Korean J Physiol Pharmacol
Vol 6: 139–142, June, 2002

Analysis of a Sphingosine 1-phosphate Receptor hS1P$_3$ in Rat Hepatoma Cells

Dong-Soon Im

Laboratory of Pharmacology, College of Pharmacy, Pusan National University, Busan 609–735, Korea

To examine intracellular signaling of human S1P$_3$ (hS1P$_3$), a sphingosine 1-phosphate (S1P) receptor in plasma membrane, hS1P$_3$ DNA was transfected into RH7777 rat hepatoma cell line, and the inhibition of forskolin-induced cAMP accumulation and activation of MAP kinases by S1P were tested. In hS1P$_3$ transformants, S1P inhibited forskolin-induced activation of adenylyl cyclase activity by about 80% and activated MAP kinases in dose-dependent and pertussis-toxin (PTX) sensitive manners. In oocytes expressing hS1P$_3$ receptor, S1P evoked Cl^- conductance. These data suggested that PTX-sensitive G proteins are involved in hS1P$_3$-mediated signaling, especially the positive action of S1P in cell proliferation. The potential advantages of rat hepatoma cells for the research of sphingosine 1-phosphate receptor are discussed.

Key Words: Sphingosine 1-phosphate, Lysophosphatidic acid, S1P$_3$, G protein-coupled receptor, cAMP, MAP kinase, Proliferation

INTRODUCTION

S1P is highly abundant in platelets and is released from activated platelets (Yatomi, 2000), and has been shown to be involved in a variety of cellular functions, including cell growth, differentiation, and programmed cell death (Meyer zu Heringdorf, 1997; Hla, 2001). S1P acts as a first messenger through the G protein-coupled receptors, termed S1P$_1$, S1P$_2$, S1P$_3$, S1P$_4$, and S1P$_5$ (Lee, 1998; Gonda, 1999; Okamoto, 1999; Im, 2000a; Van Brocklyn, 2000; Yamazaki, 2000) and also as a second messenger through hither-to-unclear mechanisms in certain cell types (Spiegel, 1996). G protein-couplings and intracellular signaling of each plasma membrane receptors have been investigated by using recombinant DNAs, especially S1P$_1$ (Lee, 1998; Okamoto, 1998; Zondag, 1998) and S1P$_2$ (Gonda, 1999; Van Brocklyn, 1999). S1P$_1$ predominantly couples to G$_i$ and Ras-Raf-MAP kinase pathways and also induces morphogenetic differentiation in a C3 exoenzyme-sensitive manner (Lee, 1998; Okamoto, 1998). S1P$_2$ increases intracellular Ca^{2+} concentration via Gq-PLC pathway and activates MAP kinases in a PTX-sensitive manner (An, 1999; Gonda, 1999). Furthermore, S1P$_2$ induces cell rounding and neurite retraction via presumably G$_{12/13}$ (Van Brocklyn, 1999).

S1P-induced Ca^{2+} efflux and SRE-driven transcription have been shown in S1P$_3$ expressing oocytes and Jurkat T cells (An, 1997). Studies with several mammalian cell lines indicated that S1P$_3$ couples to PTX-insensitive G proteins (G$_{q/11}$) and activated PLC/Ca^{2+} system (An, 1999; Okamoto, 1999; Sato, 1999). SRE-driven transcription requires activation of both G$_i$-Ras-Raf-MAP kinase and Rho GTPase pathways (An, 1997). Thus, S1P$_3$ receptor must couple to G$_i$ and induce cell shape change. In S1P$_3$-expressing HEK293 cells and PC12 cells, S1P-induced cell rounding and neurite retraction have been demonstrated (Okamoto, 1999; Van Brocklyn, 1999), however, PTX-sensitive G protein-couplings and subsequent intracellular signaling (e.g. inhibition of adenylyl cyclase and activation of MAP kinase) of S1P$_3$ have poorly been investigated and contradictory. Kon et al (1999) observed stimulation of cAMP accumulation in CHO-S1P$_3$ cells. On the other hands Okamoto et al (1999) reported that forskolin-induced increase of cellular cAMP contents was inhibited by S1P in CHO-S1P$_3$ cells. In this communication, S1P signaling through PTX-sensitive G proteins was shown in S1P$_3$-expressing rat hepatoma cells.

METHODS

Materials

Sphingosine 1-phosphate (S1P) was obtained from Biomol (Plymouth, PA), and 1-oleoyl-lysophosphatidic acid (LPA) was purchased from Avanti Pola Lipid (Alabaster, AL). [γ-^{32}P]ATP was obtained from ICN biochemicals (Costa Mesa, CA), MAP kinases assay kit from Amersham (Piscataway, NJ), and pcDNA-3 plasmid from In Vitrogen (Carlsbad, CA). All other chemicals were from Sigma.

Stable transfection

RH7777 cell monolayers were transfected with the S1P$_3$

Corresponding to: Dong-Soon Im, Laboratory of Pharmacology, College of Pharmacy, Pusan National University, San 30, Jang-Jeon-dong, Geum-Jeong-gu, Busan 609-735, Korea. (Tel) +82-51-510-2817, (Fax) +82-51-513-6754, (E-mail) imds@pusan.ac.kr

ABBREVIATIONS: S1P, sphingosine 1-phosphate; LPA, lysophosphatidic acid; PTX, pertusssis toxin; SRE, serum response element; PLC, phospholipase C; MAP kinases, mitogen-activated protein kinases; HKRB, HEPES-Krebs-Ringer-bicarbonate.

plasmid DNA using the calcium phosphate precipitate method, and clonal populations expressing the neomycin phosphotransferase gene were selected by the addition of geneticin (G418) to the culture media. The RH7777 cells were grown in monolayers at 37°C in a 5% CO_2/95% air atmosphere in growth media consisting of 90% MEM, 10% fetal bovine serum, 2 mM glutamine and 1 mM sodium pyruvate.

cAMP accumulations and MAP kinases assay

Cells were plated on 24-well dishes on sub-confluent population. After 24 h, they were washed twice with phosphate-buffered saline and incubated in HKRB for 10 min. Cells were then stimulated for 15 min with different concentrations of S1P in the presence of 1 μM forskolin and 1 mM IBMX. The reaction was stopped by adding 1 N HCl. After centrifugation, cAMP in the supernatant was measured by an automated immunoassay (Gamma flow). For MAP kinases assay, the cells plated in 35-mm dishes were stimulated with S1P or LPA in HKRB for 5 min. After changing the HKRB to ice-cold lysis buffer, the reaction was terminated by rapidly immerging the dishes into liquid nitrogen. After centrifugation, MAP kinases activity in the supernatant of the lysate was measured by using Amersham kit. PTX treatment of the cells was performed by adding the toxin (100 ng/ml) to the culture medium 24 h before the experiments.

Oocyte expression

Using the T7 RNA polymerase and the $S1P_3$ pcDNA3 DNA as a template, $S1P_3$ mRNA was transcribed *in vitro* in the presence of a capping analog. This mRNA was injected into de-folliculated stage V~VI *Xenopus laevis* oocytes. After about 48 hours, responses to S1P applied were recorded with individual oocytes held under two electrodes voltage clamp. The preparation of the oocytes and the conditions for the recordings were described previously (Im, 2000b).

RESULTS

S1P and LPA have similar chemical structures and their receptors form a sub-family of G protein-coupled receptors, formerly called as Edg (Endothelial differentiation gene) (Hla & Maciag, 1990). $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ are S1P preferring receptors (Lynch & Im, 1999; Hla, 2001) and LPA_1, LPA_2, and LPA_3 are LPA specific receptors (Lynch & Im, 1999; Im, 2000b). Among them, the present study focused on the role of $S1P_3$ signaling, especially PTX-sensitive G protein-mediated. To examine intracellular signaling of human $S1P_3$, $hS1P_3$ DNA was stably transfected into RH7777 rat hepatoma cell line, which does not have mRNAs of $S1P_1$, $S1P_2$ and $S1P_3$ (Zhang, 1999), and effect of S1P on adenyly cyclase activity was tested. In non-transfected or vector-transfected RH7777 cells, 1 μM S1P inhibited forskolin-induced cAMP accumulation by about 40%. In $hS1P_3$ transformants, S1P-induced adenylyl cyclase inhibition was about 80%, which was greater than that in vector-transformants. Potency of S1P action in $hS1P_3$ transformants was increased. Fig. 1 shows dose-response curve of S1P inhibition of cAMP accumulation. EC_{50} for S1P in $hS1P_3$ transformants was about 5 nM, and

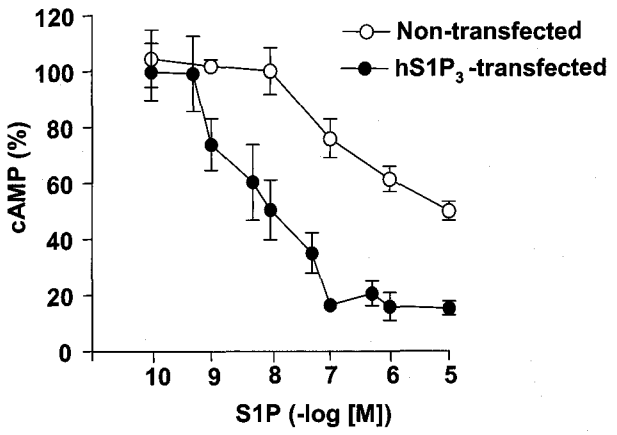

Fig. 1. Effect of S1P on cAMP accumulation in $hS1P_3$ expressing RH7777 cells. In the presence of 1 μM forskolin and 1 mM IBMX, cAMP accumulations for 15 min by different concentration of S1P were measured. Dose response curves of S1P inhibition of cAMP accumulation in $hS1P_3$ expressing (closed circles) and non-transfected RH7777 cells (open circles).

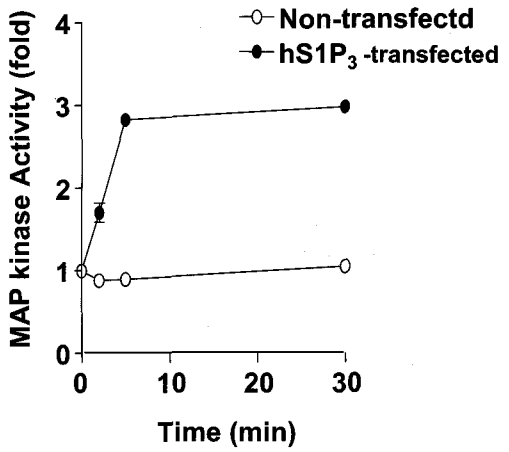

Fig. 2. S1P activation of MAP kinases in $hS1P_3$ transfected RH7777 cells. Detailed procedures are described in Experimental methods section. Time courses of MAP kinase activity with 1 μM S1P in $hS1P_3$ transfected (closed circles) or non-transfected (open circles) RH7777 cells.

that in non-transfected RH7777 cells was about 100 nM. Although endogenous response to S1P was observed in the non-transfected RH7777 cells (Fig. 1, open circle), which were reported to be null responsive to S1P and LPA (Zhang, 1999), the increased potency of S1P inhibition of cAMP accumulation strongly suggested that the $S1P_3$ was an S1P receptor, which was coupled to PTX-sensitive G proteins.

Next, MAP kinases activity was tested, an important step for cell proliferation, to study whether $S1P_3$ mediated the stimulatory action of S1P in cell growth, as shown in several types of cells (Olivera & Spiegel, 1993; Wu, 1995; An, 2000; Pebay, 2001). As shown in Fig. 2, S1P activated MAP kinases in $hS1P_3$ transformants about 2.5-fold from 5 min to 30 min, however, no activation in vector transformants or non-transfected RH7777 cells. The activation was PTX-sensitive (Fig. 3). Also, treatment with PTX (100

ng/ml, 24 hr) completely abolished the S1P-induced inhibition of adenylyl cyclase (data not shown), together with the above suggesting that $G_{i/o}$ type G proteins were involved in $S1P_3$ signaling. In $mLPA_1$ transformants, LPA inhibited adenylyl cyclase activity in dose-dependent and PTX-sensitive manners (data not shown), confirming the earlier observations (An, 1998; Im, 2000b). EC_{50} of S1P in MAP kinases activation was 5 nM. This result strongly suggested that S1P-induced MAP kinases activation and resulting cell proliferation in some cell types could be triggered via $S1P_3$ receptor.

Gq-PLC system was tested by micro-injecting $hS1P_3$ mRNA into *Xenopus laevis* oocytes. After 48 h of the injection, S1P (1~10 μM) induced significant Cl^- current, suggesting intracellular calcium mobilization through Gq-PLC system, hoever, the Cl^- current was not induced in $S1P_1$ or $S1P_2$ mRNA-injected oocytes (up to 10 μM) (Fig. 4). S1P activation of PLC and calcium mobilization through $S1P_3$ has also been shown in several different cell systems (An, 1997; An, 1999; Okamoto, 1999; Sato, 1999). Furthermore, calcium transients by S1P in $S1P_2$- and $S1P_3$-expressing RH7777 cells was observed, but not in $S1P_1$- or $S1P_5$-expressing RH7777 cells (data not shown).

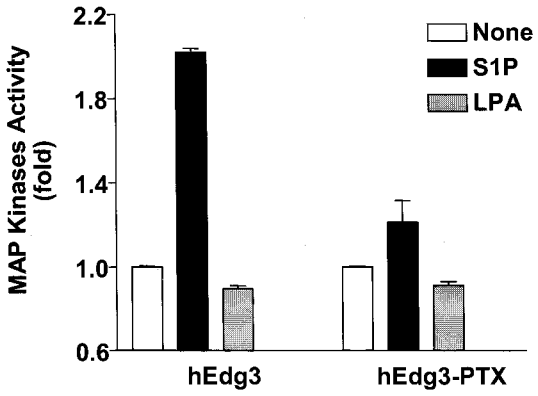

Fig. 3. Effect of PTX on S1P- or LPA-induced MAP kinase activation in $hS1P_3$ transfected RH7777 cells. Treated with PTX (100 ng/ml) for 24 hr before the experiments.

DISCUSSION

An et al (1997) showed SRE transcription in Jurkat T cells, however, S1P/$S1P_3$-mediated actions through PTX-sensitive G proteins were not fully addressed. In the present study, $G_{i/o}$ protein-mediated signaling pathways of $S1P_3$ were investigated, and inhibition of adenylyl cyclase and activation of MAP kinases by S1P were shown in recombinant $hS1P_3$ expressing RH7777 cells. As implicated in $S1P_3$-mediated SRE transcription, which requires both MAP kinases and Rho GTPase activations (An, 1997), $hS1P_3$ coupled to PTX-sensitive G proteins by S1P. The activated G proteins inhibited adenylyl cyclase activity (Fig. 1) and activated MAP kinases (Fig. 2).

Considering these results together with the observations of cell shape changes by S1P/$S1P_3$ (Van Brocklyn, 1999), $S1P_3$ receptor was most likely coupled to multiple type G proteins, i.e. $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ and induced a variety of signaling and cell responses, i.e. inhibition of cyclase, Ca^{2+} mobilization, MAP kinases activation, cell shape changes, cell proliferation and so on (Kon, 1999). In the past, three groups of investigators described the inhibition of adenylyl cyclase through $S1P_3$. An et al (1999) reported an observation of S1P inhibition of cyclase in HTC4 hepatoma cell line expressing $S1P_3$, however, no data presented. Okamoto et al (1999) reported S1P inhibition of cAMP accumulation in CHO-$S1P_3$ cells. However, Okajima and his collegues could not obtain any evidence of G_i coupling in CHO-$S1P_3$ cells, although they observed positive coupling of $S1P_3$ to Gq-PLC system and S1P calcium mobilization in these cells (Kon, 1999; Sato, 1999). On the contrary, they observed a stimulatory coupling of $S1P_3$ to adenylyl cyclase in CHO-$S1P_3$ cells (Kon, 1999). In $hS1P_3$-RH7777 cells, the coupling of $S1P_3$ to $G_{i/o}$ proteins was observed, in support of the previous observations. The reason of why a group observed stimulatory effect of $S1P_3$ receptor on cAMP accumulation

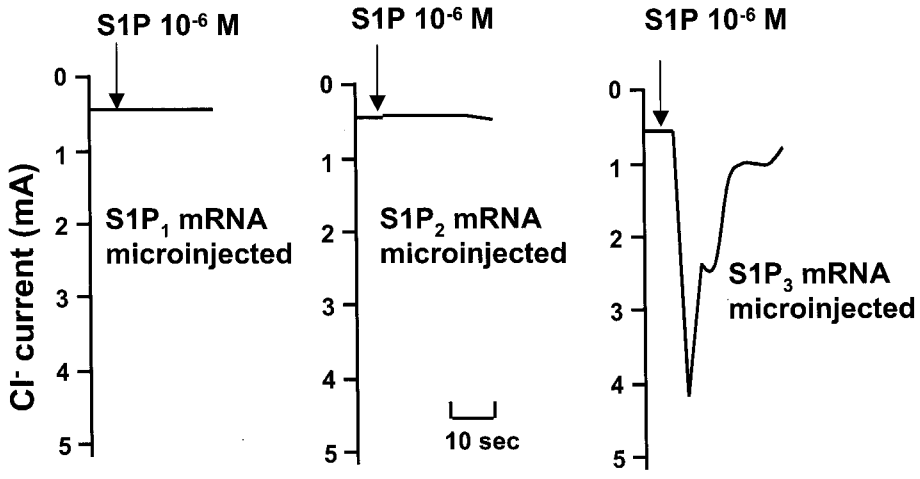

Fig. 4. Responses of oocytes injected with $S1P_1$, $S1P_2$, and $S1P_3$ mRNAs to S1P. Individual frog oocytes were held under two electrode voltage clamp and challenged with 10 μM S1P.

is not clear. It might be due to difference of transformed cells or to differential modulation of $S1P_3$ signaling in different cells. This difference might suggest diverse physiological roles of $S1P_3$ receptor, depending on the types of cells. Recently, couplings of $S1P_3$ to G_{i2}, G_q, and G_{13} type G proteins, but not to Gs, were shown in broken Sf9 insect cell assay system (Windh, 1999), in support of the present observation made in intact mammalian cells.

Usually, HEK293 and CHO cells have popularly been used for the transfection of G protein-coupled receptor DNAs. However, in the case of lysophospholipids receptors, i.e. S1P receptors and LPA receptors, rat hepatoma cell line RH7777 has been useful for several reasons. First, it is devoid of at least 6 members out of 8 lysophospholipid receptors (Zhang, 1999). Second, strong coupling to G_i proteins was observed and quite well reproduced (Im, 2000a). Third, intracellular calcium mobilization with S1P and LPA was also measurable, although a large number of cells are required (Im, 2000b).

ACKNOWLEDGMENTS

I am grateful to Dr. Kevin R. Lynch (Department of Pharmacology, University of Virginia) for his support of the present research in his laboratory.

REFERENCES

An S, Bleu T, Huang W, Hallmark OG, Coughlin SR, Goetzl EJ. Identification of cDNAs encoding two G protein-coupled receptors for lysosphingolipids. *FEBS Lett* 417: 279–282, 1997

An S, Bleu T, Zheng Y. Transduction of intracellular calcium signals through G protein-mediated activation of phospholipase C by recombinant sphingosine 1-phosphate receptors. *Mol Pharmacol* 55: 787–794, 1999

An S, Bleu T, Zheng Y, Goetzl EJ. Recombinant human G protein-coupled lysophosphatidic acid receptors mediate intracellular calcium mobilization. *Mol Pharmacol* 54: 881–888, 1998

An S, Zheng Y, Bleu T. Sphingosine 1-phosphate-induced cell proliferation, survival, and related signaling events mediated by G protein-coupled receptors Edg3 and Edg5. *J Biol Chem* 275: 288–296, 2000

Gonda K, Okamoto H, Takuwa N, Yatomi Y, Okazaki H, Sakurai T, Kimura S, Sillard R, Harii K, Takuwa Y. The novel sphingosine 1-phosphate receptor AGR16 is coupled via pertussis toxin-sensitive and -insensitive G-proteins to multiple signalling pathways. *Biochem J* 337 (Pt 1): 67–75, 1999

Hla T, Lee MJ, Ancellin N, Paik JH, Kluk MJ. Lysophospholipids--receptor revelations. *Science* 294: 1875–1878, 2001

Hla T, Maciag T. An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. *J Biol Chem* 265: 9308–9313, 1990

Im DS, Heise CE, Ancellin N, O'Dowd BF, Shei GJ, Heavens RP, Rigby MR, Hla T, Mandala S, McAllister G, George SR, Lynch KR. Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. *J Biol Chem* 275: 14281–14286, 2000a

Im DS, Heise CE, Harding MA, George SR, O'Dowd BF, Theodorescu D, Lynch KR. Molecular cloning and characterization of a lysophosphatidic acid receptor, Edg-7, expressed in prostate. *Mol Pharmacol* 57: 753–759, 2000b

Kon J, Sato K, Watanabe T, Tomura H, Kuwabara A, Kimura T, Tamama K, Ishizuka T, Murata N, Kanda T, Kobayashi I, Ohta H, Ui M, Okajima F. Comparison of intrinsic activities of the putative sphingosine 1-phosphate receptor subtypes to regulate several signaling pathways in their cDNA-transfected Chinese hamster ovary cells. *J Biol Chem* 274: 23940–23947, 1999

Lee MJ, Van Brocklyn JR, Thangada S, Liu CH, Hand AR, Menzeleev R, Spiegel S, Hla T. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science* 279: 1552–1555, 1998

Lynch KR, Im DS. Life on the edg. *Trends Pharmacol Sci* 20: 473–475, 1999

Meyer zu Heringdorf D, van Koppen CJ, Jakobs KH. Molecular diversity of sphingolipid signalling. *FEBS Lett* 410: 34–38, 1997

Okamoto H, Takuwa N, Gonda K, Okazaki H, Chang K, Yatomi Y, Shigematsu H, Takuwa Y. EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to multiple signaling pathways, including phospholipase C activation, Ca2+ mobilization, Ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition. *J Biol Chem* 273: 27104–27110, 1998

Okamoto H, Takuwa N, Yatomi Y, Gonda K, Shigematsu H, Takuwa Y. EDG3 is a functional receptor specific for sphingosine 1-phosphate and sphingosylphosphorylcholine with signaling characteristics distinct from EDG1 and AGR16. *Biochem Biophys Res Commun* 260: 203–208, 1999

Olivera A, Spiegel S. Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature* 365: 557–560, 1993

Pebay A, Toutant M, Premont J, Calvo CF, Venance L, Cordier J, Glowinski J, Tence M. Sphingosine-1-phosphate induces proliferation of astrocytes: regulation by intracellular signalling cascades. *Eur J Neurosci* 13: 2067–2076, 2001

Sato K, Kon J, Tomura H, Osada M, Murata N, Kuwabara A, Watanabe T, Ohta H, Ui M, Okajima F. Activation of phospholipase C-Ca2+ system by sphingosine 1-phosphate in CHO cells transfected with Edg-3, a putative lipid receptor. *FEBS Lett* 443: 25–30, 1999

Spiegel S, Foster D, Kolesnick R. Signal transduction through lipid second messengers. *Curr Opin Cell Biol* 8: 159–167, 1996

Van Brocklyn JR, Graler MH, Bernhardt G, Hobson JP, Lipp M, Spiegel S. Sphingosine-1-phosphate is a ligand for the G protein-coupled receptor EDG-6. *Blood* 95: 2624–2629, 2000

Van Brocklyn JR, Tu Z, Edsall LC, Schmidt RR, Spiegel S. Sphingosine 1-phosphate-induced cell rounding and neurite retraction are mediated by the G protein-coupled receptor H218. *J Biol Chem* 274: 4626–4632, 1999

Windh RT, Lee MJ, Hla T, An S, Barr AJ, Manning DR. Differential coupling of the sphingosine 1-phosphate receptors Edg-1, Edg-3, and H218/Edg-5 to the Gi, Gq, and G12 families of heterotrimeric G proteins. *J Biol Chem* 274: 27351–27358, 1999

Wu J, Spiegel S, Sturgill TW. Sphingosine 1-phosphate rapidly activates the mitogen-activated protein kinase pathway by a G protein-dependent mechanism. *J Biol Chem* 270: 11484–11488, 1995

Yamazaki Y, Kon J, Sato K, Tomura H, Sato M, Yoneya T, Okazaki H, Okajima F, Ohta H. Edg-6 as a putative sphingosine 1-phosphate receptor coupling to Ca(2+) signaling pathway. *Biochem Biophys Res Commun* 268: 583–589, 2000

Yatomi Y, Ohmori T, Rile G, Kazama F, Okamoto H, Sano T, Satoh K, Kume S, Tigyi G, Igarashi Y, Ozaki Y. Sphingosine 1-phosphate as a major bioactive lysophospholipid that is released from platelets and interacts with endothelial cells. *Blood* 96: 3431–3438, 2000

Zhang G, Contos JJ, Weiner JA, Fukushima N, Chun J. Comparative analysis of three murine G-protein coupled receptors activated by sphingosine-1-phosphate. *Gene* 227: 89–99, 1999

Zondag GC, Postma FR, Etten IV, Verlaan I, Moolenaar WH. Sphingosine 1-phosphate signalling through the G-protein- coupled receptor Edg-1. *Biochem J* 330 (Pt 2): 605–609, 1998