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The Sphingosine 1-Phosphate Receptor $S1P_4$ Regulates Cell Shape and Motility via Coupling to G_i and $G_{12/13}$

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Abstract Sphingosine 1-phosphate (S1P) receptors represent a novel subfamily of G-protein-coupled receptors binding S1P specifically and with high affinity. Although their in vivo functions remain largely unknown, in vitro extracellular application of S1P induces distinct S1P receptor-dependent cellular responses including proliferation, differentiation, and migration. We have analyzed signaling pathways engaged by $S1P_4$, which is highly expressed in the lymphoid system. Here we show that $S1P_4$ couples directly to $G\alpha_i$ and even more effectively to $G\alpha_{12/13}$-subunits of trimeric G-proteins, but not to $G\alpha_q$ unlike other S1P receptors. Consequently, CHO-K1 cells ectopically expressing $S1P_4$ potently activate the small GTPase Rho and undergo cytoskeletal rearrangements, inducing peripheral stress fiber formation and cell rounding, upon S1P stimulation. Overexpression of $S1P_4$ in Jurkat T cells induces pertussis toxin-sensitive cell motility even in the absence of exogenously added S1P. In addition, $S1P_4$ is internalized upon binding of S1P. The capacity of $S1P_4$ to mediate cellular responses, such as motility and shape change through $G\alpha_i$- and $G\alpha_{12/13}$-coupled signaling pathways may be important for its in vivo function which is currently under investigation. J. Cell. Biochem. 89: 507–519, 2003.

Key words: sphingosine 1-phosphate; endothelial differentiation gene; G protein-coupled receptor; lymphocyte; immune system

Sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) are known as cell mitogens that are capable of inducing diverse cellular responses like proliferation and differentiation, chemotaxis, cell rounding, and tumor invasion (reviewed in Chun et al., 1999; Moolenaar, 1999). Recently, eight G protein-coupled receptors could be identified that bind specifically S1P and LPA as ligands [Hecht et al., 1996; An et al., 1997, 1998a; Lee et al., 1998; Bandoh et al., 1999; Im et al., 2000; Van Brocklyn et al., 2000]. Five of them ($S1P_{1-5}$) bind S1P [An et al., 1997; Lee et al., 1998; Im et al., 2000; Van Brocklyn et al., 2000], whereas three of them (LPA_{1-3}) are receptors for LPA [Hecht et al., 1996; An et al., 1998a; Bandoh et al., 1999]. Since most cells express more than one receptor for S1P or LPA, heterologous overexpression systems and antisense-RNA studies were used to distinguish between signaling events and cellular responses of each of these receptors [Goetzl et al., 1999; Kon et al., 1999].

The S1P receptors analyzed so far are able to activate the mitogen-activated protein kinases (MAP kinases) ERK1/2 and trigger the

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release of calcium from intracellular stores by activating phospholipase C (PLC) [Okamoto et al., 1998, 1999; An et al., 1999; Gonda et al., 1999; Sato et al., 1999; Im et al., 2000; Van Brocklyn et al., 2000; Yamazaki et al., 2000]. Upon stimulation, $S1P_1$ and $S1P_5$ exert a negative effect on the level of cyclic AMP (cAMP) [Zondag et al., 1998; Im et al., 2000], whereas binding of S1P to $S1P_2$ and $S1P_3$ raise the cAMP level [Kon et al., 1999]. In addition, it has been shown that $S1P_1$ undergoes agonist-induced receptor internalization that is thought to be part of the desensitization/resensitization cycle after binding of the specific ligand S1P [Lee et al., 1998; Liu et al., 1999].

With regard to phenotypical responses it has been demonstrated that $S1P_1$ and $S1P_3$ induce cell migration towards an S1P stimulus whereas $S1P_2$ inhibits cell migration and membrane ruffling [Kon et al., 1999; Okamoto et al., 2000]. $S1P_1$ is involved in angiogenesis as well as in other developmental processes like formation of the embryonic skeletal system and neuronal differentiation [Hla and Maciag, 1990; Liu and Hla, 1997; Liu et al., 2000]. The expression of $S1P_2$ is enhanced in rat brain during embryogenesis, suggesting a role in early differentiation of neuronal cells and axon development [MacLennan et al., 1997].

Recently we isolated $S1P_4$ from in vitro differentiated dendritic cells [Gräler et al., 1998]. Because of its predominant expression in cells and tissues of the lymphoid system we suggest that $S1P_4$ may play an important role in immune surveillance [Gräler et al., 1999]. Here we show the direct coupling of $S1P_4$ to $G\alpha_i$ and $G\alpha_{12/13}$-subunits of trimeric G-proteins. We provide evidence for pertussis toxin (Ptx)-sensitive PLC activation and cytoskeleton rearrangements as corresponding downstream signaling events. It turned out that $S1P_4$ is a potent activator of $G\alpha_{12/13}$ and of the small GTPase Rho, respectively. Stimulation of $S1P_4$ expressing cells induces enhanced peripheral stress fiber formation and cell rounding. In addition, an increased Ptx-sensitive cell motility of stably transfected unstimulated Jurkat cells as well as an agonist-induced internalization of $S1P_4$ was observed.

MATERIALS AND METHODS

Materials

DMEM, RPMI, and FCS were purchased from Biochrom (Terre Haute, IN), cell culture supplements, Ptx, and G418 were from Gibco/BRL (Rockville, MD). PBS was from Seromed (Berlin, Germany), other chemicals were purchased from Merck (Poole, Dorset, UK). *myo*-[^{3}H]inositol (18.6 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NJ), [2,8-^{3}H]adenine and [α-^{32}P]GTP (3,000 Ci/mmol) were purchased from NEN Life Sciences (Boston, MA). Wildtype- and N19-RhoA constructs in pcDNA3.1 were a kind gift from Dr. Yi Zheng, University of Tennessee, TN. S1P and LPA were purchased from Sigma-Aldrich (St. Louis, MO). Other sources are mentioned in the text.

Cell Culture and Transfection

Chinese hamster ovary cells (CHO-K1, ATCC CCL-61) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FCS). Human embryonic kidney cells (HEK293, ATCC CRL-1573) were cultured in DMEM containing 10% FCS. The human leukemia T-cell line Jurkat (ATCC TIB-152) was cultured in RPMI medium containing 2 mM L-glutamine and 10% FCS. The $S1P_4$ expression plasmid RC/CMV (Invitrogen, Carlsbad, CA) containing the C-terminal *myc*-epitope-tagged human or murine $S1P_4$ or wildtype-RhoA and N19-RhoA constructs in pcDNA3.1 (Invitrogen) were transfected into CHO-K1 or Jurkat cells by electroporation (20 μg DNA; 5×10^6 cells in 800 μl PBS-d; CHO-K1: 250 V, 1,070 μF; Jurkat: 280 V, 1,070 μF) and into HEK293 cells by calciumphosphate precipitation as described previously [Emrich et al., 1993]. Transfection efficiencies were typically 20–35%, checked by fluorescence-activated cell sorting (FACS) analysis.

Fluorescence-Activated Cell Sorting (FACS) Analysis

Construction, expression, and FACS-analysis of the *myc*-epitope (spacer-epitope: PGGSGP-EQKLISEEDLL) with the murine anti-*myc*-epitope antibody 9E10 was performed as described previously [Emrich et al., 1993]. The murine $S1P_4$ receptor construct was also detected with the newly generated monoclonal rat antibodies 2A1, 6D7, and 8B4, directed against the N-terminal part of the murine $S1P_4$ receptor. Goat-anti-rat-R-phycoerythrin (PE) and donkey-anti-mouse-PE antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) were used for the detection of unlabeled antibodies from rat and mouse.

Photolabeling of Receptor-Activated G-Proteins

Photolabeling of membrane G-proteins derived from CHO cells and immunoprecipitation were performed as described previously [Grosse et al., 2000]. In brief, [α-^{32}P]GTP azidoanilide was synthesized and purified as described [Offermanns et al., 1991]. CHO-K1 membranes (200 μg of protein per assay tube) were incubated at 30°C in a buffer containing 0.1 mM EDTA, 10 mM $MgCl_2$, 30 mM NaCl, 1 mM benzamidine, 50 mM HEPES (pH 7.4), and for $G\alpha_i$ coupling also with 10 μM GDP. Samples were incubated for 3 min ($G\alpha_i$) or 30 min ($G\alpha_{q/11}$, $G\alpha_{12/13}$, $G\alpha_{15/16}$) with 10 nM [α-^{32}P]GTP azidoanilide (130 kBq per tube) in the absence or presence of 1 μM S1P. The final assay volume was 120 μl. After stopping the reaction by cooling the samples on ice, samples were centrifuged at 4°C for 5 min at 12,000*g* and pellets were resuspended in 60 μl of the buffer described above supplemented with 2 mM glutathione. Suspended membranes were irradiated for 10 s at 4°C with a 254 nm UV-lamp (Vilber Lourmat, Torcy, France) [Offermanns et al., 1991]. For immunoprecipitation, photolabeled membranes were pelleted and solubilized in 40 μl of 2% SDS at room temperature. Thereafter, 120 μl of precipitating buffer (1% Nonidet P-40, 1% desoxycholate, 0.5% SDS, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 mM Tris/HCl, pH 7.4) was added. Solubilized membranes were centrifuged at 4°C for 10 min at 12,000*g* to remove insoluble material, and 50 μl of 10% protein A-Sepharose beads (Sigma-Aldrich) was added for preclearing of the lysates. After 1 h, Sepharose beads were removed by centrifugation, and 30 μl of the following antisera were added to the supernatants: Z811 ($G\alpha_{q/11}$), AS233, and AS343 ($G\alpha_{12/13}$), AS266 ($G\alpha_{i,\ common}$) [Laugwitz et al., 1994], and anti-$G\alpha_{15/16}$. After overnight incubation of samples at 4°C at constant rotation, 60 μl of 10% protein A-Sepharose beads were added, and samples were incubated for additional 3 h. After washing of Sepharose beads, immunoprecipitated G-protein α-subunits were separated on 13% polyacrylamide gels and visualized by autoradiography of dried gels with Kodak X-Omat AR-5 films (Eastman Kodak, Rochester, NY) or with a phosphorimaging screen (Fuji, Stamford, CT).

Measurement of Intracellular Inositol Phosphate and cAMP Accumulation

For cAMP measurements, CHO-K1 cells were seeded into six-well plates (8×10^5 cells/well) 2 days prior to functional assays. [2,8-^{3}H]adenine (2 μCi/ml) was added to the growth medium. After a labeling period of 24 h, cells were washed once in PBS, followed by a 20 min preincubation with serum-free DMEM containing 1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich). Subsequently, cells were stimulated with 1 μM S1P or 50 μM forskolin for 1 h. Reactions were terminated by addition of 2 ml 5% trichloracetic acid. For IP determinations, CHO-K1 cells were seeded into 12-well plates (3×10^5 cells/well) 3 days prior to functional assays. CHO-K1 cells were incubated with 2 μCi/ml of *myo*-[^{3}H]inositol for 18 h. Thereafter, cells were washed once with serum-free DMEM containing 10 mM LiCl. Accumulation of intracellular inositol phosphate and cAMP were determined as described previously [Grosse et al., 2000].

GST-Rhotekin Fusion Protein

Cultures of *Escherichia coli* (DH5α) transformed with the plasmid pGEX-rhotekin were grown to an A_{600} of 0.5, and fusion protein expression was induced by addition of 0.3 mM isopropyl-1-thio-β-D-galactoside (Sigma-Aldrich). After 3 h, cells were collected by centrifugation, and the pellet was resuspended in ice-cold TBS containing 5 mM $MgCl_2$, 1 mM DTT, and 5 mM phenylmethylsulfonyl fluoride. After sonification of lysates, Triton X-100 was added to a final concentration of 1%, and lysates were incubated at 4°C for 30 min. Lysates were then centrifuged (12,000*g*, 10 min at 4°C), and glycerol was added to a final concentration of 10%. The fusion protein preparations were stored in aliquots at −80°C for up to 8 weeks.

Determination of Rho Activity

Activation of Rho was measured as previously described with minor modifications [Ren et al., 1999]. After stimulation of CHO-K1 cells stably transfected with RC/CMV vector (Invitrogen) or the human $S1P_4$-myc construct in 6 cm dishes, monolayers were washed with ice-cold TBS and lysed for 15 min by the addition of 300 μl of Rho-RIPA buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, 0.1 μM aprotinin, 1 μM

leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Lysates were cleared by centrifugation and incubated for 30 min at 4°C with GST-rhotekin precoupled to glutathione-Sepharose 4B (Amersham Pharmacia Biotech). Sepharose beads were collected by centrifugation (5,000 rpm, 1 min at 4°C), washed three times with RIPA buffer, and proteins were denatured in SDS sample buffer. Precipitates were resolved by SDS–PAGE on 15% acrylamide gels, and proteins were subsequently transferred to Biotrace polyvinylidene difluoride membranes (Pall Gelman Laboratory, Ann Arbor, MI). Rho proteins were probed with the monoclonal Rho antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a secondary horseradish peroxidase-conjugated anti-mouse IgG antiserum (Sigma-Aldrich) and visualized on X-ray film (Eastman Kodak) by enzyme-linked chemiluminescence (Amersham Pharmacia Biotech).

Cell Staining

CHO-K1 cells were seeded on coverslips with about 10% confluency 1 day before they starved serum-free for 16 h. They were washed three times with PBS-d (Gibco/BRL) and fixed by adding 5% paraformaldehyde (Sigma-Aldrich) for 15 min on ice. After three additional washing steps and permeabilization with 0.5% Triton-X-100 (Merck) for 5 min at room temperature, cells were incubated with 5% milk powder for 1 h. To investigate cytoskeleton rearrangements, Alexa-fluor-488 labeled phalloidin (Molecular Probes, Eugene, OR) was added for 1 h. For visualization of $hS1P_4$-myc expression, cells were incubated for 1 h with the murine anti-*myc*-epitope antibody 9E10 (Roche Molecular Biochemicals, Indianapolis, IN). After three washing steps with PBS-d, cells were incubated for 1 h with donkey-anti-mouse-Cy3 antibodies (Jackson Immunoresearch Laboratories). Cells were analyzed by fluorescence microscopy as described previously [Kusch et al., 2000].

Chemotaxis Assays

Transwell chambers (6.5 mm diameter, pores with 5 μm diameter, Costar) were incubated in 20 μg/ml mouse collagen type IV (BD Biosciences, San Diego, CA) at 4°C overnight and washed two times with PBS-d. Jurkat cells were grown overnight in RPMI medium supplemented with 2 mM L-glutamine and 1% FCS. After resuspending the cells in RPMI containing the appropriate stimulus, 2 mM L-glutamine, 25 mM HEPES, pH 7.3, and 0.1% fatty acid-free bovine serum albumin (Sigma-Aldrich), 100 μl of the cell suspension (1×10^7 cells/ml) was added to the upper chamber. Four hundred and fifty microlitres of the same medium was used for the lower chamber. Cells were incubated for 4 h under normal cell culture conditions. Subsequently, 350 μl of the lower chamber was concentrated to 50 μl, and cells were counted for 1 min by flow cytometry (BD Biosciences).

Receptor Internalization Experiments

After serum-free starvation for 16 h prior to analysis, cells were briefly trypsinized and resuspended in DMEM containing 25 mM HEPES and 0.1% fatty acid-free bovine serum albumin. The appropriate stimulus was added, and the cells were incubated at 37°C. After the corresponding time, 100 μl of the cell suspension was added to 1.4 ml of ice-cold FACS-buffer (PBS-d, 4% FCS, 10 mM EDTA, pH 7.3). Cells were kept on ice and analyzed by FACS.

Generation of Monoclonal Antibodies Against the Murine $S1P_4$ Receptor

The N-terminus (aa 2-56) of the murine $S1P_4$ receptor ($mS1P_4$) was expressed as a C-terminal hexahistidine fusion protein using the pQE60 vector system (Qiagen, Valencia, CA) according to the manufacturer's protocol under denaturing conditions. Immunization of rats and the generation of hybridoma cells was done according to standard protocols. Thirty four different hybridoma supernatants from one fusion were positive in a sandwich-ELISA using the same hexahistidine fusion protein that was also used for immunization. Five of these supernatants were also positive in a FACS-analysis with transiently $mS1P_4$ transfected CHO-K1 cells, three of which (2A1, 6D7, and 8B4) could be established by limiting dilution and grown as single clones. The antibodies of all three supernatants match the IgG 2Aκ immunoglobulin subtype as determined by standard protocols.

RESULTS

Generation of Cell Lines Stably-Expressing $S1P_4$

Since many cultured cells respond to an S1P challenge through endogenous S1P receptors, it was necessary to establish a heterologous

expression system with low background activity in order to elucidate the specific signaling pathways triggered by the human S1P4 receptor ($hS1P_4$). Previous studies have already demonstrated that CHO-K1 cells give a low background response upon stimulation with S1P [Kon et al., 1999], and PCR-studies of Jurkat cells showed only a low amount of *S1P4*-template present in these cells [Motohashi et al., 2000]. CHO-K1 and Jurkat cell lines stably expressing $hS1P_4$ were generated with a C-terminal *myc*-epitope tag (CHO-K1/$hS1P_4$-myc and Jurkat/$hS1P_4$-myc). Both transfected cell lines were tested for $S1P_4$ expression by FACS analysis (Fig. 1A). Surface expression of $S1P_4$ on stably transfected CHO-K1 cells was also confirmed by confocal microscopy (Fig. 1B). Both cell lines were used to determine the

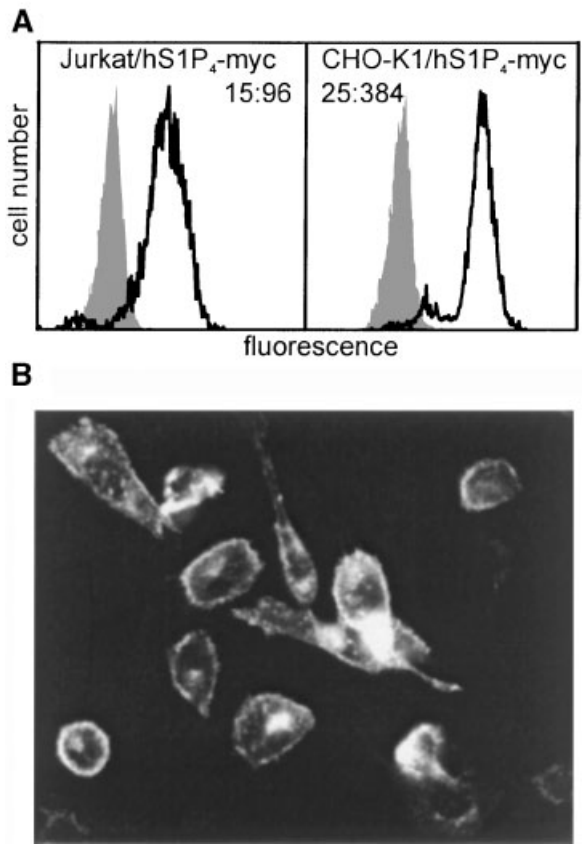

Fig. 1. Stable expression of $hS1P_4$-myc on CHO-K1 and Jurkat cell lines. **A**: FACS-analysis of stably $hS1P_4$-myc transfected CHO-K1 and Jurkat cell lines. Cells were fixed and permeabilized prior to detection of the intracellular C-terminal *myc*-epitope tag with the specific monoclonal anti-*myc*-epitope antibody 9E10. Vector transfected control cells are shown in gray, and linear means are given by numbers. **B**: Surface expression of $hS1P_4$-myc on transfected CHO-K1 cells, detected with the anti-*myc*-epitope antibody 9E10.

functional characteristics of $S1P_4$ with regard to its signaling properties and with reference to $S1P_4$ mediated cellular responses.

G-Protein-Coupling of the $S1P_4$ Receptor

First we examined the direct coupling of Gα-subunits of trimeric G-proteins to the $S1P_4$ receptor. For this purpose, membrane preparations of CHO-K1/$hS1P_4$-myc cells were incubated with the GTP analog [α-^{32}P]GTP azidoanilide. Following stimulation with S1P, [α-^{32}P]GTP azidoanilide binds to the corresponding activated Gα-subunits but cannot be hydrolyzed efficiently. Therefore, activated Gα-subunits bound to [α-^{32}P]GTP azidoanilide accumulate and can be selectively detected on an autoradiogram after immunoprecipitation with specific anti-Gα antisera and subsequent SDS–PAGE. Antisera directed against $G\alpha_{15/16}$, $G\alpha_{12/13}$, $G\alpha_q$, and $G\alpha_i$ were used to determine the specific Gα-coupling of $S1P_4$. These studies revealed that $S1P_4$ activates $G\alpha_i$ and $G\alpha_{12/13}$-subunits of trimeric G-proteins (Fig. 2A,B). $G\alpha_q$ is not affected (Fig. 2A,B). In particular, the $G\alpha_{12/13}$-subunits are activated very potently. $G\alpha_{15/16}$ which has recently been suggested to be linked to $S1P_4$ because of their genetic proximity [Contos et al., 2002] is not activated by $S1P_4$. As a control CHO-K1 cells stably transfected with gonadotropin-releasing hormone (GnRH) receptor [Grosse et al., 2000] did not activate any of the Gα-subunits examined after stimulation with S1P (Fig. 2A,B). The specificity of the antisera and the expression of the tested Gα-subunits in CHO cells have already been demonstrated [Grosse et al., 2000].

Effect of $S1P_4$ on PLC Activation

$S1P_4$ activates the MAP kinase Erk1/2 [Van Brocklyn et al., 2000]. This signaling pathway is Ptx-sensitive and therefore dependent on the activation of $G\alpha_i$-subunits of trimeric G-proteins. To examine other downstream signaling properties of $S1P_4$ linked to G-proteins, we assessed activation of the PLC by determining inositol phosphate accumulation in CHO-K1/$hS1P_4$-myc cells. As shown in Figure 3A, more than a twofold increase in PLC activation was observed after stimulation with 1 μM S1P in $S1P_4$ transfected CHO-K1 cells, whereas only a marginal response was detected in untransfected control cells. The $S1P_4$ mediated PLC activation is Ptx-sensitive and therefore dependent on $G\alpha_i$ activation. In addition, cells were

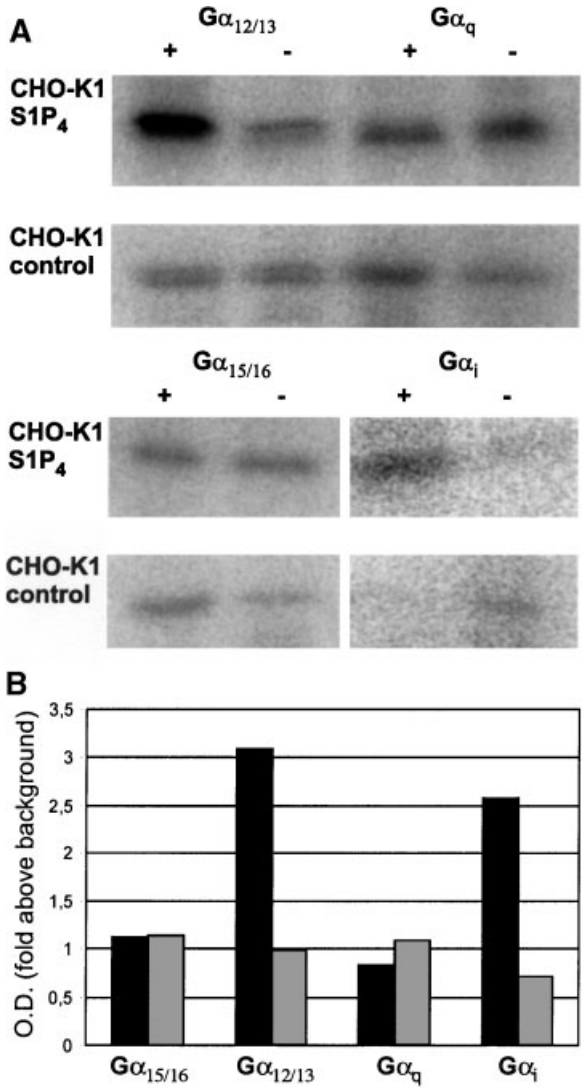

Fig. 2. Activated G-proteins in membrane preparations of CHO-K1/hS1P$_4$-myc cells. Membrane preparations were photolabeled with [α-^{32}P]GTP azidoanilide in the absence (−) or presence (+) of 1 μM S1P and immunoprecipitated with the G$\alpha_{15/16}$, G$\alpha_{12/13}$, Gα_q, and Gα_i antisera as described in ''Materials and Methods.'' **A**: Precipitated proteins were resolved in SDS–PAGE and visualized by autoradiography. **B**: Densitometric analysis of the corresponding signals on the autoradiogram normalized towards the expression in control cells. S1P-stimulated cell preparations are shown in black, unstimulated controls are shown in gray.

stimulated with 1 mM ATP. The corresponding ATP receptors couple to G$\alpha_{q/11}$-subunits of trimeric G-proteins that are much more potent in activating PLC than the $\beta\gamma$-subunits responsible for the Gα_i-induced PLC activation resulting in an up to fivefold increase in inositol phosphate production (Fig. 3A). The ATP-induced PLC activation is Ptx-insensitive and therefore not linked to Gα_i-coupled signaling pathways (Fig. 3A).

S1P$_4$ Does not Induce cAMP Accumulation

The adenylyl cyclase is known to be an important effector of trimeric G-proteins mainly activated by Gα_s subunits. Therefore, we were interested whether stimulation of S1P$_4$ would lead to an increase of the intracellular cAMP level or not. For this purpose, we stimulated CHO-K1/hS1P$_4$-myc cells and vector transfected control cells with 1 μM S1P for 1 h and tested for cAMP accumulation as described in

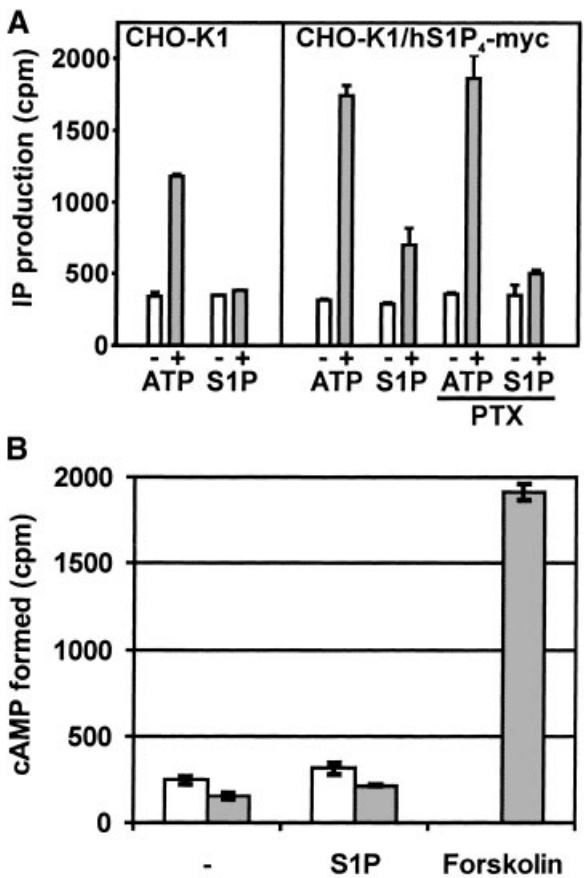

Fig. 3. Second messenger production in CHO-K1/hS1P$_4$-myc cells. **A**: Ptx-sensitive inositol phosphate accumulation induced by the S1P$_4$ receptor. Non-transfected CHO-K1 cells and CHO-K1/hS1P$_4$-myc cells were seeded in 12-well plates and incubated in the presence of Ptx (0.1 μg/ml, 18 h prior to stimulation) as indicated. Inositol phosphate accumulation was determined after 45 min of incubation with vehicle (−), 1 μM S1P, and 1 mM ATP (+) respectively. Data are means + SE of two independent experiments, each performed in triplicate. **B**: cAMP formation in vector transfected (white) versus hS1P$_4$-myc (gray) expressing CHO-K1 cells. Cells were incubated with 1 μM S1P or 50 μM forskolin for 1 h as indicated. Shown are means ± SE of triplicates.

"Materials and Methods." The $hS1P_4$ expressing CHO-K1 cells as well as the control cells show a slight increase in cAMP production upon S1P stimulation, but no $S1P_4$ specific effect could be detected (Fig. 3B). Forskolin as a positive control directly activates the adenylyl cyclase and produced more than a tenfold increase in cAMP accumulation in CHO-K1/$hS1P_4$-myc cells (Fig. 3B). Thus, $S1P_4$ does not activate the adenylyl cyclase via the $G\alpha_s$ subunit of trimeric G-proteins.

$S1P_4$ Mediated Effect on Cell Motility

The migration of lymphocytes, macrophages, and dendritic cells determines their correct trafficking and homing. By examining the migratory capacity of $S1P_4$ overexpressing cells, an enhanced motility of Jurkat/$hS1P_4$-myc cells was observed even in the absence of S1P (Fig. 4). $S1P_4$ receptor-mediated cell motility was sensitive to Ptx, indicating that this effect is linked to the $G\alpha_i$-subunit of trimeric G-proteins (Fig. 4). Interestingly, primary mouse splenocytes also show a reduced cell motility after Ptx treatment (Fig. 4). $S1P_4$ is highly expressed on these cells [Gräler et al., 2002], suggesting that $S1P_4$ induces the observed increase in cell motility also on primary lymphocytes. Vector transfected control cells did not exhibit an increased motility (Fig. 4). Jurkat/$hS1P_4$-myc cells as well as control cells show a 1.5-fold increase of cell motility in the presence of 10 nM S1P (Fig. 4). This effect is barely seen on primary mouse splenocytes and not dependent on $hS1P_4$-myc expression (Fig. 4), indicating that it is not linked to $S1P_4$-induced signaling pathways. S1P did not elicit migration of Jurkat/$hS1P_4$-myc cells compared with vector transfected control cells (Fig. 4). In addition, we did not detect migration of CHO-K1/$hS1P_4$-myc cells using S1P concentrations between 0.1 nM and 1 μM. Furthermore, expression and stimulation of $S1P_4$ did not affect the migratory capacity of Jurkat cells endogenously expressing the CXCR4 chemokine receptor towards its specific ligand SDF-1 (data not shown).

$S1P_4$ Regulates Rho Activation

The small GTPase Rho can be activated by a specific subgroup of trimeric G-proteins and is involved in several cellular responses like cytoskeleton rearrangements, integrin-mediated adhesion, and actin-dependent effector responses. We performed a Rho-assay to examine the

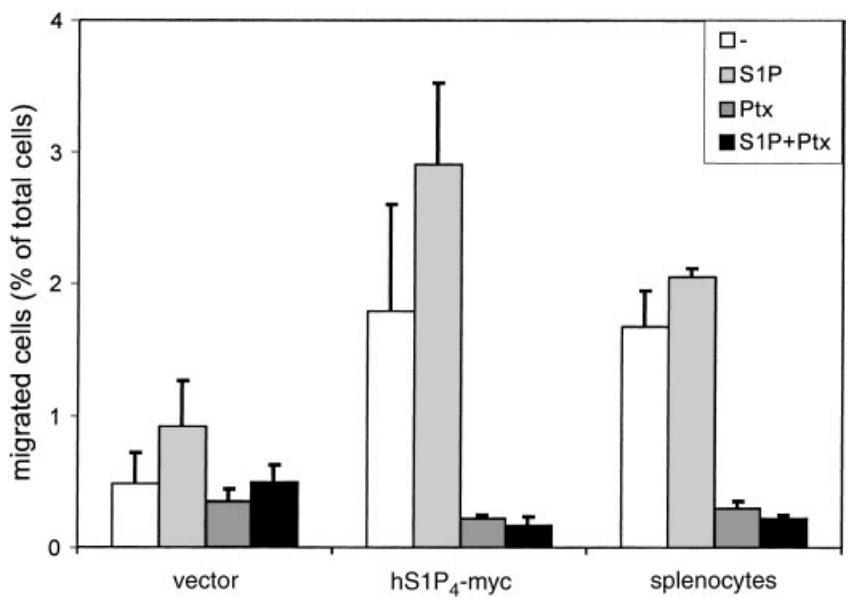

Fig. 4. Increased agonist-independent Ptx-sensitive cell motility of stably $hS1P_4$-myc transfected Jurkat cells. Stably vector transfected or stably $hS1P_4$-myc transfected Jurkat cells as well as freshly isolated mouse splenocytes were seeded in Transwell-chambers with or without simultaneous S1P stimulus (10 nM) in the upper and in the lower chamber as described in ''Materials and Methods.'' Cells were incubated with 0.1 μg/ml Ptx for 2 h prior to the experiment when indicated. After 4 h, cells were harvested and counted. Shown are means + SE, $n \geq 4$.

regulatory capacity of $S1P_4$ on Rho activation. Whereas vector transfected control cells do not show any significant increase in Rho-activation upon S1P stimulation, CHO-K1/h$S1P_4$-myc cells rapidly activate Rho within 1 min after S1P stimulation (Fig. 5A,B). The activity of Rho declines within 20 min after S1P stimulation, indicating the short half-life of $S1P_4$-induced Rho activation. Thus, $S1P_4$ is a very rapid and potent activator of the small GTPase Rho.

$S1P_4$ Mediated Cytoskeleton Rearrangements

An important event with respect to changes in adhesion and motility of cells are alterations within their cytoskeleton to promote a higher degree of mobility for managing new circumstances. To examine the influence of $S1P_4$ in this connection, we looked for S1P-induced cytoskeleton rearrangements in stably transfected CHO-K1 cells. No significant increase in the amount of peripheral stress fibers and cell rounding can be seen in control cells (Fig. 6A). Addition of S1P to CHO-K1/h$S1P_4$-myc cells however induces a significant increase in the amount of peripheral stress fibers and rounded cells (Fig. 6B). The amount of rounded cells in the total cell population as determined by three independent cell counts was $3 \pm 1\%$ SE for unstimulated CHO-K1 control cells and CHO-K1/h$S1P_4$-myc cells, $5 \pm 2\%$ SE for S1P-stimulated

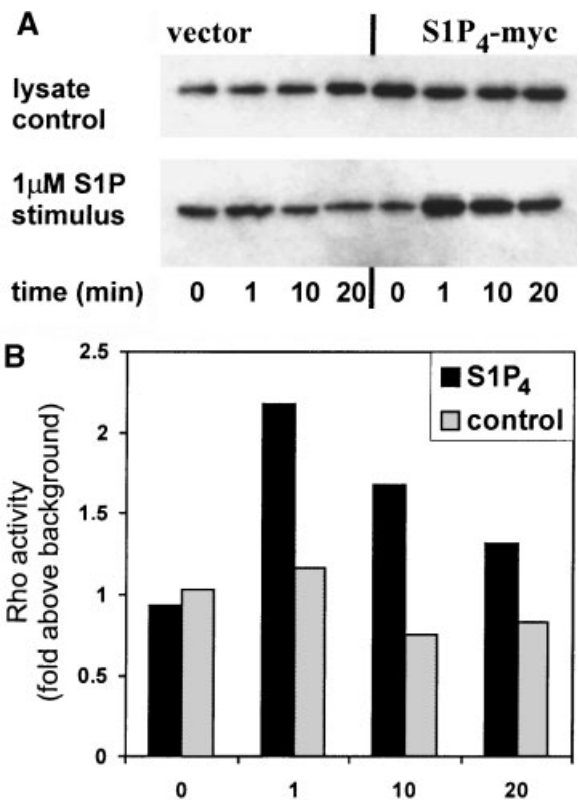

Fig. 5. Activation of Rho in CHO-K1/h$S1P_4$-myc cells. **A**: Vector transfected CHO-K1 cells and CHO-K1/h$S1P_4$-myc cells starved with 0.5% serum overnight prior to stimulation with 1 μM S1P for 1, 10, and 20 min. GTP-bound RhoA was analyzed applying a pull down assay using SEPHAROSE conjugated Rhotekin-GST fusion protein. The lysate control shows equal amounts of Rho expressed in each cell preparation. Shown is one representative out of two similar results. **B**: Densitometric quantification of the signals shown in (A). Vector transfected cells are shown in gray, h$S1P_4$-myc transfected cells are shown in black.

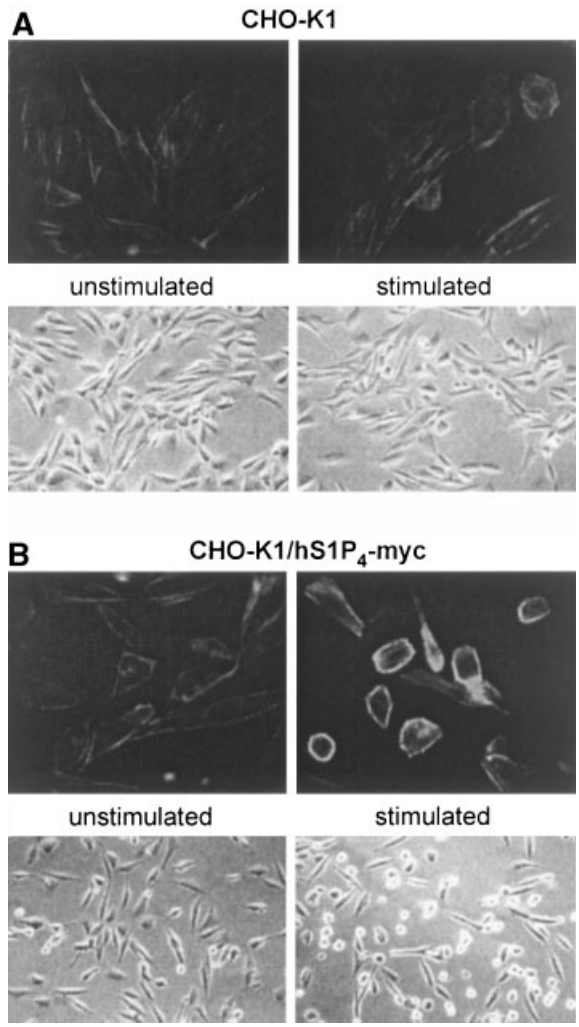

Fig. 6. Cytoskeleton rearrangements in CHO-K1/h$S1P_4$-myc cells. **A**: Non-transfected CHO-K1 cells and (**B**) CHO-K1/h$S1P_4$-myc cells were seeded on cover-slips in six-well plates, starved serum-free for 16 h, and were subsequently stimulated for 20 min with 1 μM S1P as indicated. In the **upper row** cells were stained with Alexa-fluor-488 phalloidin and analyzed by confocal microscopy, in the **lower row** the cell shape is shown by light microscopy.

control cells, and 53 ± 7% SE for S1P-stimulated CHO-K1/$hS1P_4$-myc cells.

Agonist-Induced Internalization of $S1P_4$

An important aspect of functional studies is the correct expression and presentation of the receptor on the cell surface. Using the $hS1P_4$-myc construct we provided already in an earlier study evidence that $hS1P_4$ is inserted into the plasma membrane on the cell surface in the expected orientation with the N-terminus facing the cells exterior and the C-terminus inside the cell [Van Brocklyn et al., 2000]. For the purpose of further functional studies we generated monoclonal antibodies against murine $S1P_4$ ($mS1P_4$, see "Materials and Methods"). These antibodies are suitable to detect $mS1P_4$-myc, but not $hS1P_4$-myc on the surface of transiently transfected HEK293 cells by flow cytometry (Fig. 7A). Western-blots of membrane protein extracts from these cells revealed a specific signal around 42 kDa that was not found on Interleucin-8 receptor A transfected HEK293 cells (Fig. 7B). A second weaker signal appears at about 46 kDa (Fig. 7B). Since there is no need of cell permeabilization in order to recognize the antibody epitope we used these antibodies to monitor the kinetics of $mS1P_4$ surface expression upon addition of S1P. Within minutes after stimulation with 1 μM S1P, the $mS1P_4$ specific signal on these cells significantly decreases and reaches a minimum of approximately 65% compared to the unstimulated control cells after 45 min (Fig. 7C). This effect is specific for S1P and cannot be seen with other stimuli like LPA (Fig. 7C). However, Ptx does not interfere with receptor internalization, and our monoclonal antibodies against $mS1P_4$ are unable to trigger the internalization process (data not shown). Cells that were permeabilized after S1P stimulation for the specified time points show the same receptor signal as unstimulated cells (data not shown). This demonstrates that the complete amount of detected $mS1P_4$ does not change. Therefore, $mS1P_4$ receptors are not degraded, and S1P binding to $mS1P_4$ does not interfere with antibody binding.

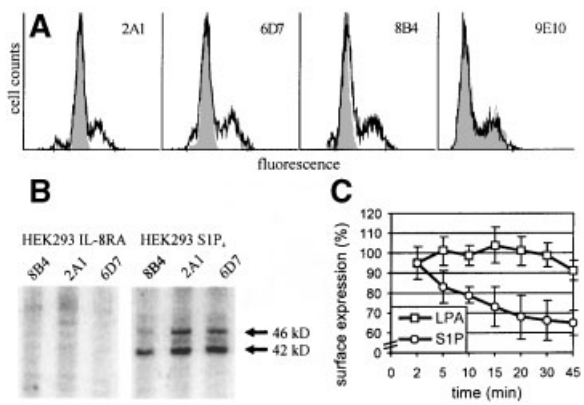

Fig. 7. Detection of the murine $S1P_4$ receptor on transiently transfected HEK293 cells. **A**: FACS-analysis of anti-mouse-$S1P_4$ monoclonal antibodies 2A1, 6D7, and 8B4 on mouse $S1P_4$-myc (black line) and on human $S1P_4$-myc (filled gray) transfected HEK293 cells. The anti-myc-epitope antibody 9E10 is shown as a control. **B**: Western-blot with membrane preparations of Interleucin-8 receptor A and mouse $S1P_4$-myc transfected HEK293 cells. **C**: Detection of $mS1P_4$-myc on the surface of transiently transfected HEK293 cells using the anti-murine $S1P_4$ antibodies 8B4, 2A1, and 6D7 by FACS-analysis. After addition of 1 μM S1P, the murine $S1P_4$ surface expression decreases within 45 min (circles). One micromolar LPA has no significant influence on the surface expression of $S1P_4$ (rectangles). The FACS signal was correlated to unstimulated cells at the corresponding time point. Shown are means ± SE, $n \geq 3$.

DISCUSSION

The responses of diverse target cells to low molecular weight lipid compounds, such as S1P and LPA have been shown to be mediated in large part by G protein-coupled receptors which bind these lipids and thus mediate their physiological functions. We have cloned recently one member of the S1P receptor family, $S1P_4$, and showed that it specifically binds S1P as an agonist [Gräler et al., 1998; Van Brocklyn et al., 2000]. However, apart from its obvious field of activity, the immune system, little is known about the importance and physiology of $S1P_4$ in this distinct compartment. This report provides information regarding the signal cascade and cellular responses triggered by the $S1P_4$-S1P interaction.

One of the first events with regard to GPCR signaling is the activation of certain G-proteins. We demonstrate in this study that $S1P_4$ activates the G-protein subunits $G\alpha_i$ and $G\alpha_{12/13}$ by two different methods: (1) By direct labeling of the activated Gα-subunits and (2) by analyzing functional downstream signaling events. $S1P_4$ activates $G\alpha_{12/13}$ very potently without affecting $G\alpha_q$ (Fig. 2). Other S1P receptors such as $S1P_2$ and $S1P_3$ couple with both, $G\alpha_{12/13}$ and $G\alpha_q$ respectively [Windh et al., 1999]. All S1P receptors currently known signal via the $G\alpha_i$-subunit of trimeric G-proteins [An et al., 1998b; Windh et al., 1999]. Thus, $S1P_4$ differs from these S1P

receptors concerning its selective G protein-coupling. $S1P_4$ is also not inducing an increase in cAMP accumulation (Fig. 3B). Therefore, it is unlikely that $S1P_4$ couples to the $G\alpha_s$-subunit of trimeric G proteins. Other S1P receptors like $S1P_2$ and $S1P_3$ are able to activate the adenylyl cyclase very potently resulting in cAMP levels up to 10 times higher than vector controls [Kon et al., 1999] although direct coupling studies performed with [^{35}S]GTPγS did not show any specific $G\alpha_s$-coupling [Windh et al., 1999]. A recent study suggests that $S1P_4$ is linked to $G\alpha_{15/16}$ signaling because of their genomic proximity, a fact that we already published in 1999 [Gräler et al., 1999]. This hypothesis does not stand a closer look because $G\alpha_{15/16}$ does not couple to $S1P_4$ (Fig. 2A,B), and the human transcript for $G\alpha_{16}$ does not show the same expression pattern as $S1P_4$ (data not shown).

Some downstream signaling events induced by $S1P_4$ like the activation of the mitogen-activated protein-kinases (MAP kinases) ERK1/2, the activation of PLC, or the ability to open intracellular calcium stores have already been investigated [Van Brocklyn et al., 2000; Yamazaki et al., 2000]. We confirmed the $S1P_4$-induced Ptx-sensitive PLC activation in our CHO-K1 cell system as a functional $G\alpha_i$-coupled signaling pathway (Fig. 3A). Our results concerning $S1P_4$-induced Rho activation (Fig. 5) and cytoskeleton rearrangements (Fig. 6) form a link to the $G\alpha_{12/13}$ directed signal transduction because former studies have shown that stress fiber formation is coupled via Rho to $G\alpha_{12/13}$ activation [Buhl et al., 1995]. $G\alpha_{13}$ is able to directly stimulate the p115 RhoGEF-catalysed guanine nucleotide exchange on Rho [Hart et al., 1998]. In addition, the described effects on cell rounding and peripheral stress fiber formation suggest an involvement of $S1P_4$ on the cell shape that could be important for its in vivo function (Fig. 6B). Similar results concerning cell rounding obtained from studies of $S1P_2$ and $S1P_3$ overexpressing HEK293 and pheochromocytoma PC12 rat cells [Van Brocklyn et al., 1999] as well as from $S1P_2$ overexpressing CHO cells with respect to stress fiber formation [Gonda et al., 1999] may be taken as an indication for a physiological feature commonly induced by S1P receptors.

It may be surprising that the ectopic expression of $S1P_4$ in Jurkat cells (Fig. 3) per se is already sufficient to direct cells towards a phenotype observed later on when its ligand S1P is added. This could be interpreted to mean that $S1P_4$ has a high intrinsic activity in terms of some independence from ligand binding for signaling or that T cells produce S1P at a level sufficient for signaling. However, this observation may well be a consequence of the ubiquitous presence of low amounts of ligand produced by the cells thus initiating autocrine stimulation. This renders the study of S1P receptors inevitably complicated because it is difficult to establish suitable cell-based test systems. Given the widespread distribution of S1P receptors and their ligand S1P in tissues it is worth speculating that an apparently unstimulated stage of an observed cell should be more precisely defined as a low level-induced stage, at least from the point of view of S1P-based cell physiology. In keeping with this hypothesis, other reports have shown that LPA_2 is able to activate $G\alpha_{i1}$ in the baculovirus expression system in the absence of the appropriate ligand [Yoshida and Ueda, 1999]. Moreover $S1P_2$ and $S1P_3$ induce cell rounding in pheochromocytoma PC12 rat cells in a ligand-independent fashion [Van Brocklyn et al., 1999]. Therefore, a ligand-independent basal activity could not only be observed with $S1P_4$, but also with other LPA and S1P receptors like LPA_2, $S1P_2$, and $S1P_3$, indicating that the mere expression of these receptors can significantly activate the cells even in the absence of the specific ligand. In this connection, it could be important for the cell to regulate S1P receptor trafficking as an additional control for their signaling capacities and for S1P receptor selectivity. As already shown for $S1P_1$ [Lee et al., 1998; Liu et al., 1999], $S1P_4$ undergoes as well ligand-induced trafficking. Surface expression of $S1P_4$ on HEK293 cells decreases within minutes after S1P stimulation (Fig. 7C), indicating that the receptor undergoes ligand-induced internalization or sequestration. This process is supposed to be an important step in receptor resensitization rather than desensitization after activation of the receptor [Ferguson and Caron, 1998]. The finding of an incomplete receptor clearance may be explained in part by the onset of counteracting process, i.e., the recycling of internalized $S1P_4$ to the cell surface. It may also be important for cells to keep a certain level of $S1P_4$ receptor expressed on the surface to maintain a constitutive signaling since lymphocytes expressing $S1P_4$ are constantly exposed to physiological S1P levels in blood [Igarashi and Yatomi, 1998].

The second weaker signal at about 46 kDa in the Western-blot with $mS1P_4$ transfected HEK293 cells (Fig. 7B) may derive from a different posttranslational receptor processing. A partially more efficient glycosylation of the transfected receptor for example could account for these two separate signals. And it has been shown recently that N-glycosylation of $S1P_1$ facilitates ligand-induced receptor internalization [Kohno et al., 2002].

With regard to the immune system as the main compartment of $S1P_4$ expression, it is known that another class of GPCRs, the chemokine receptors, play a pivotal role for the trafficking and homing of lymphocytes [Förster et al., 1996, 1999]. It is conceivable that $S1P_4$ may also be an important player in this scenario influencing the activation and migration behavior of B- and T-lymphocytes and probably the microenvironment of lymphoid organs. This hypothesis is supported by our finding that overexpression of human $S1P_4$ in the T-cell line Jurkat resulted in an increased cell motility which can be augmented further by the presence of the ligand S1P (Fig. 4). Remarkably $S1P_1$ and $S1P_3$ have already been shown to induce cell migration towards S1P [Kon et al., 1999]. In contrast to $S1P_1$ and $S1P_3$, an inhibitory effect on cell migration as well as on Rac activation and membrane ruffling has recently been demonstrated for $S1P_2$ [Okamoto et al., 2000]. Thus, S1P receptors could be important for the fine tuning of migratory properties of cells leading to an increased or decreased migration and motility respectively. The analysis of $S1P_1$ deficient mice makes clear that these cellular responses are able to affect critical functions in vivo [Liu et al., 2000] suggesting a comparable influence of $S1P_4$ in immune surveillance.

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