Sphingosine 1-Phosphate Regulates Melanoma Cell Motility through a Receptor-Coupled Extracellular Action and in a Pertussis Toxin-Insensitive Manner†

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ABSTRACT: Our previous work showed that sphingosine 1-phosphate (Sph-1-P) inhibits the cell motility of mouse melanoma B16/F10, and other types of cells at 10−100 nM concentrations. In the present paper, we have identified and characterized specific cell surface binding sites for Sph-1-P in F10 cells. Sph-1-P immobilized on controlled pore glass beads inhibited the motility of F10 cells, suggesting that Sph-1-P acts on the cells from the outside. Binding assays with [^{3}H]Sph-1-P revealed the presence of specific cell surface binding sites for Sph-1-P in F10 cells. Scatchard analysis demonstrated a single class of binding sites for Sph-1-P. The binding of [^{3}H]Sph-1-P to F10 cells was inhibited by the addition of excess unlabeled Sph-1-P but not other natural sphingolipids. The specific binding was also sensitive to treatment with a protease. Using Sph-1-P-immobilized affinity chromatography, we, for the first time, identified 41-kDa and 79-kDa Sph-1-P binding proteins on the melanoma cell surface, although the 41-kDa protein was less specific to Sph-1-P. We demonstrated that pertussis toxin (PTX) treatment did not abolish the motility inhibition by Sph-1-P, suggesting that no PTX-sensitive G-protein is involved in the signaling. Furthermore, Sph-1-P was found to be specifically released from mouse BALB/3T3 clone A31 cells and F10 cells. Collectively, these results strongly suggest that Sph-1-P regulates melanoma cell motility through an extracellular action by specific binding to cell surface receptor protein(s), which is independent of PTX-sensitive G-protein.

Many metabolites of lipids have been demonstrated to function as second messengers or modulators in signal transduction (*1*). Sphingolipid-derived metabolites such as ceramide (Cer)[1] and sphingosine (Sph) have been demonstrated to be lipid second messengers of transmembrane signaling (*2*−*4*). Sphingosine 1-phosphate (Sph-1-P) is the initial product of Sph catabolism by Sph kinase (*5*), and is cleaved by Sph-1-P lyase to ethanolamine phosphate and palmitaldehyde (*6*). Sph-1-P has also been reported to be a lipid second messenger. It stimulates growth of quiescent Swiss 3T3 fibroblasts by a pathway which is independent of protein kinase C (PKC) (*7*, *8*), and some of its biological functions have been reported to be associated with mitogenic effects: it enhances cytoplasmic Ca^{2+} release (*9*−*11*), stimulates phospholipase D activity (*12*), and enhances DNA binding activity of AP-1 (*13*). These mitogenic signaling pathways involved the pertussis toxin (PTX)-sensitive guanine nucleotide binding protein (G-protein) (*14*, *15*). On the other hand, sphingosine kinase, which produces Sph-1-P in the cells, was found to be regulated by platelet-derived growth factor (PDGF) in Swiss 3T3 cells (*16*) and by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in BALB/3T3 clone A31 cells (*17*), and to be associated with signaling by the FcεRI antigen receptor (*18*). Also, we recently found that Sph-1-P is a platelet-activating lipid which is released from activated platelets (*19*, *20*).

Furthermore, we found that Sph-1-P inhibits the chemotactic motility of mouse melanoma B16, mouse fibroblast BALB/3T3 clone A31, and other types of cells at very low concentrations (10−100 nM) (*21*, *22*). No other Sph-related compounds are known to inhibit cell motility. Sph-1-P does not affect cell proliferation in mouse melanoma B16 or human arterial smooth muscle cells. In smooth muscle cells, Sph-1-P alters phosphatidylinositol turnover, Ca^{2+} mobilization, and actin filament disassembly, and also inhibits cell motility (*22*). Sph-1-P inhibits integrin-dependent motility of mouse melanoma B16 cells, but it does not reduce integrin-dependent adhesion to the extracellular matrix (*23*). Sph-1-P inhibits actin nucleation and pseudopodium formation in B16 cells (*24*). However, a more precise mechanism

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[1] Abbreviations: BCS, bovine calf serum; BSA, bovine serum albumin; C2-Cer, C2-ceramide (*N*-acetylsphingosine); CM, conditioned medium; CPG, controlled pore glass beads; DMEM, Dulbecco's modified Eagle's medium; DMS, *N*,*N*-dimethylsphingosine; DMS-1-P, *N*,*N*-dimethylsphingosine 1-phosphate; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; LPA, lysophosphatidic acid; PBS, phosphate-buffered saline; PTX, pertussis toxin; SPC, sphingosylphosphocholine; Sph, sphingosine; Sph-1-P, sphingosine 1-phosphate; TLC, thin-layer chromatography; TMS, *N*,*N*,*N*-trimethylsphingosine; TMS-1-P, *N*,*N*,*N*-trimethylsphingosine 1-phosphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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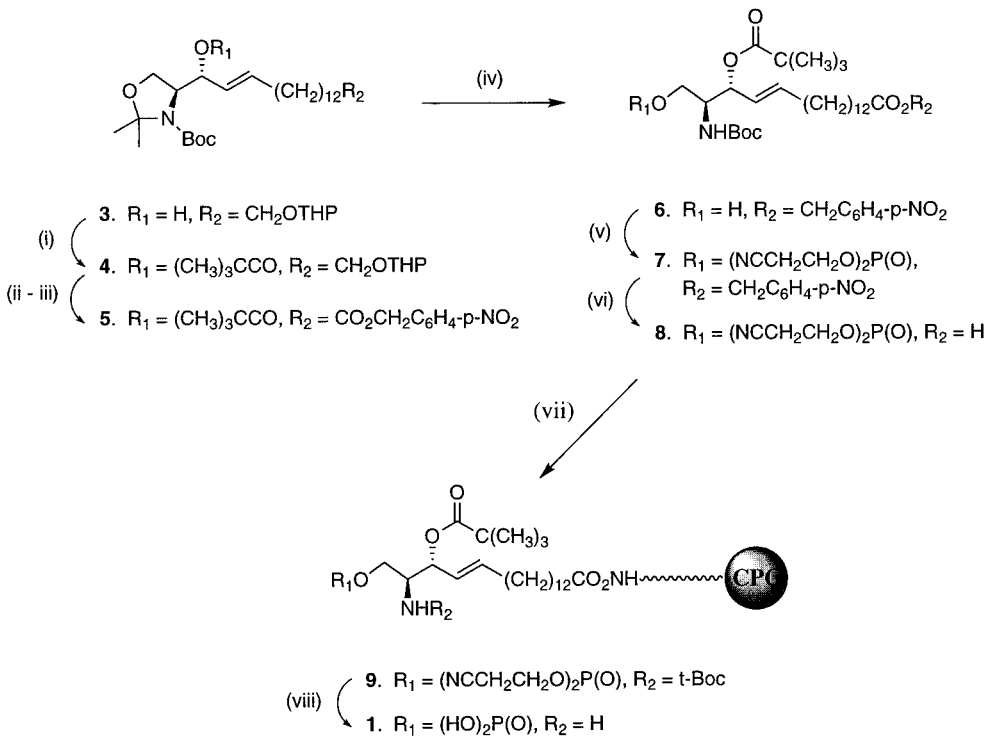
Scheme 1[a]



[a] Reagents: (i) pivaloyl chloride, pyridine; (ii) Jones reagent, acetone; (iii) p-$NO_2C_6H_4CH_2Cl$, NaI, $NaHCO_3$, DMF; (iv) p-TsOH, CH_3OH; (v) (a) $(NCCH_2CH_2O)_2PN(i\text{-}Pr)_2$, tetrazole, CH_2Cl_2, (b) MCPBA, CH_2Cl_2; (vi) zinc dust, HOAc/H_2O (9:1); (vii) (a) CPG, HOBt, DIC, CH_2Cl_2, DMF, (b) HOAc; (viii) (a) NaOH, EtOH, dioxane, (b) TFA, CH_2Cl_2.

of inhibition of cell motility is unclear. Here, we identify and characterize specific cell surface binding sites for Sph-1-P, demonstrating that Sph-1-P may act through a binding protein (receptor) in the cell motility regulation. PTX did not abolish the inhibitory effect of Sph-1-P on cell motility, suggesting that the signaling pathways involve no PTX-sensitive G-protein. Sph-1-P itself is found to be released into the culture medium from the cells upon serum stimulation, suggesting further that Sph-1-P can act as an intercellular modulator or messenger in the regulation of cell motility.

MATERIALS AND METHODS

Materials. Sph-1-P was synthesized chemically (*23*). Sph, *N*,*N*-dimethylsphingosine (DMS), and *N*,*N*,*N*-trimethylsphingosine (TMS) were synthesized as previously described (*25*, *26*). C2-ceramide (C2-Cer) was synthesized according to the literature (*27*). DMS-1-P and TMS-1-P were synthesized in our lab.[2] These lipids were dissolved in ethanol/water (1:1), and control experiments were performed using ethanol/water (1:1) as vehicle. [3-^{3}H]Sph (22.0 Ci/mmol) was purchased from DuPont−New England Nuclear (Boston, MA). [3-^{3}H]Sph-1-P was prepared enzymatically from [3-^{3}H]Sph with crude Sph kinase obtained from BALB/3T3 clone A31 cells (*17*). IODO-BEADS were purchased from Pierce (Rockford, IL). Suramin and [^{32}P]NAD were obtained from ICN Pharmaceuticals (Costa Mesa, CA). All other reagents were purchased from Sigma (St. Louis, MO).

Cell Culture. Mouse melanoma B16/F10 cells were obtained from Dr. I. J. Fidler (M. D. Anderson Cancer Center, University of Texas, Houston, TX) and cultured in DMEM supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT), 2 mM L-glutamine, 110 mg/L pyruvic acid, 4.5 mg/mL D-glucose, 100 units/mL penicillin G, and 100 μg/mL streptomycin. Mouse BALB/3T3 clone A31 cells were obtained from the American Type Culture Collection and cultured in the same medium, except that bovine calf serum (BCS) (HyClone, Logan, UT) was used.

Preparation and Application of Sph-1-P and Sph Immobilized on Controlled Pore Glass Beads. The designed structure of conjugates **1** and **2** (Figure 1) is the ω-carboxyl group of the sphingolipid moiety covalently linked to the surface of controlled pore glass beads (CPG Inc., Fairfield, NJ) through a long-chain alkyl amine. The synthesis of **1** is summarized in Scheme 1. Basically, the allylic alcohol **3**, readily obtained by our approach (*28*), was protected to afford **4**. Treatment of **4** with the Jones reagent, followed by esterification, yielded **5**. Sequential selective cleavage of the acetal moiety in **5**, phosphorylation of the resulting primary hydroxyl group of **6**, and reductive removal of the *p*-nitrobenzyl group provided the desired protected ω-carboxyl Sph-1-P, **8**. The condensation of the carboxylic acid **8** to the amino groups of CPG followed by capping of the remaining free amino groups gave **9**. Final deprotection of **9** afforded the desired conjugate **1**. The preparation of **2** was accomplished as previously described (*28*). Quantitative Kaiser assay indicated that the substitution level of **1** and **2** used in our studies was 22 and 39 μmol/g, respectively. Direct acetylation of the amino groups on the CPG particles gave the control sample for this studies.

Cells were briefly trypsinized and washed, and the glass beads were added into the cell suspensions. The cells were cultured with the glass beads for 2 days. The viability of

[2] F. Ruan, S. Hakomori, and Y. Igarashi, unpublished data.

cells which adhered to the glass beads was checked by the trypan blue exclusion assay. The interaction between cells and the glass beads was studied with scanning electron microscopy (*19*) or light microscopy.

Cell Motility Assay and Ca^{2+} Mobilization. Haptotactic cell motility of F10 cells was assayed using Transwell chambers (Costar, Cambridge, MA) with 6.5 mm diameter polycarbonate filters (8 μm pore size) coated with human plasma fibronectin (5 μg/filter) (*23*). Briefly, the lower surface of the filter was coated with human plasma fibronectin (5 μg/filter) and dried at 37 °C overnight. The cells were harvested after brief exposure to 0.02% EDTA in PBS (Irvine Scientific, Santa Ana, CA), and resuspended to a concentration of 4×10^5 cells/mL in DMEM supplemented with 0.25% BSA. Cells (100 μL of the suspension) were seeded in the upper compartment. DMEM (0.6 mL) with 0.25% bovine serum albumin (BSA) was placed in the lower chamber, which was then connected to the upper chamber. After incubation for 18 h (unless otherwise indicated) at 37 °C, cells on the lower surface of the filter were stained and counted.

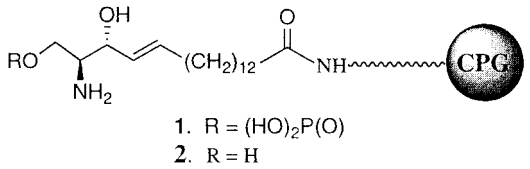
Intracellular Ca^{2+} mobilization was measured using the Ca^{2+}-sensitive fluorophore fura2 as described (*19*).

Binding Assay. Binding of Sph-1-P to the cell surface was assayed as described previously (*29*) with modifications. Briefly, F10 cells were harvested with 0.02% EDTA in PBS. Cells were resuspended (1.2×10^6 or 4×10^5 cells/mL) in DMEM and incubated with [3-^{3}H]Sph-1-P at 4 °C for the indicated time. The cells were washed 3 times with 1 mL of 2 mg/mL BSA, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5. Radioactivity of the cells was counted with a scintillation counter. The amount of specifically bound [3-^{3}H]Sph-1-P was determined by subtracting the radioactivity bound in the presence of unlabeled Sph-1-P (50 μM) from the radioactivity bound in the absence of unlabeled Sph-1-P. Scatchard analysis was conducted according to the literature (*30*). For protease experiments, F10 cells (1×10^7 cells) were incubated at room temperature for 10 min in 5 mL of PBS containing 0.02% of protease (type XXV), and binding assays were performed as described above.

Preparation of Cell Membranes. F10 cells were washed twice with PBS and scraped into buffer A [10 mM HEPES (pH 7), 5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM PMSF] (*31*). The cell suspensions were homogenized in a Dounce homogenizer in buffer A at 4 °C, and cenrifuged at 1000*g* for 15 min at 4 °C, and the pellet was resuspended in buffer A.

ADP-Ribosylation of Membranes by Pertussis Toxin. F10 cells were cultured overnight in DMEM supplemented with 0.25% BSA in the presence or absence of 100 ng/mL PTX (Sigma). Membranes were prepared as described above from PTX-treated and control cells. The reaction mixture containing cell membranes (50 μg of total protein), 100 mM Tris-HCl (pH 7.5), 20 μg/mL preactivated PTX, 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 1 mM $MgCl_2$, 0.5 mM EDTA, and 20 μM [^{32}P]NAD was incubated at 30 °C for 30 min (*31*). Aliquots were loaded and run on 10% SDS−polyacrylamide gel electrophoresis (SDS−PAGE). The gel was dried and subjected to autoradiography.

Iodination of Cell Surface Proteins. One hundred microcuries of $Na^{125}I$ and three IODO-BEADS (PIERCE, Rockford, IL) were added to F10 cells (1×10^6 cells) in 0.5 mL of 50 mM Tris-HCl, pH 7.5. The sample was incubated at 4 °C for 15 min. The reaction was stopped by removing the IODO-BEADS. The cells were washed 3 times with 0.5 mL of 50 mM Tris-HCl, pH 7.5, and then sonicated in 0.5 mL of buffer B (50 mM Tris-HCl, pH 7.5, 0.5% CHAPS, 1 mM EDTA, 1 mM PMSF, and 10 μg/mL aprotinin) at 4 °C for 10 min. The lysate was centrifuged at 4 °C for 30 min. Fifty microliters of the supernatant was incubated with 100 μM Sph-1-P, Sph, or no compound at room temperature. Another 50 μL of the supernatant was heated at 90 °C for 3 min. These four samples were incubated with 1 mg of immobilized Sph-1-P on glass beads at room temperature for 30 min. The glass beads were washed 3 times with buffer B and then incubated for 30 min with 100 μL of 100 μM Sph-1-P in buffer B at room temperature. The eluted proteins were analyzed on 10% SDS−PAGE.



1. R = $(HO)_2P(O)$
2. R = H

FIGURE 1: Structure of the conjugates of Sph-1-P, **1**, and Sph, **2**, with controlled pore glass beads (CPG) used in this study.

Metabolism of [3-^{3}H]Sph. Cells in a 60-mm-diameter dish were incubated with 1.5 μM [3-^{3}H]Sph (0.3 μCi) in 2.5 mL of DMEM supplemented with 10% calf serum. The culture medium was collected, and the cells were washed with 0.5 mL of fresh medium which was then combined with the recovered medium. The cells were washed twice with 2.5 mL of PBS, and scraped with 0.5 mL of ice-cold methanol twice. One milliliter chloroform, 10 μL of concentrated HCl, and 1 mL of methanol were added to the cell suspension (final chloroform:methanol:concentrated HCl ratio was 100:200:1). For extraction, 7.5 mL of chloroform/methanol/concentrated HCl (100:200:1) was added to 2 mL of the medium. Lipids were extracted from the cell suspension or medium by the method of Bligh and Dyer (*32*). The lower chloroform phase sample was dried and resuspended in small volume of chloroform/methanol (2:1). A portion of the lipids obtained from the lower phase was applied to silica gel 60 HPTLC plates (Merck, Darmstadt, Germany), and the plate was developed with butanol/acetic acid/water (3:1:1). After enhancer (Resolution TLC; E. M. Corp., Chestnut Hill, MA) treatment of the plate, autoradiography was conducted with Kodak X-Omat film (Eastman Kodak, Rochester, NY) at −80 °C for 3−7 days. The silica gel areas which contained the radiolabeled sphingolipids were scraped off, and the radioactivity was counted with a scintillation counter (Beckman).

RESULTS

Effects of Immobilized Sph-1-P on Cell Motility. To investigate if Sph-1-P acts on cells from the outside, we first immobilized Sph-1-P on glass beads by covalently linking the ω-carboxyl group of the Sph-1-P derivative to a long-chain alkyl amine on the surface of controlled pore glass beads (Figure 1). Immobilized Sph-1-P maintained its functions but could not be internalized by the cells. For comparison, we also immobilized Sph on glass beads by the same method (*28*) or acetylated the amino group on the surface of these beads as the control glass beads.

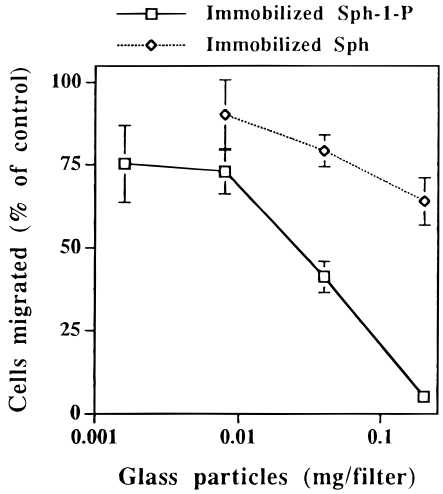


FIGURE 2: Effect of immobilized sphingolipids on F10 cell motility. Haptotactic cell motility of F10 cells was assayed using Transwell chambers as described under Materials and Methods. Glass beads with immobilized sphingolipids were added to the upper compartment with the cells. Data represent percent of motility assayed with control glass beads [mean ± SD (n = 3)].

Cell motility assays were then conducted with the immobilized sphingolipids. Figure 2 shows the effect of the immobilized Sph-1-P on the cell motility of F10 cells. As the total amount of the immobilized Sph-1-P increased, the inhibitory effect on cell motility increased. At 0.2 mg/filter (4.4 nmol of Sph-1-P/filter) of the glass beads, the immobilized Sph-1-P inhibited the cell motility almost completely, but the control glass beads did not. The immobilized Sph glass beads at 0.2 mg/filter (7.8 nmol/filter) inhibited the cell motility by ca. 35% of that seen with control glass beads. This may be due to concentrated Sph on the surface of the particles since 10 μM Sph has been shown to inhibit cell motility completely (*21*). Upon analysis of the medium by thin-layer chromatography (TLC), we observed no release of Sph-1-P or Sph from the glass beads during the cell motility assay. These results suggest that Sph-1-P acts on these cells from the outside.

Upon the addition of the immobilized Sph-1-P to a suspension of F10 cells, cells adhered to the glass beads instantly and retained their round shapes even after 2 days in culture. This was in contrast to a percentage of cells which did not come into contact with the immobilized Sph-1-P and which then adhered normally to the dish (Figure 3A). Upon the addition of the immobilized Sph, few F10 cells adhered to the glass beads, and also retained their round shapes (Figure 3B); however, the control glass beads elicited no interaction with the cells (Figure 3C). On the other hand, K562 cells, previously shown to have no response to Sph-1-P (*33*), did not interact with the immobilized Sph-1-P (Figure 3D). We examined the viability of the round-shaped

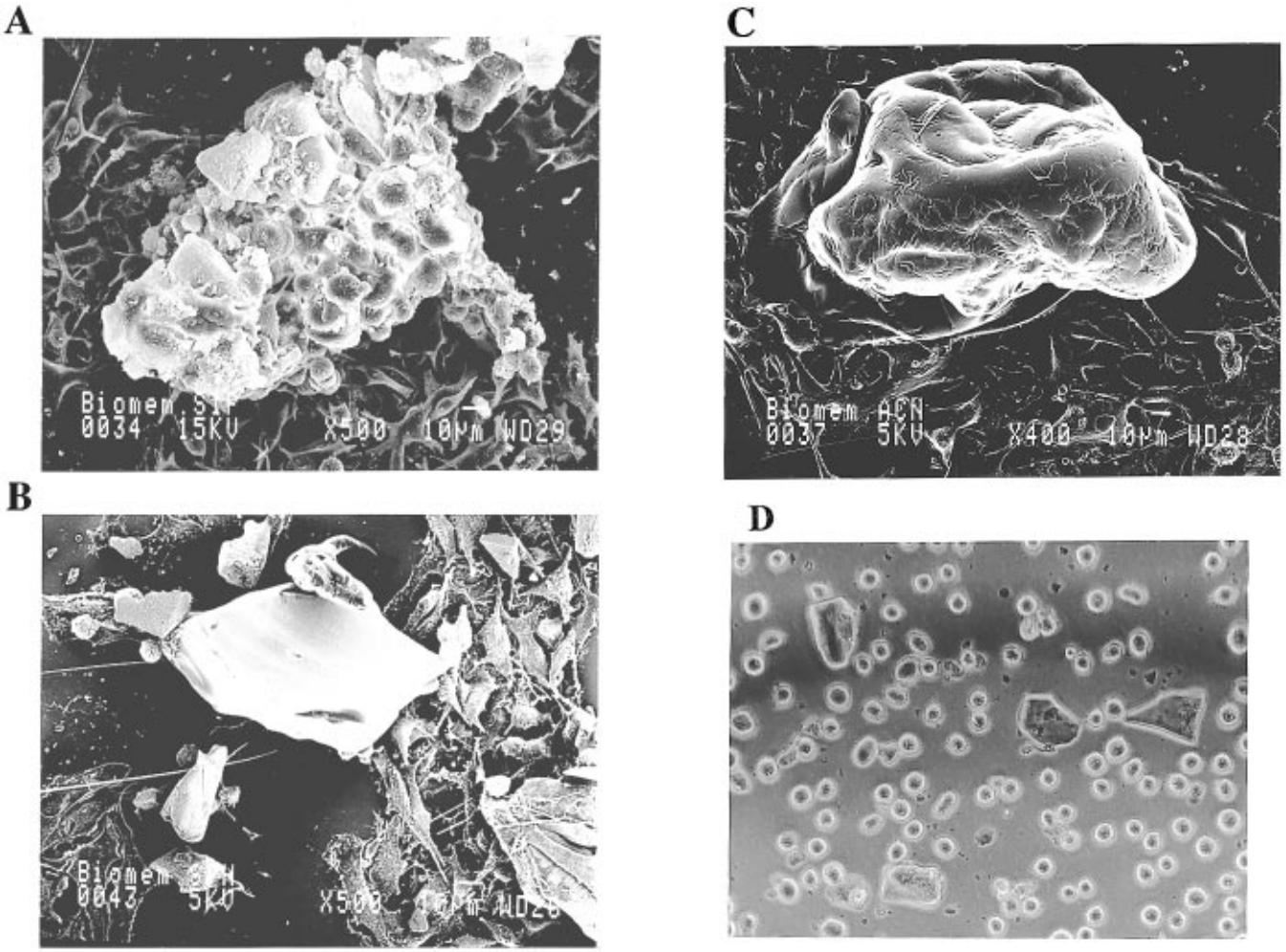


FIGURE 3: Interaction of cells with immobilized sphingolipids. Glass beads were added to the cell suspension, and interaction of the cells with the glass beads was studied by scanning electron microscopy or light microscopy. (A) F10 cells with immobilized Sph-1-P. (B) F10 cells with immobilized Sph. (C) F10 cells with control glass beads. (D) K562 cells with immobilized Sph-1-P.

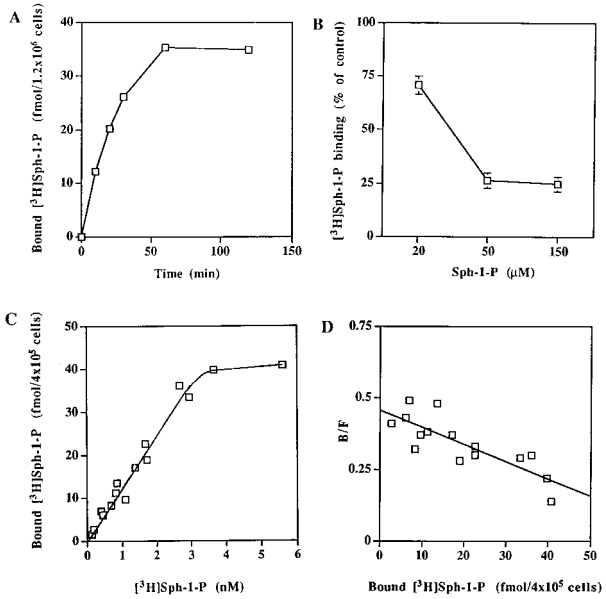


FIGURE 4: Specific binding of [^{3}H]Sph-1-P to the F10 cell surface. The specific binding was determined as described under Materials and Methods. (A) Time course of specific binding of [^{3}H]Sph-1-P. F10 cells (1.2×10^6 cells) were incubated with 2.7 nM [^{3}H]Sph-1-P at 4 °C for the indicated time. (B) Effect of unlabeled Sph-1-P on [^{3}H]Sph-1-P binding. F10 cells (1.2×10^6 cells) were incubated with 2.7 nM [^{3}H]Sph-1-P at 4 °C for 1 h in the presence of the indicated concentrations of unlabeled Sph-1-P. (C) Dose curve of specific binding of [^{3}H]Sph-1-P. F10 cells (4×10^5 cells) were incubated with various concentrations of [^{3}H]Sph-1-P at 4 °C for 1 h. (D) Scatchard analysis of specific binding of [^{3}H]Sph-1-P to F10 cells using the data in panel C. Data are typical from three independent experiments.

cells on the immobilized Sph-1-P or Sph after 2 days of culture and found cell viability to be greater than 95% for each culture.

Specific Binding of Sph-1-P to the Surface of F10 Cells. We assayed the binding of Sph-1-P to the F10 cell surface using [3-^{3}H]Sph-1-P. Figure 4A shows the time course of specific binding of [^{3}H]Sph-1-P at 4 °C. The specific binding depended on time, reached equilibrium within 1 h, and remained at equilibrium for at least 1 h. Excess amounts of unlabeled Sph-1-P reduced the binding of [^{3}H]Sph-1-P to F10 cells (Figure 4B). This effect increased with the concentration of unlabeled Sph-1-P, reaching a constant at 50 μM where unlabeled Sph-1-P reduced the binding by ca. 75%. The specific binding was saturated around 3 nM of the ligand (Figure 4C). The concentration of unlabeled Sph-1-P (50 μM) required for the replacement of [^{3}H]SpH-1-P that bound to the F10 cell surface was very high, 10 000 times that of the radioactive compound. However, this high value seems not to be uncommon when the binding assays of various lipophilic compounds are performed. In the binding assays of LPA (*34*), platelet activating factor (PAF) (*35*), or antagonist of PAF (*36*, *37*), the replacement of radioactive lipids needed a large molar excess, 10 000 times that of unlabeled lipids, due to the highly lipophilic nature of these lipids which cause and exhibit high levels of nonspecific binding.

Scatchard analysis of these data revealed a single class of low-affinity binding sites for Sph-1-P (Figure 4D). The K_d value was estimated to be 60−170 nM, and the number of binding sites was $(4 \times 10^4)-(1 \times 10^5)$/cell. K562 cells showed only 5−10% specific binding of [^{3}H]Sph-1-P, as compared with that of F10 cells. We also assayed for binding of [^{3}H]Sph to F10 cells, and found no specific binding.

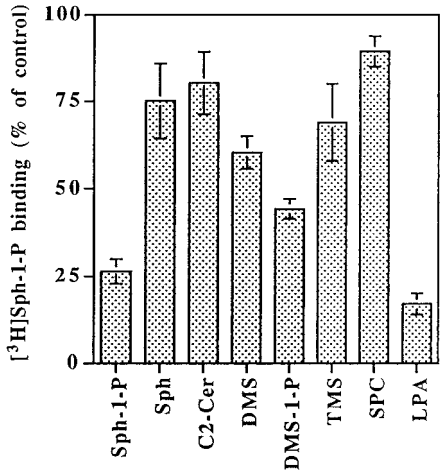


FIGURE 5: Effect of various lipids on [^{3}H]Sph-1-P binding to F10 cells. F10 cells (1.2×10^6 cells) were incubated with 2.7 nM [^{3}H]-Sph-1-P at 4 °C for 1 h in the presence and the absence of various lipids (50 μM). The binding was determined as described under Materials and Methods. Data represent mean ± SD ($n = 3$).

Figure 5 shows results of competition binding experiments with unlabeled lipids which have similar structures to Sph-1-P. Sph, C2-Cer, TMS, and sphingosylphosphocholine (SPC) only slightly inhibited the binding of [^{3}H]Sph-1-P. SPC has been reported to activate K^+ conductance in guinea pig atrial myocytes through the same putative receptor as that of Sph-1-P (*38*); however, our result indicates that their receptor may be distinct in F10 cells. DMS inhibited the binding by ca. 35%, and DMS-1-P by ca. 55%, suggesting that the phosphate group is required for specific binding. LPA, a structurally similar compound to Sph-1-P, remarkably reduced the binding (by ca. 85%), suggesting the possibility that LPA shares the binding site with Sph-1-P. We also examined the effect of a protease on the specific binding. Treatment of F10 cells with a nonspecific protease (type XXV) remarkably reduced the specific binding of Sph-1-P by 81.2 ± 8.5 ($n = 3$), indicating that the binding sites are proteins which are located on the surface of the cells. During these binding assays at 4 °C, [^{3}H]Sph-1-P was found not to be converted to any other compound by analysis with TLC. Taken together, these results indicate the presence of specific cell surface binding sites for Sph-1-P.

Relation between Cell Surface Binding and Motility Inhibition. Because DMS-1-P and LPA reduced the binding of Sph-1-P in the competition binding experiments (Figure 5), we tested the effects of these compounds on cell motility (Figure 6). LPA (1 μM) had a strong inhibitory effect on F10 cell motility, inhibiting by ca. 85% and ca. 70% for 18 h (Figure 6A) and 8 h incubations (Figure 6B), respectively, but at 10 nM LPA inhibited motility only by ca. 20% (Figure 6A) and ca. 10% (Figure 6B). In contrast, the maximum effect of Sph-1-P appeared at 10 nM where it inhibited cell motility by ca. 80% (Figure 6A) and ca. 90% (Figure 6B). Higher concentrations of Sph-1-P above 1 μM did not show a significant inhibitory effect as we reported previously (21), and the reason for this bell shape is unknown at present. Thus, the dose-dependent manner of LPA inhibition of cell motility was quite different from that of Sph-1-P. Measurement of intracellular Ca^{2+} mobilization revealed that prior

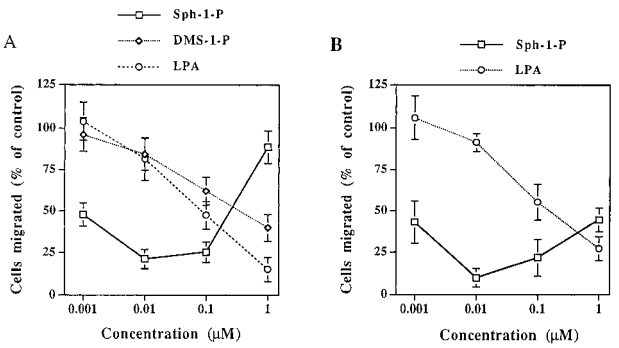


FIGURE 6: Effect of Sph-1-P, DMS-1-P, and LPA on F10 cell motility. Haptotactic cell motility of F10 cells was assayed using Transwell chambers as described under Materials and Methods. The test compound was added in the lower chamber. Data represent mean ± SD ($n = 3$). Incubation time: (A) 18 h; (B) 8 h.

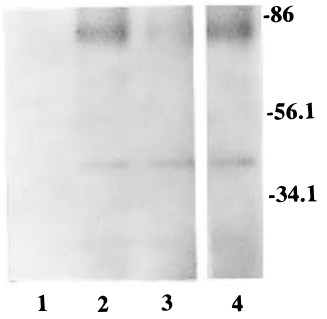


FIGURE 7: Identification of the Sph-1-P binding protein. The surface proteins of F10 cells were labeled with ^{125}I. The cells were lysed, and proteins were denatured by heating (lane 1) or were incubated with 100 μM Sph (lane 2), 100 μM Sph-1-P (lane 3), or no compound (lane 4). The proteins were applied to the Sph-1-P immobilized on glass beads. The bound proteins on the glass beads were eluted with 100 μM Sph-1-P, and were analyzed on 10% SDS−PAGE. Values on the figure indicate molecular mass (kDa).

challenge of Sph-1-P (20 μM) did not abolish the response to LPA (20 μM) (data not shown). On the other hand, in the competition binding experiments, LPA inhibited binding of [^{3}H]Sph-1-P as effectively as Sph-1-P (Figure 5). These results together suggest that LPA may be able to bind to sites distinct from those of Sph-1-P in addition to Sph-1-P binding sites (see Discussion).

DMS-1-P at 0.1 μM and 1 μM inhibited the cell motility by ca. 40% and 60%, respectively (Figure 6A), presumably by binding to the Sph-1-P binding site. SPC did not inhibit F10 cell motility at low concentrations. At 0.01 μM SPC, cells migrated were 117.0 ± 18.7% of control ($n = 3$), indicating that SPC binding sites are most likely distinct from those of Sph-1-P. Sph, C2-Cer, TMS, and DMS have been shown to have no significant effect on cell motility (*21*−*23*). These results indicate that the binding seen in Figure 4 well associates, except LPA, with inhibition of cell motility.

Effects of Suramin and Pertussis Toxin on the Inhibitory Effect of Sph-1-P on Cell Motility. We tested the effect of suramin, a known inhibitor of ligand−receptor interactions (*39*), on the inhibition of cell motility by 100 nM Sph-1-P. F10 cells were preincubated with 1 mg/mL suramin for 30 min or untreated, and haptotactic cell motility was assayed with or without 1 mg/mL suramin in both upper and lower chambers (8 h incubation). In the absence of suramin, migrated cells were 9.1 ± 3.7% ($n = 3$) of control, but in the presence of suramin, migrated cells were 50.9 ± 16.7% ($n = 3$). Suramin reduced the inhibitory effect of 100 nM Sph-1-P by ca. 40% of the control. This finding further indicates the presence of a cell surface receptor for Sph-1-P.

A PTX-sensitive G-protein has been shown to mediate Sph-1-P-induced signaling pathways for cell proliferation and intracellular Ca^{2+} mobilization (*14*, *40*). To study mediation of the G-protein, we assayed the haptotactic cell motility of F10 cells in the presence of 1−100 ng/mL PTX with a 30 min preincubation. Although [^{32}P]ADP-ribosylation experiments with 100 ng/mL PTX indicated that greater than 90% of the total PTX-sensitive G-proteins (possibly G_i and G_o based on their molecular weights) were ADP-ribosylated (data not shown), PTX did not abolish the inhibitory effect of Sph-1-P on cell motility (data not shown). This suggests that PTX-sensitive G-proteins are not involved in the signaling pathways of Sph-1-P inhibition of cell motility.

Identification of a Cell Surface Binding Protein for Sph-1-P. To identify the cell surface Sph-1-P binding protein, we labeled the surface of F10 cells with ^{125}I. The iodinated proteins were applied to the Sph-1-P immobilized on glass beads in the presence of Sph-1-P, Sph, or no compound. The bound proteins were eluted with Sph-1-P, and analyzed by SDS−PAGE. Two radioactive bands with molecular masses of ca. 41 and 79 kDa were observed, but after denaturing by heating, no band was apparent (Figure 7, lane 1). Furthermore, among the two Sph-1-P binding proteins, the intensity of the 79-kDa band was remarkably reduced by the addition of Sph-1-P (Figure 7, lane 3) but not by that of Sph (Figure 7, lane 2) in comparison with the control (Figure 7, lane 4). The intensity of the 41-kDa band was less sensitive to the addition of Sph-1-P (Figure 7, *lane 3*). ^{125}I-Labeling of the surface of K562 cells, which showed only a small amount of the specific binding, revealed no 41- or 79-kDa protein that binds to Sph-1-P (data not shown). These results suggest that the 79-kDa protein is a specific binding protein for Sph-1-P on the F10 cell surface.

Release of Sph-1-P from Cells. Having demonstrated the presence of a cell surface binding protein (or a receptor) for Sph-1-P, we raised the possibility that Sph-1-P acts as an intercellular modulator or messenger. To test this possibility, we examined if Sph-1-P is released from the cells into the culture medium upon stimulation. Figure 8 shows the metabolism of [3-^{3}H]Sph in A31 cells and the subsequent release of ^{3}H-labeled Sph-1-P into the medium in the presence of serum. Exogenously added [^{3}H]Sph was rapidly taken up within 3 min, reaching a plateau at 8 min. [^{3}H]-Sph was quickly phosphorylated to ^{3}H-labeled Sph-1-P (Figure 8A,C) with ceramide and sphingomyelin (SM) also being formed. The level of Sph-1-P peaked at 8 min and then decreased. Analysis of lipids in the medium showed that Sph-1-P was released from the cells, but ceramides or other lipids produced in the cells were not (Figure 8B). At 3 min, Sph-1-P was slightly detectable in the medium (Figure 8C). At 60 min, 28.5 ± 3.8% ($n = 3$) of Sph-1-P was in the medium and decreased thereafter. The decrease of Sph-1-P in the medium can be ascribed to internalization or binding to the cells. At 180 min, the cell viability was ca. 98%. In the control experiments (in the absence of serum), Sph-1-P was scarcely released from the cells into the medium which contained only 0.1% BSA.

F10 cells showed a similar metabolism of [3-^{3}H]Sph to that in A31 cells. Exogenously added [^{3}H]Sph was rapidly

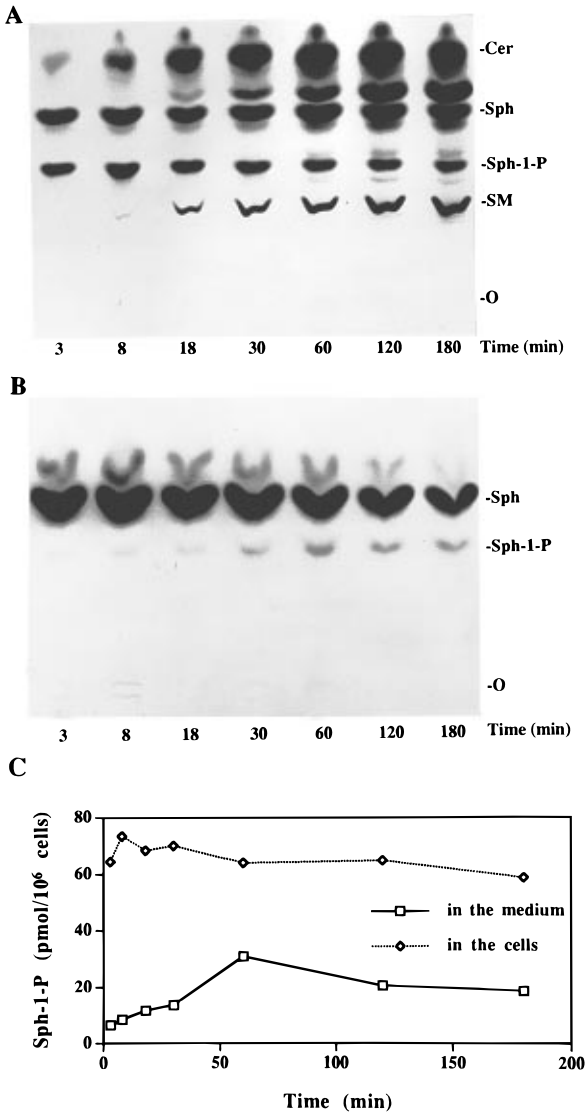


FIGURE 8: Metabolism of [3-^{3}H]Sph in A31 cells and the release of ^{3}H-labeled Sph-1-P into the medium. Confluent cultures of A31 cells were incubated with 1.5 μM [^{3}H]Sph for the indicated period. The lipids in the cells (A) and the medium (B) were extracted and analyzed by TLC as described under Materials and Methods. (C) Radioactive bands of Sph-1-P on the TLC plate shown in panels A and B were scraped and quantified.

incorporated into the cells, and ^{3}H-labeled Sph-1-P was quickly formed, and the maximum level of Sph-1-P appeared at 18 min. The analysis of the medium showed that a small amount of Sph-1-P was released into the media (ca. 2.5% at 60 min) in the presence of serum (data not shown).

DISCUSSION

Sph-1-P has been regarded as an intracellular messenger of PDGF- or serum-stimulated cell proliferation in Swiss 3T3 cells (*16*). On the other hand, Sph-1-P at low concentrations (10−100 nM) inhibits the motility of mouse melanoma B16/F1 and F10 (EC_{50} = 3 nM) (*21*, *23*) and human arterial smooth muscle cells (*22*), yet it has no mitogenic effects on these cells. This inhibition of cell motility by Sph-1-P has been explained by the intracellular actions of Sph-1-P, including the induction of phosphatidylinositol bisphosphate (PIP_2) hydrolysis, calcium mobilization, increase in cyclic AMP levels, and the activation of cAMP-dependent protein kinase (*22*). In contrast to the intracellular actions of Sph-1-P, Sph-1-P-induced signaling pathways involving a specific cell surface receptor have also been suggested (*14*, *15*, *39*, *40*).

Although a Sph-1-P receptor has been implicated in these studies, no direct evidence has been reported for the presence of a Sph-1-P receptor. In the present study, we identify and characterize the presence of specific binding sites for Sph-1-P on the surface of mouse cells. First, Sph-1-P immobilized on glass beads interacted with the cells and inhibited their motility, indicating that Sph-1-P acts on the cells from the outside, and that the sites of action for Sph-1-P are on the surface of the plasma membranes. Second, the binding studies with [^{3}H]Sph-1-P showed the presence of specific binding sites for Sph-1-P on the cell surface of F10 cells. These binding sites were found to have a rather low-affinity nature. Competition experiments with various Sph-1-P analogs indicated that a core structure of Sph and the phosphate group are both required for the specific binding. Cell motility assays with various compounds revealed that the binding is associated with inhibition of cell motility. Treatment with a protease disrupted the specific binding, suggesting that the binding sites are proteins which are located on the surface of the cells. Third, suramin, an inhibitor of ligand−receptor interactions (*39*), reduced the inhibitory effect of Sph-1-P on cell motility. Finally, we identified the Sph-1-P binding site as a 79-kDa protein by labeling the cell surface proteins with ^{125}I followed by affinity chromatography using Sph-1-P-immobilized glass beads. Taken together, these results suggest the possibility that the 79-kDa protein might be a specific cell surface receptor for Sph-1-P and that Sph-1-P may regulate cell motility through this specific cell surface binding site.

LPA has structural similarity to Sph-1-P (*33*) and elicits various biological effects on such phenomena as cell proliferation and differentiation, through its receptor, coupled with PTX-sensitive G-proteins (*41*). In relation to cell motility, LPA has also been reported to enhance cancer cell motility and invasiveness, through Rho-mediated protein tyrosine phosphorylation (*42*, *43*), and through actin polymerization by a PTX-sensitive mechanism (*44*). In our present study, in competition binding experiments, LPA abolished binding of [^{3}H]Sph-1-P as effectively as did Sph-1-P itself (Figure 5). However, LPA was found to be a rather weak inhibitor of cell motility compared to Sph-1-P. The dose dependence of the inhibition was quite distinct from that of Sph-1-P, and the effective inhibitory concentration of LPA was 100-fold higher than Sph-1-P (Figure 6). These results suggest that LPA may act on both Sph-1-P binding sites and its own receptor, which have distinct, or opposite, effects on cell motility. Our observation is somewhat inconsistent with the recent findings that a Sph-1-P receptor is completely distinct from that of LPA in various cells (*39*, *40*, *45*). However, cross-desensitization between Sph-1-P and LPA was observed in *Xenopus* oocytes (*33*), in atrial myocytes (*38*), and very recently in human platelets (*20*) and in FRTL-5 thyroid cells (*46*). These observations strongly suggest the existence of cell-type specific, or different types of receptors for Sph-1-P with distinct affinities for LPA. Very recently, interestingly, the cloning of a LPA receptor of neocortical neuroblast cell lines was reported, showing its molecular mass to be 41−42 kDa (*47*).

Many known receptors transduce signals through G-proteins sensitive to PTX which ADP-ribosylates and

inactivates G_α-proteins. Sph-1-P induces signaling pathways of cell proliferation or Ca^{2+} mobilization that are mediated by PTX-sensitive G-proteins (*14*, *15*, *40*). Very recently, in FRTL-5 thyroid cells, Sph-1-P-induced responses have been reported to be mediated by both PTX-sensitive and PTX-insensitive G-proteins (*46*). In the present study, PTX treatment did not attenuate the inhibitory effect of Sph-1-P on F10 cell motility, indicating that PTX-sensitive G-proteins are not involved in the signaling pathways of cell motility regulation. In this regard, the signaling pathways of Sph-1-P in motility inhibition of F10 cells are very unique among the reported signaling mechanisms induced by Sph-1-P in other systems.

Recent studies showed that the focal adhesion kinase (FAK) plays an important role in cell migration, as it is localized to focal adhesion sites and is phosphorylated and activated by integrin/ligand interactions (*48*, *49*). The small G-protein Rho also has been implicated in the regulation of actin stress fiber formation, motility, adhesion, and morphology of cells (*50*, *51*). Sph-1-P was found to induce Rho-dependent neurite retraction in neuronal cells (*39*). It is possible that Sph-1-P controls cell motility through its receptor by affecting these factors and regulating the actin filament formation. The studies along these lines are proceeding in our laboratory.

Recently, we found that Sph-1-P stored in platelets is released upon agonist stimulation (*19*, *20*), and that human plasma contains 50−350 nM Sph-1-P (*52*). Major portions of plasma Sph-1-P seemed to originate from activated platelets, although the possibility remained that cells other than platelets may release Sph-1-P into the extracellular environment. In this study, we found that Sph-1-P was specifically released from F10 melanoma and A31 fibroblast cells into the culture medium in response to serum stimulation. This implies that Sph-1-P released from the cells might regulate cell motility in an autocrine or a paracrine fashion, through the proposed receptor mechanisms. If 30% of cellular Sph-1-P is released (Figure 8), the locally concentrated Sph-1-P in the extracellular environment would possibly be enough to regulate cell motility. At present, the mechanisms by which Sph-1-P is secreted from the cells are unknown and remain to be determined.

In summary, we demonstrate and characterize for the first time the specific binding protein for Sph-1-P on the cell surface, using the conjugate Sph-1-P with controlled pore glass beads (Figure 1). We further illustrate that Sph-1-P regulates cell motility, at least partly, by binding to the cell surface receptor which triggers a PTX-sensitive G-protein-independent signaling mechanism, and that Sph-1-P is specifically released from the cells into the extracellular environment, suggesting a role as an autocrine or a paracrine intercellular modulator or messenger which regulates cell motility. The purification of Sph-1-P binding proteins (receptors) from the cell surface and the cloning of its genes are crucial to further elucidate the precise roles of Sph-1-P in the regulation of cell motility.

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