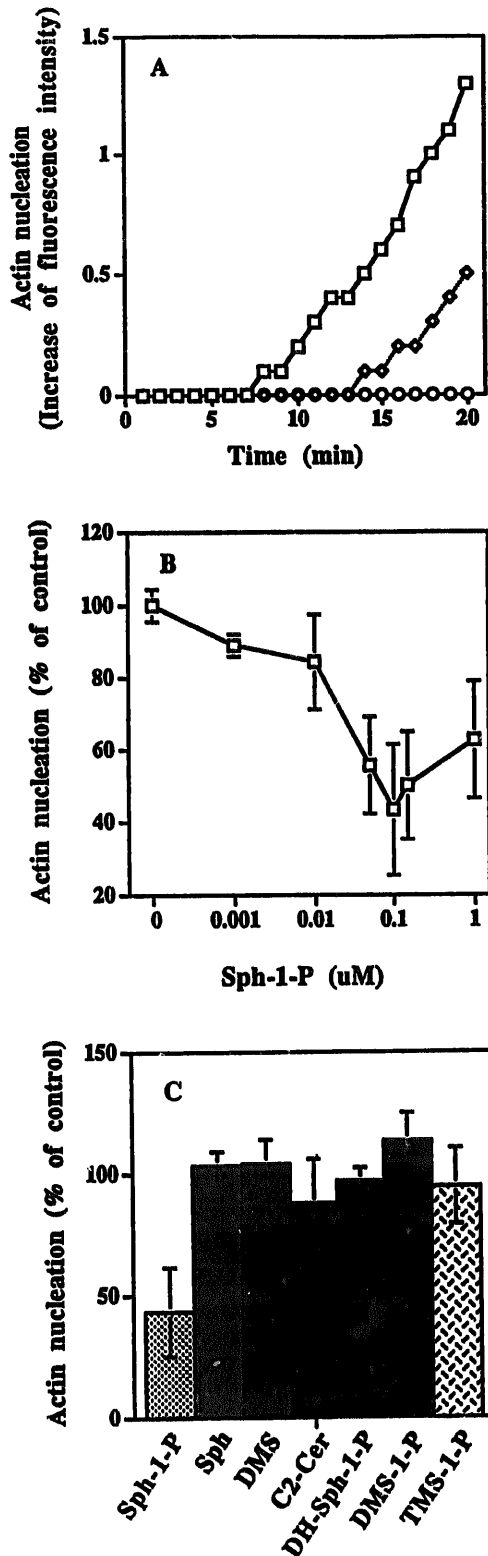
Fig. 3. Effects of Sph-1-P and other sphingolipids on actin nucleation mediated by F1 cell plasma membranes. (A) Time course of actin nucleation. Actin concentration was 8 µM (10% labeled with pyrene), and membrane concentration was 7 µg/ml. □, without Sph-1-P. ◇, 100 nM Sph-1-P. ○, without membranes. (B) Dose dependence of Sph-1-P inhibition of actin nucleation. Concentrations of actin and membranes were the same as those in panel A. Increase of fluorescence intensity at 15 min was compared with that of control (without Sph-1-P). (C) Comparative effects of sphingolipids (100 nM). Concentrations of actin and membranes were the same as those in panel A. Increase of fluorescence intensity at 15 min was compared with that of control (without Sph-1-P). The results are presented as mean ± SE of percent relative to control (n = 3 or 4).

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2.8. Measurement of intracellular Ca²⁺

F1 cells, harvested with brief trypsin-EDTA treatment and resuspended in DMEM with 2% FCS, were added to Lab-Tek cover glass chambers precoated with Matrigel, and cultured overnight. Cultures were washed with PBS and labeled with 3 µM Indo-1/AM (Molecular Probe) at 37°C for 60 min. Cells were washed with PBS supplemented with 1 g/l glucose, rinsed in 0.5 ml of buffer, and subjected to simul-
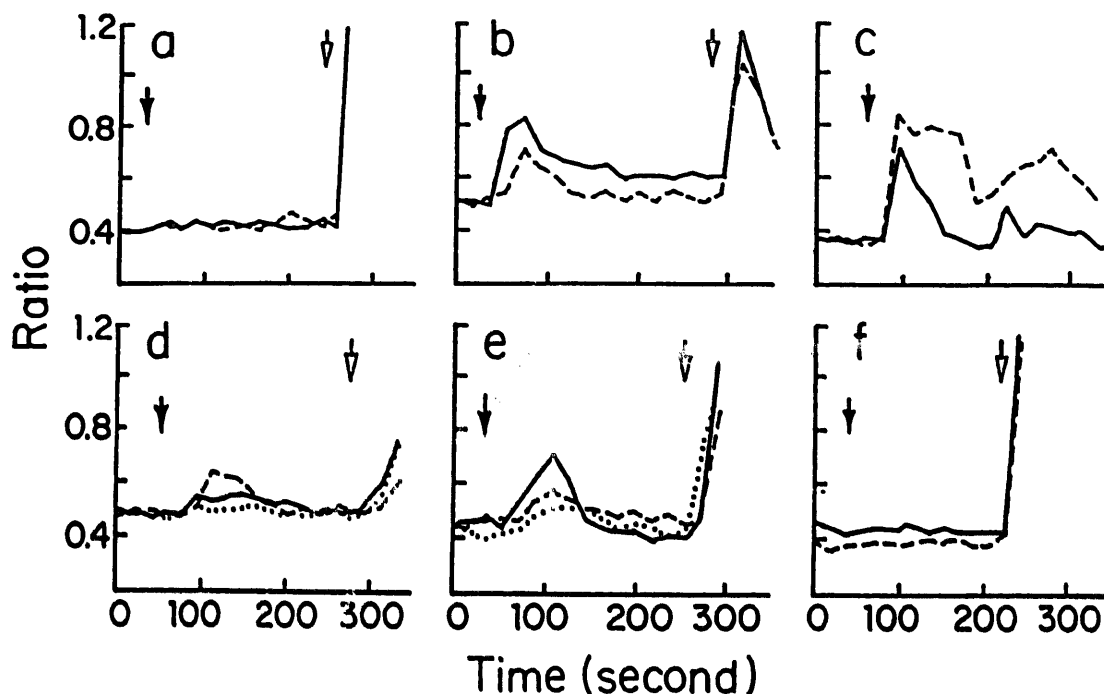
taneous dual wavelength detection imaging with an ACAS 470 Interactive Laser Cytometer (Meridian Instruments, Okemos, MI). Effector lipids were dissolved in the buffer at 37°C, and 0.5 ml of the diluted lipid was added to the chamber. Changes in intracellular Ca^{2+} levels were measured as changes in fluorescence ratio by measuring the fluorescent emission at 405 and 480 nm with two-dimensional scans.

3. Results and discussion

F1 cells on Matrigel-coated plates stimulated by CM produced pseudopodia or flat, extended, 'ruffled' cytoplasmic edges (Fig. 1a). Cell spreading and pseudopodium formation in response to CM and Matrigel were significantly enhanced by addition of TPA (10 nM), which induced elongated, stalk-like processes (Fig. 1b). TMS (1 µM), a strong PKC inhibitor, did not inhibit pseudopodium formation even in the presence of TPA. However, in the presence of Sph-1-P (1 µM), cells kept the elongated shape but exhibited no formation of pseudopodium (Fig. 1c,d). These results strongly agreed with those of cell motility inhibition by these compounds [2,3]. The morphological changes induced by Sph-1-P appeared entirely different from those induced by colcemid and cytochalasin D, in which cell polarization and spreading disappeared due to complete disorganization of the cytoskeletal structures of microtubules (colcemid) and microfilaments (cytochalasin D) (Fig. 1e,f).

The polarization of actin was clearly observed after staining with FITC-labeled phalloidin. FITC-phalloidin staining demonstrated marked F-actin deposition at the active edge of pseudopodia (Fig. 2a–c). Some cells (9–20%) showed a circular arrangement of F-actin filaments (Fig. 2b), similar to the previously reported 'circular membrane ruffling' of fibroblasts exposed to platelet-derived growth factor [26]. Thin stress fibers were also seen along the direction of cell strength (Fig. 2a,c). F-actin deposition with specific organization at the edges of pseudopodia was enhanced by addition of TPA (Fig. 2d,e). Sph-1-P-treated cells exhibited stress fibers extending in various directions, but showed neither F-actin deposition at the cell periphery nor circular staining patterns (Fig. 2f–h). Quantitative determination of F-actin in F1 cells revealed a 24% decrease (SE = 9.8%, n = 3) in BODIPY-phallacidin staining after Sph-1-P treatment. This phenomenon was reversible, i.e. subtraction of Sph-1-P from culture medium led to reappearance of actin rings and pseudopodia and restoration of motility.

To test whether Sph-1-P inhibits in vitro actin nucleation, we used a fluorescence assay with pyrene-labeled actin [24,25]. In this assay, fluorescence intensity increases as actin nuclea-

Fig. 4. Intracellular changes of Ca^{2+} levels in F1 cells in the presence of Sph-1-P and related compounds. (a) 1 μM Sph-1-P. (b) 5 μM Sph-1-P. (c) 10 μM Sph-1-P. (d) 40 μM dihydro-Sph-1-P. (e) 40 μM SPC. (f) 20 μM C2-Cer. The test compound was added at the time indicated by solid arrows. 1 μM ionomycin (a calcium ionophore) was added at the time indicated by open arrows.

tion proceeds. Fig. 3A shows time course of actin nucleation mediated by F1 cell plasma membranes. No actin nucleation was observed without F1 cell plasma membranes, but with the plasma membranes, actin nucleation was observed as reported for plasma membranes from Dictyostelium discoideum [27,28]. The onset of nucleation was characterized by a lag phase which may result from the slow processes of monomer activation [29]. Sph-1-P at 100 nM inhibited actin nucleation mediated by F1 cell plasma membranes. Sph-1-P delayed initiation of the nucleation: in the presence of 100 nM Sph-1-P, the nucleation started at 13 min (compared to 7 min in the absence of Sph-1-P) (Fig. 3A). This inhibition of the nucleation was dose-dependent (Fig. 3B). At the maximal inhibitory concentration of 100 nM, Sph-1-P reduced the nucleation 43 ± 18% (n = 3) at 15 min. Likewise, Sph-1-P had an inhibitory effect on cell motility at 10 nM-1 μM concentrations. The inhibitory effect of Sph-1-P was compared with those of structurally-related Sph derivatives (Fig. 3C), however, none of these compounds inhibited the actin nucleation.

Intracellular Ca^{2+} release from F1 cells was induced by 5 mM Sph-1-P, but was not affected by 0.01–0.1 μM Sph-1-P, the concentration range in which cell motility was strongly inhibited, suggesting that the motility-inhibitory effect of Sph-1-P may not be due to changes of intracellular Ca^{2+} mobilization, although further studies are necessary. Dihydro-Sph-1-P and SPC induced Ca^{2+} mobilization at 40 μM, but not at lower concentrations. C2-ceramide induced no Ca^{2+} mobilization (Fig. 4).

Cell motility is an important component for development, immunity, metastasis and wound healing, but the molecular mechanisms and signaling pathways which mediate cell motility are largely unknown. The cellular response to chemotactic factors is attributed to a dynamic cycle of actin assembly and

disassembly causing reconstruction of the actin network in leading lamellae under control of transmembrane signals [30]. Chemotactic factors trigger the formation of actin nucleation sites through receptors on the cell surface. The rapid response of actin nucleation suggests that plasma membranes are involved in the signal transduction, but the intracellular site of the actin nucleation is unknown. A fluorescence assay with pyrene-labeled actin demonstrated that F1 cell plasma membranes are necessary for actin nucleation which can be inhibited by Sph-1-P. Because Sph-1-P did not interact with actin directly (Yamamura, Hakomori and Igarashi, unpublished), these results indicate that Sph-1-P in the nM range interacts with actin nucleation sites or receptors on the plasma membranes or other molecules which regulate actin nucleation, resulting in inhibition of actin nucleation, polymerization and reorganization of pseudopodium formation, thereby controlling cell motility.

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