Article

Involvement of Sphingosine 1-Phosphate (S1P) Receptor Type 1 and Type 4 in Migratory Response of Mouse T Cells toward S1P

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Sphingosine 1-phosphate (S1P), a pleiotropic lysophospholipid, regulates signal transduction pathway *via* G-protein-coupled receptors termed $S1P_{1-5}$ in several types of the cells including lymphocytes. Higher levels of $S1P_4$ mRNA as well as $S1P_1$ mRNA are expressed in lymphoid tissues such as the spleen, thymus, lymph nodes, and Payer's patches. In contrast to $S1P_1$ that plays an essential role in lymphocyte egress, little is known about the role of $S1P_4$ in immune system. In this study, we found that S1P at 10 to 100 nM significantly induced the cell migration and the significant levels of $S1P_1$ and $S1P_4$ mRNA were expressed in mouse CD4 T cells, D10.G4.1 mouse Th2 cells, and EL-4.IL-2 mouse thymoma cells. In D10.G4.1 and EL-4.IL-2 cells, S1P-induced migration was almost completely inhibited by pretreatment with pertussis toxin, *Clostoridium difficile* toxin B, and (*S*)-enantiomer of FTY720-phosphate, a potent agonist at $S1P_1$ and $S1P_4$. The members of the Rho family small GTPase, Cdc42 and Rac were activated by S1P stimulation in these cells. The transfection with dominant negative or constitutively active forms of Cdc42 and Rac revealed that the activation of both Cdc42 and Rac is essential for S1P-induced migration of these cells. The immunoprecipitation assays using CHO cells co-expressing both $S1P_4$ and $S1P_1$ receptors indicated that $S1P_4$ and $S1P_1$ are associated on the cell surface. These results suggest that the association of $S1P_4$ and $S1P_1$ plays an important role in migratory response of mouse T cells toward S1P. *Cellular & Molecular Immunology*. 2006;3(6):429-437.

Introduction

Sphingosine 1-phosphate (S1P), a pleiotropic lysophospholipid mediator, is converted primarily from sphingosine by sphingosine kinase (SPHK) (1) and stimulates multiple signaling pathways that result in calcium mobilization from intracellular stores, polymerization of actin, cell proliferation and cell motility in various types of the cells (2). S1P is formed in various cells including mast cells, platelets, and macrophages in response to diverse stimuli such as growth factors, cytokines, G-protein-coupled receptor agonists and antigens. Significant amounts (100 to 300 nM) of S1P are found in plasma and S1P binds with nanomolar (nM) affinities to five related G-protein-coupled receptors, termed $S1P_{1-5}$, (formally Edg-1, -5, -3, -6, and -8) (3-9). $S1P_1$ mRNA is widely expressed in various tissues, including the heart, brain, lung, thymus, kidney, spleen adipose tissue, skin, uterus, testis, and liver (10). Recently Matloubian et al. has reported that there are no T cells in periphery in mice whose hematopoietic cells lack $S1P_1$ because mature T cells are unable to exit in secondary lymphoid tissues and the thymus (11). Thus, $S1P_1$ is expressed ubiquitously in many tissues; however, it is clearly demonstrated that $S1P_1$ is essential for lymphocyte egress from secondary lymphoid tissues and the thymus.

FTY720 (fingolimod) is a new class of immuno-modulator with a sphingosine-related chemical structure (12, 13). Its most striking feature is the induction of a marked

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decrease in the number of peripheral blood lymphocytes at doses that display an immunomodulating activity in various experimental allograft and autoimmune disease models (12-16). Our previous studies suggested that FTY720 induces the sequestration of circulating mature lymphocytes into secondary lymphoid tissues such as lymph nodes and Payer's patches, and thereby decreases the number of lymphocytes in peripheral blood, thoracic duct lymph, and spleen (14, 17). Moreover, Yagi et al. reported that FTY720 inhibits egress of mature thymocytes from the thymus to periphery in mice (18). It has been demonstrated that FTY720 is converted to an active metabolite, FTY720-phosphate (FTY720-P) by SPHK1a and SPHK2 (19), and FTY720-P acts as an agonist at S1P receptors except S1P$_2$ (20, 21). Recently, we successfully synthesized (*S*)- and (*R*)-enantiomers of FTY720-P with a high enantio-selectively (22) and demonstrated that only (*S*)-enantiomer of FTY720-P [(*S*)-FTY720-P] shows agonist activity at S1P$_1$, down-regulates S1P$_1$ on cell surface, and inhibits migration of CD4 T cells toward S1P. (16). Thus, it is likely that FTY720 reduces circulating lymphocytes by inhibiting S1P/S1P$_1$-dependent lymphocyte egress from secondary lymphoid tissues and the thymus (14, 16).

In contrast to S1P$_1$, S1P$_4$ expression is reported to be highly restricted in lymphocyte-containing tissues including the thymus, spleen, bone marrow, appendix, peripheral leukocytes, and lung (7). It has been also reported that the mRNA expressions of S1P$_1$ and S1P$_4$ are the most prominent on murine CD4 T cells (23). Furthermore, it has been demonstrated that S1P promotes cell migration through the activation of Rho family small GTPase, Cdc42, in mouse S1P$_4$-expressing CHO cells (24). Based on theses results, it is highly probable that S1P$_4$ as well as S1P$_1$ are involved in migration and recirculation of T cells; however, little is known about the immunological function of S1P$_4$. In the present study, we demonstrate that S1P induces migratory response of mouse T cell lines that express both S1P$_4$ and S1P$_1$ mRNA and that the association of S1P$_1$ and S1P$_4$ appears to play an important role in the migration of mouse T cells toward S1P.

Materials and Methods

Animals and agents
C57BL/6 mice purchased from Charles River Japan Inc. (Yokohama, Japan) were used at 8 to 12 weeks of age. (*S*)-FTY720-P was synthesized with high enantio-selectivity (> 99.5% enantio excess) in Mitsubishi Pharma Corporation according to the method as described previously (22) and dissolved in ethanol. S1P was purchased from Avanti (Alabaster, AL). Recombinant mouse (rm)-CCL19 was obtained from R&D systems (Minneapolis, MN). Pertussis toxin (PTX) and *Clostoridium difficile* toxin B (Toxin B) were obtained from Sigma (St Louis, Mo), and Calbiochem (La Jolla, CA), respectively. Anti-human S1P$_1$ Ab (H-60), anti-HA Ab (Y-11), and anti-phosphorylated extracellular-signal related kinase 1/2 (ERK1/2) Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HA Ab

(HA-7) and anti-FLAG Ab were obtained from Sigma. Anti-ERK1/2 Ab was purchased from Cell Signaling Technology (Danvers, MA). Anti-myc tag Ab was obtained from MBL (Nagoya, Japan). Anti-Cdc42 and anti-Rac Abs were purchased from BD Transduction Laboratories (Lake Placid, NY). Alexa 488-conjugated goat anti-mouse IgG (H+L) and Alexa 594-conjugated goat anti-rabbit IgG (H+L) were obtained from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were obtained from GE healthcare bioscience (Piscataway, NJ). FITC-conjugated hamster anti-mouse CD3ε mAb (145-2C11), PE-conjugated rat anti-mouse CD4 mAb (RM4-5) and PE-Cy5-conjugated rat anti-mouse CD8α mAb (53-6.7) were purchased from BD Biosciences (San Jose, CA).

Cell lines and transfection
D10.G4.1 mouse Th2 cells and EL-4.IL-2 mouse thymoma cells were obtained from ATCC (Manassas, VA). D10.G4.1 cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS, Sigma), 50 μM 2-mercaptoethanol, and 10% concanavalin A-stimulated rat spleen cell culture supernatant at 37°C in 5% CO$_2$. EL-4.IL-2 cells were cultured in RPMI 1640 medium containing 10% FCS at 37°C in 5% CO$_2$. In some experiments, EL-4.IL-2 cells were transfected with N-terminal-myc-epitope-tagged mouse V12 Cdc42, N17 Cdc42, V12 Rac, or N17 Rac constructs in pcDNA3.1(+) by nucleofector (Amaxa, Gaithersburg, MD). Chinese hamster ovary (CHO) cells purchased from Dainippon Pharmaceutical were transfected with DNAs encoding C-terminal HA-tagged human S1P$_1$ receptor or human S1P$_4$ receptor constructs in pcDNA3.1(+) using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA). Then the cells stably expressing human S1P$_1$ (hS1P$_1$-CHO cells) or human S1P$_4$ (hS1P$_4$-CHO cells) were selected in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Sigma) supplemented with 10% FCS and 600 μg/ml Geneticin (Sigma) at 37°C in 5% CO$_2$.

Preparation of CD4 T cells from mesenteric lymph nodes
Mesenteric lymph nodes were removed from mice and single cell suspensions were prepared by mincing and passing through stainless mesh. Obtained lymph node cells were suspended in RPMI 1640 medium containing 10 mM HEPES, 100 U/ml penicillin, 60 μg/ml kanamycin sulfonate, 50 μM 2-mercaptoethanol, and 0.5% fatty acid free BSA. For real-time PCR analysis, the lymph node cells were purified to > 95% of CD4 T cells by passing through mouse CD4 subset enrichment columns (R&D Systems).

Reverse transcription-polymerase chain reaction
Total RNA was extracted from mouse tissues, CD4 T cells, D10.G4.1 cells, and EL-4.IL-2 cells using RNA isolation reagent (Nippon Gene, Tokyo, Japan) and the concentration of total RNA was measured spectrophotometrically. An aliquot (0.5 μg) of total RNA was reverse transcribed to cDNA in a 50 μl volume at 25°C for 10 min, 48°C for 30 min

and 95°C for 10 min with TaqMan® reverse transcription reagents using a thermal cycler, Gene Amp® PCR System 9700 (Applied Biosystems, Branchburg, NJ). The level of mRNA for S1P receptors and GAPDH was determined using the TaqMan® polymerase chain reaction (PCR). Five micro-liters of cDNA were amplified with S1P receptors TaqMan® probe (6-carboxy-fluorescein label)/primer, GAPDH TaqMan® probe (VIC^{TM} label)/primer, and TaqMan® Universal PCR Master Mix in an ABI PRISM^{TM} 7900 Sequence Detection System (Applied Biosystems). The reaction was incubated for 2 min at 50°C, denatured for 10 min at 95°C and subjected to 40 two-step amplification cycles with annealing/extension at 60°C for 1 min followed by denaturation at 95°C for 15 sec. The detection of PCR product was monitored by measuring the increase in fluorescence caused by degradation of the probe. For every sample, the levels of S1P receptors mRNA were normalized by calculating the ratio of S1P receptors/GAPDH levels. The following primers were used: S1P$_1$ (sense 5'-AAA TGC CCC AAC GGA GAC T-3' antisense 5'-CTG ATT TGC TGC GGC TAA ATT C-3'), S1P$_2$ (sense 5'-GCC ATC GTG GTG GAG AAT CTT-3' antisense 5'-AGG TAC ATT GCT GAG TGG AAC TTG-3'), S1P$_3$ (sense 5'-GAA CTT TCC CGA CTG CTC TAC CA-3' antisense 5'-TGG CGG TGA AGA TAC TGA TGA G-3'), S1P$_4$ (sense 5'-GGC TAT GCC CAT TGT CCA GTA-3' antisense 5'-GGC TCT GAG CTA GAG AGC ATG AT-3'), S1P$_5$ (sense 5'-CAT GGC TAA CTC GCT GCT GAA-3' antisense 5'-AGC TGT TGG AGG AGT CTT GGT T-3'). The PCR product of cellular S1P receptors was separated by electrophoresis through a 3% NuSieve 3:1 agarose gel containing ethidium bromide, and visualized using Chemi Imager (Alpha Innotec, San Leandro, CA).

Migration assays
Migration assays were conducted according to the method described previously (25). After serum starvation using RPMI 1640 medium containing 0.5% fatty acid free BSA (Sigma) for 18 h at 37°C, D10.G4.1 cells or EL-4.IL-2 cells (5×10^6 cells) were added to the upper wells of 5-μm pore, polycarbonate 24-well tissue culture inserts (Transwell, Corning Costar Corp., Cambridge, MA) in 100 μl, with 600 μl of S1P or rm-CCL19 dilution (or medium) in the bottom wells. Three chemotactic wells were set up for each chemoattractant. All migration assays were conducted in RPMI 1640 medium with 0.5% fatty acid free BSA for 3 h at 37°C in 5% CO$_2$. In some experiments, the cells were pretreated with PTX at 200 ng/ml for 3 h, *Clostridium difficile* toxin B at 100 ng/ml for 3 h, or (S)-FTY720-P for 1 h. The migrated cells recovered from each well were counted using comparison to a known number of beads as an internal standard of Flow-Count fluorosphere (Becton Coulter Inc., Fullerton, CA) by flow cytometry with an Epics-XL (Beckman Coulter) and the percent migration was determined. In the migration assays for CD4 T cells, mouse lymph node cells (5×10^6 cells) were used after serum starvation for 3 h at 37°C and the migrated cells were stained with FITC-conjugated hamster anti-mouse CD3ε mAb, PE-conjugated rat anti-mouse CD4 mAb and PE-Cy5-conjugated rat anti-

mouse CD8α mAb to identify CD3$^+$CD4$^+$CD8$^-$ T cells according to the method as described (15, 25).

Electrophoresis and immunoblotting
SDS-PAGE in the reducing condition was performed on 10% or 12.5% polyacriylamid gels. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore, Bedford, MA). Blocking was performed with 5% skim milk in Tris-HCl buffer (20 mM, pH 7.5) containing 137 mM NaCl and 0.05% Tween-20 for 1 h at room temperature. The blotted PDVF membrane was incubated with appropriate Abs overnight at 4°C. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary Ab for 1 h at room temperature and developed with ECL-plus.

Assay for ERK1/2 activation
hS1P$_1$-CHO cells and hS1P$_4$-CHO cells were grown in 12-well plates for 24 h in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Sigma) supplemented with 10% FCS at 37°C in 5% CO$_2$, followed by serum starvation for 18 h. The cells were incubated with serum-free medium containing various concentrations of S1P or (S)-FTY720-P. After 3 min, the cells were washed with ice-cold PBS, and lysed with SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and analyzed by Western blot using an anti-phosphorylated ERK1/2 or anti-ERK1/2 antibody.

Pull-down assay
A pull-down assay was performed as previously described (24) using Cdc42 and Rac activation assay kit (Upstate Biotechnology, Lake placid, NY). After serum starvation for 18 h, the cells (1×10^6) were stimulated with 500 nM S1P for 5 min at 37°C. The cells were washed with ice-cold phosphate buffered saline (PBS), and lysed with HEPES buffer (25 mM, pH 7.5) containing 150 mM NaCl, 0.3 mM sodium azide, 1% Igepal CA-630, 10 mM MgCl$_2$, 1 mM EDTA, and 2% glycerol. The cell lysates were incubated with glutathione S-transferase-p21 activated kinase1-binding domain (GST-PBD, amino acids 67-150) fusion protein and glutathione Sepharose 4B at 4°C for 45 min. The precipitates were collected by centrifugation at 10,000 × g for 30 s, separated by SDS-PAGE, and were analyzed by Western blot using an anti-Cdc42 or anti-Rac antibody.

Immunoprecipitation
After serum starvation for 18 h, the cells (1×10^6) were stimulated with 500 nM S1P for 5 min at 37°C. The cells were washed with ice cold PBS, and lysed in ice-cold Tris-HCl buffer (50 mM, pH 8.5) containing 150 mM NaCl, 0.5 mM DTT, 5 mM EDTA, 1% (v/v) Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors cocktail. The cell lysates were incubated with protein A/G Sepharose fast flow. After agitation for 1 h at 4°C, the lysates were centrifuged at 10,000 × g for 30 s at 4°C, and the supernatants were incubated with protein A/G Sepharose CL4B and antibodies for 2 h at 4°C. The immunocomplex was collected by centrifugation at 10,000 × g for 30 s at 4°C.

Immunoprecipitates were washed twice with ice-cold wash Tris-HCl buffer (50 mM, pH 8.5) containing 150 mM NaCl, 0.1 mM EDTA, 1% (v/v) lubrol, 0.2 mM phenyl-methylsulfonyl fluoride, and protease inhibitors cocktail and were subjected to SDS-PAGE and Western blotting.

Immunofluorescence microscopy
hS1P$_4$-CHO cells grown on Labtek chamber slides (NUNC, Rochester, NY) were tranfected with human S1P$_1$. After 24 h of S1P$_1$-transfection, the cells were washed with PBS and fixed with 3.7% formaldehyde in PBS at room temperature for 20 min. Permeabilization was performed in PBS containing 0.5% Triton X-100 and 1 mg/ml BSA for 5 min. Immunostaining was performed by incubation with a 1:100 dilution of the anti-HA and anti-human S1P$_1$ antibody for 1 h, followed by detection with a 1:300 dilution of Alexa 488-conjugated anti-mouse IgG and Alexa 594-conjugated anti-rabbit IgG at room temperature for 30 min. The cell images were digitally captured from a confocal laser microscope (Carl Zeiss, Thornwood, NJ).

[^{32}P] S1P binding assay
[^{32}P] S1P was synthesized enzymatically using recombinant SPHK1a according to the method described previously (24). After serum starvation for 18 h, the cells (5×10^4) were washed twice with ice-cold Tris-HCl buffer (20 mM, pH 7.5) containing 100 mM NaCl, 15 mM NaF and 0.4% (w/v) fatty acid free BSA and incubated with the 1 nM [^{32}P] S1P in MultiScreen 96-well microtest plates (Nihon Millipore, Tokyo, Japan). After incubation for 60 min at 4°C, the cells were washed four times and bound [^{32}P] S1P was quantified by scintillation counting.

Results

The mRNA expressions of S1P$_4$ and S1P$_1$ in mouse lymphoid tissues
We first examined the mRNA expressions of S1P$_4$ and S1P$_1$ in the spleen, thymus, mesenteric lymph nodes, Payer's patches, kidney and liver in mice by real-time PCR analysis. As shown in Figure 1A, the mRNA expression of S1P$_4$ was relatively high level in the spleen, thymus, mesenteric lymph nodes, and Payer's patches, whereas that in kidney and liver was very low. In contrast to S1P$_4$ mRNA expression, S1P$_1$ mRNA expression was the most prominent in the spleen and mesenteric lymph nodes, lower but a significant level in the thymus, Payer's patches and liver, and very low in the kidney (Figure 1B). Thus, we confirmed that significant levels of S1P$_4$ mRNA as well as S1P$_1$ mRNA are expressed in lymphocyte-containing tissues including the spleen, thymus, lymph nodes, and Payer's patches and that S1P$_4$ mRNA expression is highly restricted in the lymphoid tissues. These results suggest that the functional amounts of both S1P$_4$ and S1P$_1$ are expressed on the cell surface of lymphocytes.

S1P-induced migration and mRNA expression of S1P receptors in mouse T cells
Because S1P can induce a migratory response in various
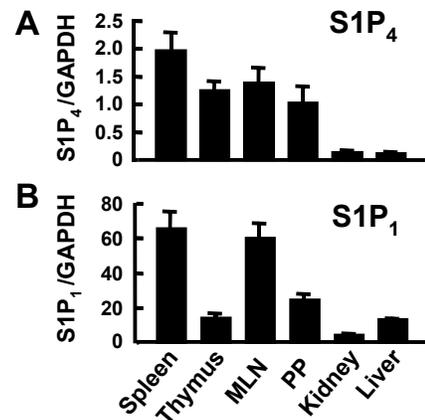
Figure 1. The mRNA expressions of S1P$_1$ and S1P$_4$ in mouse lymphoid tissues. The mRNA expressions of S1P$_4$ (A) and S1P$_1$ (B) in the spleen, thymus, mesenteric lymph nodes, Payer's patches, kidney and liver in C57BL/6 mice were analyzed by real-time PCR. The results are expressed as the mean ± S.E.M. of triplicate determinations.

types of cells including lymphocytes *via* S1P receptors, we examined the migratory response toward S1P and the expression pattern of S1P receptors in CD4 T cells prepared from mouse mesenteric lymph nodes. S1P at 10 to 100 nM induced a significant migration of CD4 T cells in chemotaxis assay, and relatively higher level of S1P$_1$ mRNA and significant level of S1P$_4$ mRNA were expressed in the mouse CD4 T cells (Figure 2). Then we examined the migratory response of several mice T cell lines toward S1P and found that S1P at 10 to 100 nM as well as CCR7-ligand chemokine, CCL19 at 100 ng/ml could induce a significant migration of
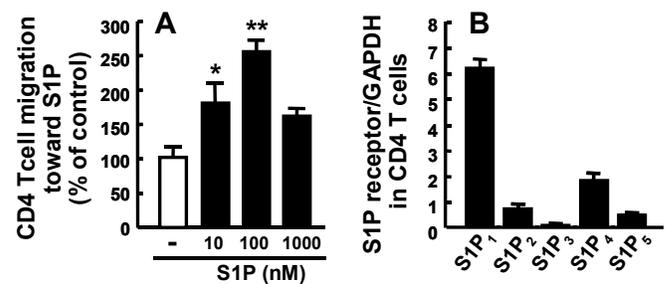
Figure 2. The migration toward S1P and mRNA expressions of S1P receptors in mouse CD4 T cells. (A) The migration of mouse CD4 T cells toward S1P was examined by chemotaxis assay using Transwell. After serum starvation for 3 h, the migration assays of mouse lymph node cells (5×10^5 cells) were performed in RPMI 1640 medium with 0.5% fatty acid free BSA for 3 h. The percent migration of CD3$^+$CD4$^+$CD8$^-$ T cells was determined by 3-color flow cytometry. Statistical differences versus control (without S1P) were calculated by Dunnett's multiple-comparison test (*, $p < 0.05$; **, $p < 0.01$). (B) The mRNA expressions of S1P receptors in mouse CD4 T cells were analyzed by real-time PCR. The results are expressed as the mean ± S.E.M. of triplicate determinations.
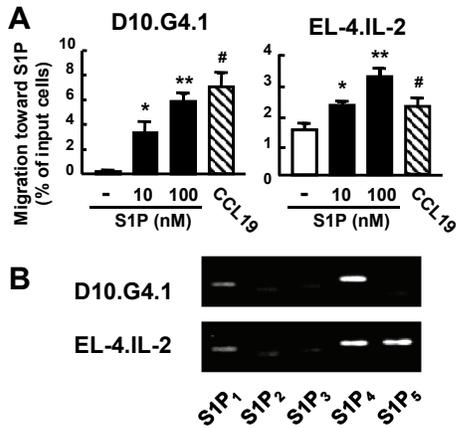
Figure 3. The migration toward S1P and mRNA expressions of S1P receptors in D10.G4.1 and EL-4.IL-2 cells. (A) After serum starvation for 18 h, the migration assays of D10.G4.1 or EL4.IL-2 cells (5×10^5 cells) toward S1P or CCL19 (100 ng/ml) were conducted in RPMI 1640 medium with 0.5% fatty acid free BSA for 3 h. The number of migrated cells was determined by flow cytometry. The results are expressed as the mean ± S.E.M. of triplicate determinations. Statistical differences versus control were calculated by Dunnett's multiple-comparison test (*, $p < 0.05$; **, $p < 0.01$) and t-test (#, $p < 0.05$). (B) The mRNA expressions of S1P receptors in D10.G4.1 and EL4.IL-2 were analyzed by semi-quantitative RT-PCR.

D10.G4.1 Th2 cells and EL-4.IL-2 thymoma cells (Figure 3A). In D10.G4.1 cells, relatively higher level of $S1P_4$ mRNA was constitutively expressed and a significant level of $S1P_1$ mRNA expression was detected by semi-quantitative RT-PCR analysis. EL-4.IL-2 cells also expressed high levels of both $S1P_4$ and $S1P_5$ mRNA and a lower but significant level of $S1P_1$ mRNA (Figure 3B). No products were obtained after omitting reverse transcription in the reaction (data not shown).

Inhibitory effect of (S)-FTY720-P on S1P-induced migration of mouse T cells

S1P-induced migration of EL-4.IL-2 cells toward 100 nM S1P was almost completely inhibited by pretreatment with Gαi-specific inhibitor, PTX or Rho family small GTPase inhibitor, Toxin B (Figure 4A). Furthermore, the pretreatment with a potent S1P receptor agonist, (S)-FTY720-P at 0.1 and 1 nM resulted in a significant inhibition of S1P-induced migration of EL-4.IL-2 cells. Similar results were obtained when D10.G4.1 cells were pretreated with PTX, Toxin B, or (S)-FTY720-P (data not shown). These results indicate that S1P-induced migration of mouse T cells is involved in Gαi and Rho family small GTPase mediated signal transduction pathways *via* S1P receptors including $S1P_4$ and $S1P_1$.

Because the migration of mouse T cells toward S1P is significantly inhibited by the pretreatment with (S)-FTY720-P, we investigated the effect of this compound on the phosphorylation of ERK1/2 in $hS1P_1$-CHO or $hS1P_4$-CHO cells. As shown in Figure 5, both S1P and (S)-
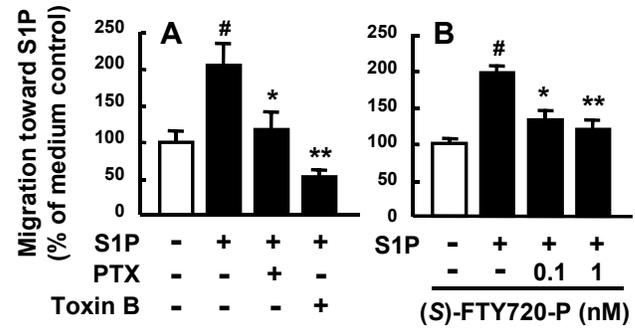
Figure 4. Inhibition of S1P-induced migration by pretreatment with PTX, Toxin B or (S)-FTY720-P in EL-4.IL-2 cells. After serum starvation for 18 h, EL-4.IL-2 cells were pretreated with (A) PTX (200 ng/ml), Toxin B (100 ng/ml) for 3 h or (B) (S)-FTY720-P (0.1 and 1 nM) for 1 h. Then, the migration assays toward S1P (100 nM) were conducted in RPMI 1640 medium with 0.5% fatty acid free BSA for 3 h. The results are expressed as the mean ± S.E.M. of quadruplicate determinations. Statistical differences versus control (without S1P) or S1P-induced migration were calculated by t-test (#, $p < 0.05$) or Dunnett's multiple-comparison test (*, $p < 0.05$; **, $p < 0.01$), respectively.

FTY720-P at 0.1 nM or higher induced a phosphorylation of ERK1/2 in $hS1P_1$-CHO cells. Moreover, we, for the first time, demonstrated that (S)-FTY720-P as well as S1P induced a phosphorylation of ERK1/2 in $hS1P_4$-CHO cells (Figure 5). From these results, it is presumed that the agonistic activities
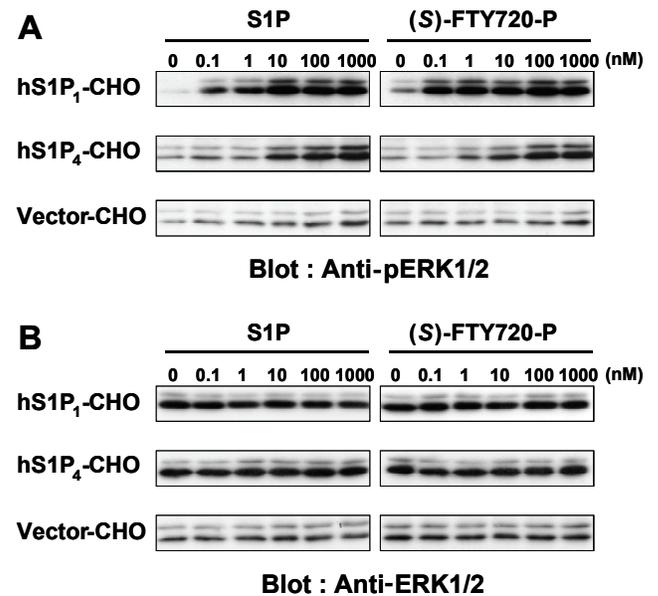
Figure 5. (S)-FTY720-P shows agonistic activities at $S1P_1$ and $S1P_4$. After serum starvation for 18 h, $hS1P_1$-CHO and $hS1P_4$-CHO cells were stimulated with S1P or (S)-FTY720-P at 0.1 to 1000 nM for 3 min. Then the proteins obtained by cell lysis were separated by SDS-PAGE and analyzed by Western blot using an anti-phosphorylated ERK1/2 (A) or anti-ERK1/2 Ab (B).

D10.G4.1   EL-4.IL-2

Cdc42-GTP

Total Cdc42

Rac-GTP

Total Rac

Control   S1P   Control   S1P
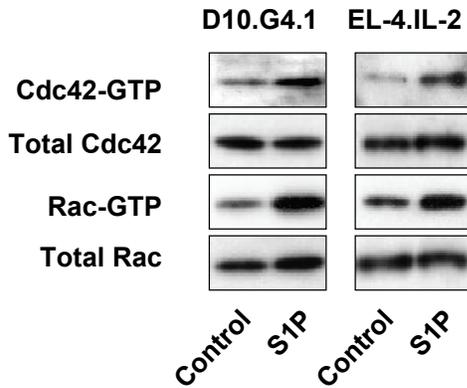
Figure 6. S1P induces the activation of Cdc42 and Rac in D10.G4.1 and EL-4.IL-2 cells. After serum starvation for 18 h, D10.G4.1 and EL-4.IL-2 cells were stimulated with S1P at 500 nM for 5 min. Then the cells were lysed and the obtained lysates were used for an affinity precipitation assay in the presence of GST-PBD and glutathione Sepharose beads. GTP-Cdc42 or GTP-Rac bound to the beads was separated on SDS-PAGE and analyzed by Western blot using anti-Cdc42 or anti-Rac Ab.

at S1P₄ as well as S1P₁ are involved in the inhibition of S1P-induced T cell migration by the pretreatment with (S)-FTY720-P.

Activation of Cdc42 and Rac is essential for S1P-induced migration of mouse T cells

It has been reported that S1P-induced cell migration is mediated by the activation of Cdc42 in mouse S1P₄-expressing CHO cells (24) or by the activation of Rac *via* S1P₁- or S1P₃-expressing CHO cells (26). Based on these results, we examined whether the activation of Cdc42 and/or Rac is involved in S1P-induced migration of D10.G4.1 and EL-4.IL-2 cells by using a pull-down assay with GST-PBD. The activated forms of Cdc42 (Cdc42-GTP) and Rac (Rac-GTP) were increased markedly by S1P stimulation in D10.G4.1 and EL-4.IL-2 cells (Figure 6). From these results, it is presumed that S1P-induced migration of mouse T cells is mediated by S1P₄ as well as S1P₁ because the activation of both Cdc42 and Rac was induced by S1P stimulation in these cells.

To determine whether the activation of both Cdc42 and Rac is essential for S1P-induced migration of mouse T cells, EL-4.IL-2 cells were transfected with the dominant negative or constitutively active form of Cdc42 and Rac. Before migration assays, we confirmed that dominant negative form (myc-N17 Cdc42 and myc-N17 Rac), constitutively active form (myc-V12 Cdc42, myc-V12 Rac), or the myc-vector was transiently expressed in each transfected EL-4.IL-2 cells (Figure 7A). EL-4.IL-2 cells transfected with V12 Cdc42, V12 Rac, or vector could migrate toward 100 nM S1P, whereas S1P-induced migration was not observed in N17 Cdc42- or N17 Rac-transfected cells (Figure 7B). Consequently, it is clarified that the activation of both Cdc42 and Rac is essential for S1P-induced migration of mouse T cells.

A

Anti-myc   Anti-myc

Anti-Cdc42   ←   Anti-Rac   ←

Vector V12 Cdc42 N17 Cdc42   Vector V12 Rac N17 Rac

B

Vector   N17 Cdc42   V12 Cdc42   N17 Rac   V12 Rac

% of control
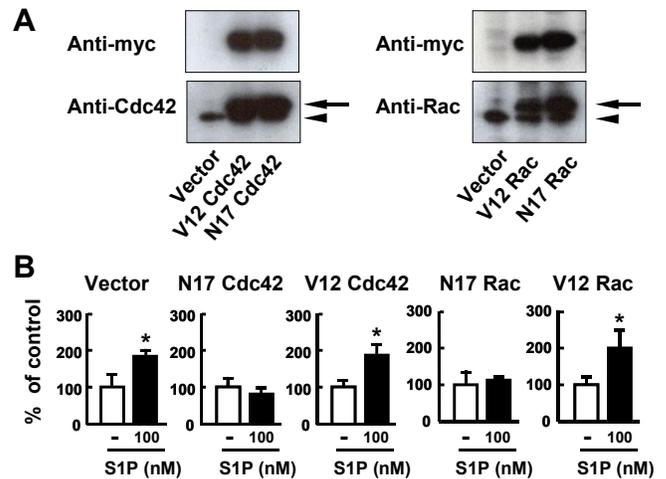
Figure 7. Cdc42 and Rac activation is required for S1P-induced migration of EL-4.IL-2 cells. (A) The expressions of Cdc42 and Rac in the transfected cells were analyzed by Western blot using an anti-myc (upper), anti-Cdc42 (lower) or anti-Rac (lower) Ab. The arrow and arrowhead indicate the myc-tagged Cdc42 or Rac and endogenous Cdc42 or Rac, respectively. (B) EL-4.IL-2 cells were transiently transfected with pcDNA3.1(+), myc-N17 Cdc42 (dominant negative), myc-V12 Cdc42 (constitutively active), myc-N17 Rac (dominant negative) or myc-V12 Rac (constitutively active). After serum starvation for 6 h, the migration assays of the transfected cells toward S1P (100 nM) were conducted in RPMI 1640 medium with 0.5% fatty acid free BSA for 3 h. The results are expressed as the mean ± S.E.M. of triplicate determinations. Statistical differences versus control (without S1P) were calculated by *t*-test (*, $p < 0.05$).

S1P₄ and S1P₁ are associated on the cell surface

Recent study by Van Brocklyn et al. suggests that S1P receptors form both homodimers as well as heterodimers with S1P₁, S1P₂ and S1P₃ (27). In the present study, our results suggest that both S1P₄ and S1P₁ are expressed on mouse T cells and both S1P₄- and S1P₁-mediated signaling is essential for S1P-induced migration of these cells. Based on these results, it is highly probable that S1P₄ and S1P₁ are associated on the cell surface of T cells and both receptors are involved in S1P-induced migratory response.

To clarify the possibility of the association of S1P₄ and S1P₁, we prepared the S1P₄ and S1P₁ co-expressing CHO cells. As shown in Figure 8A, both S1P₄ and S1P₁ are co-expressed in approximately 30% of C-terminal-HA-tagged S1P₄ (S1P₄-HA) stably expressing CHO cells transiently transfected N-terminal-FLAG-tagged S1P₁ (FLAG-S1P₁). Similar results were obtained when N-terminal-FLAG-tagged S1P₄ (FLAG-S1P₄) was transfected into C-terminal-HA-tagged S1P₁ (S1P₁-HA) stably expressing CHO cells (data not shown). Furthermore, we confirmed that approximately 2-fold amount of [³²P] S1P bound FLAG-S1P₁ expressing S1P₄-HA cells as compared with vector-transfected S1P₄-HA cells, indicating that S1P₄ and S1P₁ are co-expressed on the cell surface and possess a binding capacity for S1P (Figure 8B).

The cell lysates of S1P₄-HA cells transiently transfected
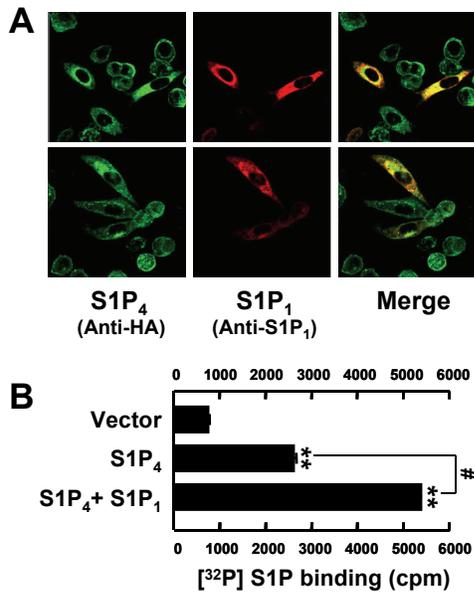
Figure 8. Co-expression of S1P$_4$ and S1P$_1$ on CHO cells. C-terminal HA-S1P$_4$ stably expressing CHO cells were transiently transfected N-terminal FLAG-S1P$_1$. (A) The cell surface expressions of S1P$_4$ and S1P$_1$ were visualized by staining with anti-HA Ab followed by Alexa 488-conjugated secondary Ab (Green) and anti-human S1P$_1$ Ab followed by Alexa 594-conjugated secondary Ab (Red), respectively. The cells co-expressing S1P$_4$ and S1P$_1$ were stained in yellow. (B) Co-expression of S1P$_4$ and S1P$_1$ was determined by [^{32}P] S1P binding assay. The results are expressed as the mean ± S.E.M. of triplicate determinations. Statistical differences were calculated by t-test when compared with vector-transfected CHO cells (**, $p < 0.01$) and only S1P$_4$-expressing CHO cells (#, $p < 0.05$).

Figure 9. The immunoprecipitation assay of CHO cells co-expressing S1P$_4$ and S1P$_1$. (A) C-terminal HA-S1P$_4$ stably expressing CHO cells were transiently transfected N-terminal FLAG-S1P$_1$ or vector alone. (B) C-terminal HA-S1P$_1$ stably expressing CHO cells were transiently transfected N-terminal FLAG-S1P$_4$ or vector alone. After serum starvation for 6 h, S1P$_4$ and S1P$_1$ co-expressing cells were treated with or without S1P (100 nM) for 5 min. Then the cell lysates were immunoprecipitated with anti-HA Ab and analyzed by Western blot using anti-FLAG Ab.

FLAG-S1P$_1$ or S1P$_1$-HA cells transfected FLAG-S1P$_4$ were immunoprecipitated using anti-HA antibody and the obtained immunoprecipitates were immunoblotted with anti-FLAG antibody. As shown in Figure 9, the immunoprecipitates from S1P$_4$-HA cells by anti-HA antibody contained FALG-S1P$_1$. By contrast, the immunoprecipitates from S1P$_1$-HA cells by anti-HA antibody contained FALG-S1P$_4$. There was no clear change in the pattern of immunoprecipitation when 100 nM S1P was added. Furthermore, we confirmed that S1P-induced ERK1/2 activation was induced not only in S1P$_1$-HA cells but also FLAG-S1P$_4$ and S1P$_1$-HA co-expressing cells (data not shown). These results indicate that S1P$_1$ and S1P$_4$ are associated or make complexes on the cell surface and that the formation of S1P$_1$/S1P$_4$ complexes is independent on S1P stimulation.

Discussion

S1P, a bioactive lysophospholipid, binds with nM-order affinities to five G-protein-coupled receptors, S1P$_{1-5}$ and stimulates multiple signaling pathways that resulted in calcium mobilization from intracellular stores, poly-

merization of actin, cell proliferation, differentiation, motility and survival in various types of the cells (2). Recently, it has been reported that S1P$_1$ is essential for lymphocyte recirculation, especially lymphocyte egress from secondary lymphoid tissues and the thymus (11) although S1P$_1$ is expressed ubiquitously in many tissues (10). In contrast to S1P$_1$, S1P$_4$ expression is reported to be highly restricted in lymphocyte-containing tissues including the thymus, spleen, bone marrow, appendix, peripheral leukocytes, and lung (28). It has been also reported that the mRNA expressions of S1P$_1$ and S1P$_4$ are the most prominent on mouse CD4 T cells (23). Furthermore, it has been demonstrated that S1P promotes cell migration through the activation of Rho family small GTPase, Cdc42, in mouse S1P$_4$-expressing CHO cells (24). However, little is known about the function of S1P$_4$ in lymphocytes.

Consistent with the results reported previously (7, 10), we confirmed in the present study that higher level of S1P$_4$ mRNA as well as S1P$_1$ mRNA is expressed in lymphoid tissues such as the spleen, thymus, lymph nodes, and Payer's

patches (Figure 1). Furthermore, we found that S1P at 10 to 100 nM significantly induced the migration of D10.G4.1 and EL-4.IL-2 cells as well as mouse CD4 T cells (Figures 2A and 3A). We also demonstrated that the significant levels of $S1P_1$ and $S1P_4$ mRNA were expressed in these cells by RT-PCR (Figures 2B and 3B). On the contrary, Wang et al. reported that no detectable level of $S1P_{1-5}$ mRNA is expressed in D10.G4.1 or EL-4.IL-2 cells, and these cells transfected with $S1P_4$ fail to induce migration toward S1P at 1 to 1000 nM (23). Thus, there is a discrepancy between the results by Wang et al. and ours in the aspect of $S1P_4$ mRNA expression and migratory response toward S1P in these cells.

In this study, we analyzed the mRNA expression pattern of S1P receptors in CD4 T cells and T cell lines separately because the mRNA expression levels of S1P receptors in D10.G4.1 and EL-4.IL-2 cells are rather lower when compared with CD4 T cells. If the mRNA expressions of S1P receptors in CD4 T cells and T cell lines are analyzed in the same assay, it may be concluded that the $S1P_4$ mRNA expression in T cell lines is extremely low level as compared with CD4 T cells. Furthermore, Wang et al. reported no migratory response of $S1P_4$-tranfected T cell lines toward S1P; however there was no description regarding serum starvation in their paper (23). D10.G4.1 and EL-4.IL-2 cells are usually grown in medium containing 10% FCS. Since it is well known that a bioactive concentration of S1P is contained in FCS (29), the serum starvation before experiments is very important for the bioassay of S1P. It is highly probable that S1P derived from FCS can induce the desensitization of S1P receptors without serum starvation and therefore additional S1P shows no effect in such a condition. From these reasons, we performed the migration assay toward S1P after sufficient serum starvation to avoid the influence of FCS-derived S1P.

In the present study, we confirmed that S1P-induced migration of D10.G4.1 cells and EL-4.IL-2 cells was almost completely inhibited by pretreatment with PTX, Toxin B, and (*S*)-FTY720-P (Figure 4). Because (*S*)-FTY720-P induces a phosphorylation of ERK1/2 in $hS1P_4$-CHO and $hS1P_1$-CHO cells (Figure 5), it is suggested that the agonistic activities at $S1P_4$ as well as $S1P_1$ are involved in the inhibition of S1P-induced T cell migration by the pretreatment with (*S*)-FTY720-P. Previous studies suggested that activation of Rho family small GTPase is an essential for S1P-induced cell motility (24, 26). In $S1P_1$-expressing CHO cells, Rac is activated by S1P and is involved in cytoskeletal rearrangement and cell migration (26). S1P also induces the activation of Rho and Rac in $S1P_3$-expressing CHO cells (26). Furthermore, it has been reported that S1P can induce the migration of mouse $S1P_4$-expressing CHO cells, and specific Cdc42 activation occurred in these cells upon stimulation with S1P (24). Our results revealed that Cdc42 and Rac are activated by S1P stimulation in D10.G4.1 and EL-4.IL-2 cells (Figure 6). The transfection with dominant negative or constitutively active form of Cdc42 and Rac indicated that S1P-induced migration of these cell lines was mediated through the activation of Cdc42 and Rac (Figure 7). Consistent with our results, it has been reported that

$S1P_4$-expressing CHO cells transfected with myc-N17 Cdc 42 (the dominant negative form) do not migrate toward S1P, whereas there is no apparent difference among vector-, wild type-, and myc-V12 Cdc42 (the constitutively active form)-transfected cells upon S1P stimulation (24). From these results, it is presumed that $S1P_4$ as well as $S1P_1$ are the functional receptors in T cells and S1P stimulation *via* $S1P_1$ and $S1P_4$ induces cell migration with activation of Cdc42 and Rac. A significant level of $S1P_5$ mRNA as well as $S1P_4$ mRNA is expressed in EL-4.IL-2 cells (Figure 3B); however the involvement of $S1P_5$ on migratory response toward S1P can be disregarded because D10.G4.1 cells that express no detectable level of $S1P_5$ mRNA show quite similar migratory response toward S1P as EL-4.IL-2 cells.

We demonstrated in the present study that the activation of Cdc42 and Rac is essential for S1P-induced migration of mouse T cells suggesting that the migration toward S1P is mediated *via* $S1P_1$ and $S1P_4$. Many types of cells express several S1P receptors, and more than one S1P receptor may be involved in mediating cellular responses. For examples, human umbilical vein endothelial cells (HUVEC) express $S1P_1$ and $S1P_3$ and both of these receptors are crucial for mediating various aspects of the angiogenic effects of S1P (30). Recent study suggested that S1P receptors, $S1P_1$, $S1P_2$ and $S1P_3$ form both homodimers as well as heterodimers with other members of S1P receptors (27). The immuno-precipitation assays using CHO cells co-expressing $S1P_1$ and $S1P_4$ receptors revealed that $S1P_1$ and $S1P_4$ are associated or make $S1P_1/S1P_4$ complexes on the surface and the formation of $S1P_1/S1P_4$ complexes is independent on S1P stimulation (Figure 9). Thus, it is highly likely that association of $S1P_1$ and $S1P_4$ plays an important role in migratory response of mouse T cells toward S1P. We are currently conducting further studies to elucidate a physiological role for $S1P_1$ and $S1P_4$ in T cells including association of $S1P_1$ and $S1P_4$.

Acknowledgements

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