



Title	Palmitoylation of the sphingosine 1-phosphate receptor S1P1 is involved in its signaling functions and internalization
Author(s)	Ohno, Yusuke; Ito, Ayako; Ogata, Ren; Hiraga, Yuki; Igarashi, Yasuyuki; Kihara, Akio
Citation	Genes to Cells, 14(8), 911-923 https://doi.org/10.1111/j.1365-2443.2009.01319.x
Issue Date	2009-08
Doc URL	http://hdl.handle.net/2115/42607
Rights	The definitive version is available at www3.interscience.wiley.com
Type	article (author version)
File Information	GtC14-8_p911-923.pdf



[Instructions for use](#)

Palmitoylation of the sphingosine 1-phosphate receptor S1P₁ is involved in its signaling functions and internalization

Yusuke Ohno^{1,†}, Ayako Ito^{2,†}, Ren Ogata¹, Yuki Hiraga², Yasuyuki Igarashi³, and Akio Kihara^{1,*}

¹*Laboratory of Biochemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita 12-jo, Nishi 6-choume, Kita-ku, Sapporo 060-0812, Japan*

²*Laboratory of Biomembrane and Biofunctional Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita 12-jo, Nishi 6-choume, Kita-ku, Sapporo 060-0812, Japan*

³*Laboratory of Biomembrane and Biofunctional Chemistry, Faculty of Advanced Life Sciences, Hokkaido University, Kita 21-jo, Nishi 11-choume, Kita-ku, Sapporo 001-0021, Japan*

[†]These authors contributed equally in this work

*Correspondence kihara@pharm.hokudai.ac.jp

Running title: Palmitoylation of S1P₁

Address correspondence to:

Akio Kihara

Laboratory of Biochemistry, Faculty of Pharmaceutical Sciences, Hokkaido University

Nishi 6-choume, Kita-ku, Sapporo 060-0812, Japan

Telephone: +81-11-706-3754

Fax: +81-11-706-4900

E-mail: kihara@pharm.hokudai.ac.jp

Abstract

The lipid mediator sphingosine 1-phosphate (S1P) regulates several cellular processes through binding to its receptors (S1P₁-S1P₅), which are heterotrimeric G protein-coupled receptors. Here, we report that all S1P receptors are palmitoylated. In S1P₁, three Cys residues in the cytoplasmic tail are palmitoylated. We examined the roles of palmitoylation of S1P₁ using model cells in which wild type S1P₁ or a non-palmitoylated mutant S1P₁ was overproduced. Compared to wild type S1P₁, the non-palmitoylated S1P₁ exhibited similar binding affinity to the natural ligand S1P but lower affinity to the synthetic ligand FTY720 phosphate (FTY720-P), the active form of the immunomodulator FTY720. However, downstream signaling of non-palmitoylated S1P₁ was similarly affected by S1P and FTY720-P stimulation. Moreover, upon stimulation with S1P, internalization of the mutant non-palmitoylated S1P₁ was retarded, compared to that of the wild type protein. This effect was much more pronounced with FTY720-P stimulation. Similar differences were observed for the phosphorylation of S1P₁ and its mutant. These findings may provide insight into the molecular mechanisms of the pharmacological effects of FTY720. Finally, palmitoylation of wild type S1P₁ increased upon treatment with S1P, suggesting that S1P₁ undergoes a palmitoylation/depalmitoylation cycle after stimulation by its ligands.

Introduction

Many proteins, especially those involved in signal transduction, are modified on their Cys residues by fatty acid acylation (*S*-acylation) (Resh 1999; Smotrys & Linder 2004). Fatty acids utilized in *S*-acylation are heterologous, but palmitic acid is the most common. Therefore, *S*-acylation is often referred to as palmitoylation. Palmitoylation plays a role in regulating protein localization, membrane association, protein-protein interaction, protein-lipid interaction, and enzyme activity (Resh 1999; Smotrys & Linder 2004). Unlike other lipid modifications such as *N*-myristoylation, prenylation, and GPI-anchoring, palmitoylation is reversible. Thus, target proteins can be manipulated via a palmitoylation/depalmitoylation cycle.

G protein-coupled receptors (GPCRs) are the most abundant class of cell surface receptors and are involved in a variety of cellular functions. Recent studies have indicated that lipid modifications play important roles in GPCR-associated signaling (Qanbar & Bouvier 2003; Escribá *et al.* 2007). Most GPCRs are modified by palmitoylation on one or more Cys residues, although G α proteins are *N*-myristoylated and/or palmitoylated, and G γ proteins are prenylated (Escribá *et al.* 2007). In contrast to myristoylation and prenylation, palmitoylation requires no strict motifs, however in GPCRs, Cys residues positioned 10-14 amino acids downstream of the last transmembrane segment are usually palmitoylated (Qanbar & Bouvier 2003; Escribá *et al.* 2007). The roles of palmitoylation vary among GPCRs. For example, palmitoylation is required for the cell surface expression of some GPCRs, such as bovine opsin (Karnik *et al.* 1993), leutropin/choriogonadotropin receptor (Zhu *et al.* 1995), and H₂ histamine receptor (Fukushima *et al.* 2001). Yet while palmitoylation is necessary for the signaling functions of some GPCRs, including the β_2 -adrenergic receptor (O'Dowd *et al.* 1989), it is not involved in the signaling of others, such as the α_{2A} -adrenergic receptor (Kennedy & Limbird 1993). Palmitoylation also can affect the phosphorylation of GPCRs, due to the close proximity of the respective amino acid residues. Normally, GPCRs are phosphorylated and then internalized during

signaling initiated by exposure to their ligands. However, a palmitoylation site mutant of the β_2 -adrenergic receptor is hyperphosphorylated even without ligand stimulation (Moffett *et al.* 1993), yet phosphorylation is suppressed in palmitoylation mutants of the V_{1a} vasopressin receptor (Hawtin *et al.* 2001) and the chemokine receptor CCR5 (Kraft *et al.* 2001). Therefore, it is important to determine the roles palmitoylation exerts on each individual GPCR.

The GPCRs S1P₁ through S1P₅ recognize the lipid mediator sphingosine 1-phosphate (S1P), which regulates a variety of cellular functions (Spiegel & Milstien 2003; Sanchez & Hla 2004; Kihara *et al.* 2007). Of these receptors, S1P₁ is the most important physiologically, especially in the vascular and immune systems (Spiegel & Milstien 2003; Sanchez & Hla 2004; Kihara *et al.* 2007). For example, S1P₁ performs a pivotal function in lymphocyte egress from the thymus and secondary lymphoid organs (Chiba *et al.* 2006; Kihara *et al.* 2007; Kihara & Igarashi 2008). In fact, a novel therapeutic (FTY720) currently undergoing Clinical Trials as an immunomodulator (Budde *et al.* 2006; Chiba *et al.* 2006; Kihara & Igarashi 2008) is actually a prodrug whose active phosphorylated form, FTY720 phosphate (FTY720-P), acts as a ligand for all S1P receptors except S1P₂ (Brinkmann *et al.* 2002; Mandala *et al.* 2002).

After ligand binding, S1P₁ is internalized, desensitizing the stimulus (Liu *et al.* 1999). When the ligand is S1P, the S1P₁ receptor is recycled back to the cell surface. However, when the stimulus is FTY720-P, S1P₁ is not recycled to the plasma membrane but is degraded, resulting in a loss of the receptor at the cell surface (Gräler & Goetzl 2004; Matloubian *et al.* 2004; Jo *et al.* 2005). To date, the molecular mechanism causing the differences between S1P and FTY720-P in internalization of S1P₁ has remained unclear.

Several post-translational modifications have been reported for S1P₁, including phosphorylation (Watterson *et al.* 2002), *N*-glycosylation (Kohno *et al.* 2002), sulfation (Fieger *et al.* 2005), and ubiquitinylation (Oo *et al.* 2007). However, palmitoylation of S1P₁ and its role have not been

previously examined. In the study presented here, we have demonstrated that three Cys residues in the C-terminal tail of S1P₁ are palmitoylated. We also report that palmitoylation of S1P₁ is involved in high affinity binding to FTY720-P but not to S1P, and in the downstream signaling of S1P₁, irrespective of S1P and FTY720-P stimulation. Palmitoylation also plays a role in the internalization of S1P₁, especially following FTY720-P stimulation. Thus, palmitoylation affects several post-stimulation processes involving S1P₁, sometimes differently following S1P stimulation versus FTY720-P stimulation. The differential effects of palmitoylation on internalization may provide important clues for understanding the molecular mechanism of the pharmacological effects of FTY720.

Results

All S1P receptors are palmitoylated

Like other GPCRs, S1P receptors contain one to three Cys residues proximal to the last transmembrane in the C-terminal tail (Fig. 1A). To investigate whether S1P receptors are palmitoylated, each receptor was cloned and transiently expressed in Human embryonic kidney (HEK) 293T cells as a C-terminally 3xFLAG-tagged protein. All S1P receptors were detected by immunoblotting as several bands, typically a broad upper band and sharp lower band(s) (Fig. 1B). Different protein bands of each S1P receptor presumably represent proteins carrying different forms of glycosylation, since all S1P receptors contain potential *N*-glycosylation sites at their N-termini, and the *N*-glycosylation of S1P₁ has already been reported (Kohno *et al.* 2002). To examine whether the S1P receptors carried high mannose- or complex-type *N*-glycosylation, we treated each protein sample with Endoglycosidase H (Endo H), which removes only high mannose-type *N*-glycosylation, and peptide:*N*-glycosidase F (PNGase F), which can remove both high mannose- and complex-types. The lower sharp bands were shifted to lower molecular weight bands upon treatment with Endo H (Fig. 1B), indicating that these proteins are modified with high mannose-type glycosylation. On the other hand, the upper broad bands were shifted to low molecular weight bands only by treatment with PNGase F (Fig. 1B), indicating that they were modified with complex-type glycosylation. Proteins destined to the plasma membrane, including GPCRs, are synthesized in the ER, where high mannose-type *N*-glycosylation occurs, then are transported to the Golgi, where the glycosylation is modified to the complex-type, and, finally, are delivered to the plasma membrane. Therefore, high mannose-type glycosylated S1P receptors may localize in the ER, while complex-type glycosylated S1P receptors may localize in the plasma membrane (or possibly in the Golgi). Detecting such substantial levels of ER-localized receptors may be due to the transient expression system, in which protein synthesis begins to increase ~24 h after transfection. The cells were transfected 24 h before use, and so the observed S1P receptor proteins

might have been newly synthesized.

Next, HEK 293T cells transiently expressing each S1P receptor were metabolically labeled with [³H]palmitic acid. As shown in Fig. 1C, all S1P receptors were radiolabeled, although the intensities of the label varied among the receptors. Labeling efficiency was highest for S1P₁ and S1P₅, and then for S1P₄. The number of potential palmitoylated Cys residues in the receptor was at least partly responsible for the high labeling efficiency (S1P₁, S1P₄, and S1P₅ each have three), however this did not always correlate with labeling efficiency. For example, S1P₃, which contains two potential palmitoylated Cys residues, was less efficiently labeled with [³H]palmitic acid than was S1P₂, which contains only one. Therefore, a higher population of S1P₃ may exist in a non-palmitoylated state compared to the other S1P receptors, or S1P₃ may be depalmitoylated more rapidly. Both high mannose-type and complex-type glycosylated S1P receptors were labeled, indicating that palmitoylation, at least partly, already occurs in the ER, although the possibility that the S1P receptors are further palmitoylated or re-palmitoylated after depalmitoylation at other organelles cannot be excluded.

Palmitoylation of the S1P receptors was further confirmed by an acyl-biotinyl exchange (ABE) assay (Drisdell & Green 2004), in which palmitic acid is removed via hydroxylamine-mediated cleavage of the palmitoyl-thioester bond and the remaining thiol group is labeled with a thiol-specific biotinylation reagent. Non-palmitoylated Cys residues were first protected by the thiol modifier *N*-ethyl maleimide (NEM), and so do not react with the thiol-specific biotinylation reagent. The biotinylated proteins were recovered by avidin-agarose and detected by immunoblotting. Using this method, all S1P receptors were again determined to be palmitoylated proteins (Fig. 1D).

S1P₁ is palmitoylated on three Cys residues in the C-terminal tail

We focused on the palmitoylation of S1P₁, since it is the physiologically most important S1P receptor.

To examine whether the Cys residues are palmitoylated as expected, we constructed

S1P₁(3CA)-3xFLAG, in which Cys-328, -329, and -331 were all changed to Ala. HEK 293T cells expressing S1P₁-3xFLAG or S1P₁(3CA)-3xFLAG were metabolically labeled with [³H]palmitic acid. In contrast to wild type S1P₁, no labeling was detected in S1P₁(3CA)-3xFLAG (Fig. 2A), indicating that S1P₁ is palmitoylated on these Cys residues.

We also created single and double Cys-to-Ala substituted mutants (C328A, C329A, C331A, C328/329A, C329/331A, and C328/331A), and subjected them to the ABE assay. Wild type S1P₁-3xFLAG and all of the single and double Cys-substituted mutants were detected as palmitoylated proteins, although the triple Cys-substituted mutant (S1P₁(3CA)-3xFLAG) was not (Fig. 2B). The band intensities were not dependent on the number of palmitoylated Cys residues. This result is reasonable, since biotinylated proteins are logically recovered by avidin beads with nearly equal efficiency, regardless of the number of biotinylated Cys residues, and we detected them with an anti-FLAG antibody, rather than an anti-biotin antibody. In conclusion, S1P₁ is palmitoylated on Cys-328, Cys-329, and Cys-331 residues.

Palmitoylation is not involved in cell surface expression of S1P₁

Palmitoylation is required for the surface expression of some GPCRs (Escribá *et al.* 2007). Therefore, we examined the intracellular localization of the wild type (S1P₁-3xFLAG) and non-palmitoylated (S1P₁(3CA)-3xFLAG) forms of S1P₁. Chinese hamster ovary (CHO) cells stably expressing wild type (S1P₁-CHO) and non-palmitoylated S1P₁ (S1P₁(3CA)-CHO) were subjected to indirect immunofluorescent microscopy using anti-FLAG antibodies. Both wild type and non-palmitoylated S1P₁ were observed in the plasma membrane (Fig. 3A), indicating that palmitoylation is not required for proper localization of S1P₁. Both S1P₁-CHO and S1P₁(3CA)-CHO cells expressed S1P₁ to similar levels judging from immunoblotting (Fig. 3B) and flow cytometry (Fig. 3C).

Palmitoylation of S1P₁ is required for efficient S1P signal transduction

We next investigated the role of palmitoylation in S1P signaling. S1P₁ couples to G_i and transduces the S1P signal to several downstream pathways including the Ras/ERK, cAMP/protein kinase A, and phosphatidylinositol 3-kinase/Akt/Rac pathways (Spiegel & Milstien 2003; Sanchez & Hla 2004; Kihara *et al.* 2007). We first examined the binding affinities of the wild type and 3CA mutant S1P₁ to S1P using a binding assay and [³²P]S1P. We found that the wild type and mutant proteins exhibited similar K_d values (39.4 nM and 31.4 nM, respectively).

We next investigated the signaling activities of the wild type and non-palmitoylated mutant S1P₁ by measuring the phosphorylation levels of ERK upon treatment with various concentrations of S1P. Maximal activation of ERK was observed in S1P₁-CHO cells at 5 nM S1P (Fig. 4A,B), and the EC₅₀ value was estimated to be 1.4 nM. Although the EC₅₀ value of the 3CA mutant was similar (2.4 nM), the maximal p-ERK/ERK value was determined to be 32% of that observed for the wild type S1P₁, after subtracting background levels (those of the vector-transfected cells). These results suggest that palmitoylation affects the signaling activity of S1P₁ without affecting its binding to S1P.

When FTY720-P, the active form of the novel immunomodulating agent FTY720 (Brinkmann *et al.* 2002; Mandala *et al.* 2002), was used for stimulation instead of S1P, wild type S1P₁ fully activated ERK even at 1 nM (EC₅₀, 0.49 nM; Fig. 4C,D). However, such full activation was observed for the 3CA mutant only at 50 nM (EC₅₀, 5.2 nM; Fig. 4C,D). Moreover, the maximal p-ERK/ERK value was ~1/2 that of the wild type S1P₁. Thus, although palmitoylation affects the signaling activity of S1P₁ similarly after S1P or FTY720-P stimulation, it affects only the high affinity binding of S1P₁ to FTY720-P, and has little or no apparent effect on S1P₁ binding to S1P.

We also examined the kinetics of ERK activation upon treatment with S1P. Phosphorylation of ERK reached maximal levels within 3 min in S1P₁-CHO cells and then gradually decreased (Fig. 4E,F). Non-palmitoylated S1P₁ also activated ERK with similar kinetics, but p-ERK levels in the

S1P₁(3CA)-CHO cells were lower than those in the S1P₁-CHO cells until 20 min post-stimulation (Fig. 4E,F).

Stimulated S1P₁ induces cell migration toward S1P by activating the small GTPase Rac, via phosphatidylinositol 3-kinase (Okamoto *et al.* 2000). We next investigated the ability of non-palmitoylated S1P₁ to promote cell migration, using a modified Boyden chamber. Inclusion of S1P in the lower chamber significantly stimulated the cell migration of the S1P₁-CHO cells, but non-palmitoylated S1P₁ was less active (Fig. 4G). The number of migrated S1P₁(3CA)-CHO cells was ~25% that of the S1P₁-CHO cells (Fig. 4G). These results also suggest that palmitoylation is needed for efficient signaling of S1P₁.

Palmitoylation affects internalization of S1P₁

After stimulation, S1P₁ is rapidly internalized (Liu *et al.* 1999). We compared the internalization of wild type and non-palmitoylated S1P₁ by flow cytometry. The overall amount of wild type S1P₁ internalized was greater than that of non-palmitoylated S1P₁ at any time point examined, although a statistically significant difference was observed only at 60 min post-stimulation (Fig. 5A). We also investigated the effect of palmitoylation on the internalization of S1P₁ induced by FTY720-P. As reported previously (Gräler & Goetzl 2004; Matloubian *et al.* 2004; Jo *et al.* 2005), FTY720-P stimulated the internalization of S1P₁ more efficiently than S1P did (Fig. 5B). The effect of stimulation on non-palmitoylated S1P₁ versus S1P₁ was likewise more pronounced with FTY720-P (Fig. 5B).

Phosphorylation of the C-terminal tail in S1P₁ by G protein-coupled receptor kinases (GRKs) facilitates binding of the adaptor protein β -arrestin, which then induces receptor desensitization and endocytosis (Watterson *et al.* 2002; Oo *et al.* 2007). To investigate whether retarded internalization of non-palmitoylated S1P₁ was due to lowered phosphorylation, we performed [³²P]orthophosphate labeling. After a 5 min treatment with S1P, phosphorylation of wild type S1P₁ was increased in a

concentration-dependent manner, reaching nearly maximum levels with 10 nM S1P (Fig. 6A,B). A similar dose-dependent curve was observed for non-palmitoylated S1P₁ (Fig. 6A,B). However, when FTY720-P was used, the wild type S1P₁ was phosphorylated at the maximum level even at 1 nM, whereas maximal phosphorylation of the 3CA mutant required 10 nM, and its phosphorylation level was ~2/3 that of wild type S1P₁ (Fig. 6A,B).

We also investigated the kinetics of phosphorylation for wild type and non-palmitoylated S1P₁ following stimulation with S1P or FTY720-P. In general, phosphorylation of wild type S1P₁ and non-palmitoylated S1P₁ increased over 60 min post-stimulation (Fig. 6C,D). However, following treatment with FTY720-P, although both proteins were phosphorylated, the phosphorylation levels for non-palmitoylated S1P₁ were lower than those for wild type S1P₁ (Fig. 6C,D). No difference in phosphorylation levels between wild type and non-palmitoylated S1P₁ were apparent following S1P treatment. This is consistent with the small difference noted in the internalization of the proteins (Fig. 5A), indicating some correlation between the phosphorylation levels and internalized amounts.

Palmitoylation of S1P₁ is increased upon treatment with S1P

It has been reported that in some GPCRs the state of palmitoylation is altered upon ligand treatment (Moffett *et al.* 1993; Hawtin *et al.* 2001). Therefore, we next performed [³H]palmitic acid labeling in the presence or absence of S1P using S1P₁-CHO cells. In the absence of S1P stimulation, the amount of labeled S1P₁ was unchanged over time (Fig. 7A). In contrast, labeling was enhanced by increasing the length of S1P treatment (Fig. 7 A). Note that most S1P₁ in S1P₁-CHO cells is already localized in the plasma membrane (Fig. 3A) and has undergone complex-type glycosylation. Therefore, palmitoylation following treatment with S1P occurs in the plasma membrane.

To determine whether this increase in S1P₁ palmitoylation could be attributed to direct activation of the receptor or to an indirect effect mediated by some non-receptor, we performed additional

[³H]palmitic acid labeling assays in the presence of pertussis toxin, which inactivates S1P₁ by ADP-ribosylation of the G_iα protein. The S1P-dependent increase in the S1P₁ palmitoylation was indeed inhibited by pertussis toxin (Fig. 7B). Thus, it is most likely that binding of the ligand to S1P₁ induces a conformational change in the structure, rendering it susceptible to palmitoylation.

Discussion

In the presented study, we determined that all S1P receptors are palmitoylated (Fig. 1C, D). Even S1P receptors with high mannose-type glycosylation are palmitoylated (Fig. 1B, C, D), so palmitoylation would seem to have already occurred in the ER. However, S1P₁, at least, is also palmitoylated in other organelles. Palmitoylation of complex-type glycosylated S1P₁, localized in the plasma membrane, was increased upon treatment with S1P (Fig. 7A). Therefore, S1P₁ in the plasma membrane seems to be subjected to a palmitoylation/depalmitoylation cycle. This cycle may be regulated during receptor activation, desensitization, internalization, and/or recycling.

Palmitoylation does not cause a large change in the affinity of S1P₁ toward S1P (wild type S1P₁: $K_d = 39.4$ nM, EC_{50} for ERK activation = 1.4 nM; 3CA mutant: $K_d = 31.4$ nM and $EC_{50} = 2.4$ nM). However, S1P₁ binding affinities toward FTY720-P seem to be affected greatly by palmitoylation, judging from the EC_{50} value for ERK activation (wild type S1P₁: 0.5 nM; 3CA mutant: 5.2 nM). It is possible that the insertion into the membrane of the palmitic acid moieties attached to the C-terminal tail of S1P₁, which generates an additional intracellular loop in S1P₁, may induce a local conformational change in the ligand binding site.

Although many GPCRs are known to be palmitoylated, the roles of their palmitoylation vary (Qanbar & Bouvier 2003; Escribá *et al.* 2007). For S1P₁, palmitoylation affects downstream signaling pathways (Fig. 4) irrespective of whether S1P or FTY720-P is the ligand. The maximum levels of p-ERK were significantly lower in cells overproducing the non-palmitoylated mutant S1P₁ compared to those overproducing the wild type S1P₁ (Fig. 4A-D). Heterotrimeric G proteins ($G\alpha\beta\gamma$) are attached to the membrane via lipid modifications on $G\alpha$ and $G\gamma$ (Escribá *et al.* 2007). It is possible that palmitoylation of S1P₁ increases its affinity for the heterotrimeric G proteins through interactions with their lipid moieties. Alternatively, palmitoylation may cause a local conformational change that affects the interactions between S1P₁ and heterotrimeric G proteins.

Like other GPCRs, ligand stimulation induces a conformational change in S1P₁, which aids not only in the GDP-GTP exchange of the heterotrimeric G protein α subunit, but also the availability of the receptor for phosphorylation by GRK and β -arrestin recruitment. Several lines of evidence suggest that S1P-bound S1P₁ and FTY720-P-bound S1P₁ differ in structure. For example, following treatment with S1P, internalized S1P₁ is recycled back to the cell surface, yet treatment with FTY720-P causes ubiquitination of S1P₁ and degradation (Gräler & Goetzl 2004; Matloubian *et al.* 2004; Jo *et al.* 2005). Therefore, ubiquitin ligase may recognize the structure of S1P₁ induced by FTY720-P but not that induced by S1P. These differences are thought to be responsible for the pharmacological effects of FTY720. However, the molecular mechanism behind the difference in the fate of S1P₁ following treatment with S1P and FTY720-P has been completely unclear. We found only a small difference in the internalization of the wild type and non-palmitoylated mutant S1P₁ upon treatment with S1P (Fig. 5A), in contrast to significant differences observed with FTY720-P treatment (Fig. 5B). Thus, palmitoylation affects the internalization of S1P₁ differently when binding S1P or FTY720-P. Phosphorylation of the mutant S1P₁ was also reduced compared to that of wild type S1P₁ following treatment with FTY720-P but not with S1P (Fig. 6). The differential effects of palmitoylation on internalization and phosphorylation provide important clues in understanding the molecular mechanism of the pharmacological effects of FTY720. We speculate that a combination of FTY720-P binding by S1P₁ and palmitoylation of S1P₁ causes the creation of high affinity binding sites for GRKs and putative ubiquitin ligases.

Although palmitoylation was first recognized over 30 years ago, identification of the responsible enzymes, palmitoyltransferases (PATs), was only recently achieved. In 2002, two yeast proteins containing the Cys-rich domain and conserved signature motif identifying them as DHHC proteins were determined to be PATs; Erf2 and Akr1 were found to palmitoylate Ras and the casein kinase Yck2, respectively (Lobo *et al.* 2002; Roth *et al.* 2002). Studies have shown that yeast and mammals

contain 7 and 23 DHHC proteins, respectively. Yeast mutant analysis has further indicated that each DHHC protein exhibits PAT activity with an obvious substrate preference, which overlaps among the enzymes (Roth *et al.* 2006). To date, PATs active toward GPCRs have not been identified. Therefore, future studies are required for identification of SIP₁ PATs.

Experimental procedures

Cell culture and transfection

HEK 293T cells and CHO cells were grown in Dulbecco's modified Eagle's medium (DMEM) (D6429; Sigma) and in Ham's F-12 medium (N6658; Sigma), respectively, each containing 10% FBS and supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin. HEK 293T cells were grown in 0.3% collagen-coated dishes. Transfections were performed using Lipofectamine Plus™ Reagent (Invitrogen) according to the manufacturer's instructions.

Plasmids

The pCE-puro 3xFLAG-4 plasmid is a derivative of the pCE-puro vector (Kihara *et al.* 2003) and is designed to produce a C-terminal triple FLAG (3xFLAG)-tagged protein. Each pCE-puro S1P_x-3xFLAG plasmid (with x representing the S1P receptor number) encodes a human S1P receptor tagged with 3xFLAG at its C-terminus. The genes of the S1P receptors were amplified by PCR using appropriate templates (*SIP*₁, human spleen cDNA (Clontech, Takara Bio, Shiga, Japan); *SIP*₂ and *SIP*₃, HEK 293T cDNA; *SIP*₄, human leukocyte cDNA (Clontech, Takara Bio); and *SIP*₅, human lung cDNA (Clontech, Takara Bio)) and primers (for *SIP*₁, 5'-AGGATCCGCCACCATGGGGCCCACCAGCGTCCCG-3' and 5'-AGGATCCCTGGAAGAAGAGTTGACGTTTCCAGAAG-3'; for *SIP*₂, 5'-GGATCCGCCACCATGGGCAGCTTGTACTCGGAGTACC-3' and 5'-GGATCCCTGACCACCGTGTTGCCCTCCAGAAACG-3'; for *SIP*₃, 5'-GCCACCATGGCAACTGCCCTCCCGCCGCG-3' and 5'-GGATCCCTGTTGCAGAAGATCCCATTCTGAAGTG-3'; for *SIP*₄, 5'-GCCACCATGAACGCCACGGGGACC-3' and 5'-AGATCTCCGATGCTCCGCACGCTGGAGATGCTGG-3'; and for *SIP*₅,

5'-GCCACCATGGAGTCGGGGCTGCTGC-3' and 5'-GGATCCGCGTCTGCAGCCGGTTCTGATAACC-3'). Each amplified fragment was first cloned into the pGEM-T Easy vector (Promega, Madison, WI), and then the *Bam*HI-*Bam*HI (*SIP*₁ and *SIP*₂), *Eco*RI-*Bam*HI (*SIP*₃ and *SIP*₅) or *Eco*RI-*Bgl*III (*SIP*₄) fragments of the resulting plasmids were cloned into the pCE-puro 3xFLAG-4 vector, generating the respective pCE-puro *SIP*_x-3xFLAG plasmids. The pCE-puro *SIP*₁(C328A)-3xFLAG, pCE-puro *SIP*₁(C329A)-3xFLAG, pCE-puro *SIP*₁(C331A)-3xFLAG, pCE-puro *SIP*₁(C328/329A)-3xFLAG, pCE-puro *SIP*₁(C329/331A)-3xFLAG, pCE-puro *SIP*₁(C328/331A)-3xFLAG, and pCE-puro *SIP*₁(3CA)-3xFLAG plasmids encode Cys-to-Ala substituted *SIP*₁ mutants. These plasmids were constructed from the pCE-puro *SIP*₁-3xFLAG plasmid by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Two primers with complementary sequences were used for each mutagenesis. The sense primers used were, for pCE-puro *SIP*₁(C328A)-3xFLAG, 5'-CATCCGGATCATGTCCGCCTGCAAGTGCCCGAGCG-3'; for pCE-puro *SIP*₁(C329A)-3xFLAG, 5'-CCGGATCATGTCCTGCGCCAAGTGCCCGAGCGGAG-3'; for pCE-puro *SIP*₁(C331A)-3xFLAG, 5'-CATGTCCTGCTGCAAGGCCCGAGCGGAGACTCTG-3'; for pCE-puro *SIP*₁(C328/329A)-3xFLAG, 5'-CATCCGGATCATGTCCGCCGCAAGTGCCCGAGCG-3'; for pCE-puro *SIP*₁(C328/331A)-3xFLAG, 5'-CATCCGGATCATGTCCGCCTGCAAGGCCCGAGCG-3'; for pCE-puro *SIP*₁(C329/331A)-3xFLAG, 5'-CCGGATCATGTCCTGCGCCAAGGCCCGAGCGGAG-3'; and for *SIP*₁(3CA)-3xFLAG, 5'-CCGGATCATGTCCGCCGCAAGGCCCGAGCGGAGACTC-3'.

Production of stable transformants

To obtain stable transformants of the *SIP*₁-3xFLAG or *SIP*₁(3CA)-3xFLAG gene, the pCE-puro

S1P₁-3xFLAG or pCE-puro S1P₁(3CA)-3xFLAG plasmid was transfected into CHO cells. Cells were subjected to puromycin selection at 10 µg/mL. One stable transformant for each plasmid, termed S1P₁-CHO and S1P₁(3CA)-CHO, expressed the highest level of S1P₁-3xFLAG and S1P₁(3CA)-3xFLAG, respectively, among the isolated clones. Although we present only the results obtained using S1P₁-CHO and S1P₁(3CA)-CHO cells here, other clones also tested gave similar results. For use as a control, Vector-CHO cells were similarly obtained using the pCE-puro 3xFLAG-4 vector.

***In vivo* labeling experiments**

[³H]Palmitic acid labeling was done as described previously (Kihara *et al.* 2006) using 0.2 mCi of [³H]palmitic acid (60 Ci/mmol; American Radiolabeled Chemical, St. Louis, MO). Prior to [³²P]orthophosphate labeling, the culture medium was changed to phosphate-free DMEM (11971-025; Gibco, Invitrogen). After 1 h, the cells were placed in phosphate-free DMEM containing 75 µCi [³²P]orthophosphate (28 kCi/mmol; PerkinElmer Life Sciences, Ontario, Canada) and 0.1% fatty acid-free BSA. After a 4 h incubation, cells were incubated with 200 nM S1P or 200 nM FTY720-P for various times. Cells were then lysed with RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.1% sodium deoxycholate), and the resulting total cell lysates were subjected to immunoprecipitation with anti-FLAG M2 agarose beads (Sigma). Beads were washed with RIPA buffer twice and with 10 mM Tris-HCl (pH 8.0) once, then proteins were eluted with 2x SDS sample buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercapoethanol, and a trace amount of bromophenol blue). Proteins were separated by SDS-PAGE and detected by autoradiography.

Deglycosylation of proteins

Endo H and PNGase F were purchased from New England Biolabs (Beverly, MA). Cells were washed

with PBS twice, suspended in buffer A (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 10% 2-mercaptoethanol), and sonicated. After a 5 min incubation at 37 °C, samples were centrifuged at 17,400 x g for 5 min at room temperature. The supernatant was subjected to deglycosylation by EndoH or PNGase F according to the manufacturer's instructions.

Immunoblotting

Immunoblotting was performed as described previously (Kihara *et al.* 2003). Anti-FLAG M2 (1 µg/mL; Stratagene), anti-extracellular signal-regulated kinase (ERK; p44/42 MAP kinase) (1:1000 dilution; Cell Signaling Technology, Beverly, MA), and anti-phospho-ERK (p-ERK) (200 ng/mL; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used as primary antibodies.

HRP-conjugated anti-mouse or anti-rabbit IgG F(ab')₂ fragment (both from GE Healthcare Bio-Sciences, Piscataway, NJ, and diluted 1:7500) were used as secondary antibodies. Labeling was detected using ECLTM Reagents or an ECL plus System for Western Blotting Detection (both from GE Healthcare).

Immunofluorescence microscopy

Microscopic immunofluorescence analysis was performed as described previously (Ogawa *et al.* 2003) with anti-FLAG M2 antibodies (0.5 µg/mL) and Alexa Fluor 488 goat anti-mouse IgG (H+L) conjugated antibody (5 µg/mL, Molecular Probes, Invitrogen). The stained cells were analyzed by fluorescence microscopy (Axioskop 2 PLUS; Carl Zeiss, Oberkochen, Germany).

Flow cytometry

Cells were detached from the culture surface by treatment with Trypsin/EDTA (0.25% Trypsin and 0.02% EDTA) solution, washed twice with FACS buffer (0.5% fatty acid-free BSA in PBS), and

suspended in FACS buffer containing a human S1P₁ antibody (anti-hEDG1; 20 µg/mL; R&D Systems, Minneapolis, MN). After a 30 min incubation at 4 °C, cells were washed twice with FACS buffer, then treated with Alexa Fluor 488 goat anti-mouse IgG (H+L) conjugated antibody (10 µg/mL) for 30 min at 4 °C. Cells were washed twice with FACS buffer then suspended in FACS buffer, and the intensity of cell fluorescence was determined by a FACScan cytometer (BD Biosciences, San Jose, CA). Data was analyzed by CellQuest™ 3.0 software.

ABE Assay

An ABE assay was done as described elsewhere with modifications (Drisdell & Green 2004). Cells were washed twice with PBS and lysed on ice using RIPA buffer containing 1x Complete™ protease inhibitor mixture (EDTA free; Roche Diagnostics, Indianapolis, IN), 1 mM phenylmethylsulfonyl fluoride, and 10 mM NEM. After sonication, cell debris was removed by centrifuging at 17,400 x g for 5 min at 4 °C. The resulting supernatant was incubated overnight at 4 °C with anti-FLAG M2 agarose by rotating the sample tubes. Beads were washed twice with 1 mL RIPA buffer containing 10 mM NEM, and suspended in buffer B (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 0.1 % SDS) containing 50 mM NEM. After rotating the sample tube for 1 h at room temperature, beads were washed twice with 1 mL buffer B and once with 1 mL 10 mM Tris-HCl (pH 7.4), then suspended in buffer C (10 mM Tris-HCl (pH 7.4), 0.2% Triton X-100, 150 mM NaCl, and 0.2 mM EZ-Link™ Biotin-HPDP (Pierce, Rockford, IL) containing 1 M hydroxylamine (pH 7.4) or 50 mM Tris-HCl (pH 7.4), and incubated for 1 h at room temperature with rotation. Beads were washed twice with 1 mL buffer B and once with 1 mL 10 mM Tris-HCl (pH 7.4), and bound proteins were eluted with 80 µL elution buffer (50 mM Tris-HCl (pH 7.4), 2% SDS, and 5 mM EDTA). Samples were then diluted with 20 volumes of buffer D (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100) and incubated with Immunopure Immobilized Avidin (Pierce) overnight

at 4 °C with rotation. Beads were washed twice with 1 mL buffer B and once with 1 mL 10 mM Tris-HCl (pH 7.4), and bound proteins were eluted with 2x SDS sample buffer and subjected to immunoblotting with anti-FLAG M2 antibodies.

Cell migration assay

Trypsinized cells were suspended in F-12 medium containing 0.1% fatty acid-free BSA then added to the upper chambers of Transwell filters (polycarbonate membrane, pore size of 8.0 µm; Corning, New York, NY) coated with 10 µg/mL fibronectin. Medium containing 200 nM S1P or FTY720-P was added to the lower chamber. The cultures were incubated for 4 h at 37 °C. Cells on the upper surface of the Transwell filter were removed with a cotton swab, and those on the lower side of the filter were fixed with cold methanol and stained with 1% crystal violet in 2% ethanol. Cells within 4 randomly selected regions were counted under a phase-contrast microscope (IX70-S8F2; Olympus, Tokyo, Japan).

Acknowledgements

We are grateful to Dr. E. A. Sweeney for scientific editing of the manuscript. We thank Ayaka Okuda for technical support. This work was supported by a Grant-in-Aid for Exploratory Research (20659007) (A.K.) and by a grant from the Japan Society for the Promotion of Sciences (Y.O.).

References

- Brinkmann, V., Davis, M.D., Heise, C.E., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumruker, T., Hiestand, P., Foster, C.A., Zollinger, M. & Lynch, K.R. (2002) The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J. Biol. Chem.* **277**, 21453-21457.
- Budde, K., Schütz, M., Glander, P., Peters, H., Waiser, J., Liefeldt, L., Neumayer, H.H. & Böhrer, T. (2006) FTY720 (fingolimod) in renal transplantation. *Clin. Transplant.* **20 Suppl 17**, 17-24.
- Chiba, K., Matsuyuki, H., Maeda, Y. & Sugahara, K. (2006) Role of sphingosine 1-phosphate receptor type 1 in lymphocyte egress from secondary lymphoid tissues and thymus. *Cell. Mol. Immunol.* **3**, 11-19.
- Drisdel, R.C. & Green, W.N. (2004) Labeling and quantifying sites of protein palmitoylation. *Biotechniques* **36**, 276-285.
- Escribá, P.V., Wedegaertner, P.B., Goñi, F.M. & Vögler, O. (2007) Lipid-protein interactions in GPCR-associated signaling. *Biochim. Biophys. Acta* **1768**, 836-852.
- Fieger, C.B., Huang, M.C., Van Brocklyn, J.R. & Goetzl, E.J. (2005) Type 1 sphingosine 1-phosphate G protein-coupled receptor signaling of lymphocyte functions requires sulfation of its extracellular amino-terminal tyrosines. *FASEB J.* **19**, 1926-1928.
- Fukushima, Y., Saitoh, T., Anai, M., Ogiwara, T., Inukai, K., Funaki, M., Sakoda, H., Onishi, Y., Ono, H., Fujishiro, M., Ishikawa, T., Takata, K., Nagai, R., Omata, M. & Asano, T. (2001) Palmitoylation of the canine histamine H2 receptor occurs at Cys(305) and is important for cell surface targeting. *Biochim. Biophys. Acta* **1539**, 181-191.
- Gräler, M.H. & Goetzl, E.J. (2004) The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J.* **18**, 551-553.
- Hawtin, S.R., Tobin, A.B., Patel, S. & Wheatley, M. (2001) Palmitoylation of the vasopressin V_{1a} receptor reveals different conformational requirements for signaling, agonist-induced receptor

- phosphorylation, and sequestration. *J. Biol. Chem.* **276**, 38139-38146.
- Jo, E., Sanna, M.G., Gonzalez-Cabrera, P.J., Thangada, S., Tigyi, G., Osborne, D.A., Hla, T., Parrill, A.L. & Rosen, H. (2005) S1P₁-selective *in vivo*-active agonists from high-throughput screening: off-the-shelf chemical probes of receptor interactions, signaling, and fate. *Chem. Biol.* **12**, 703-715.
- Karnik, S.S., Ridge, K.D., Bhattacharya, S. & Khorana, H.G. (1993) Palmitoylation of bovine opsin and its cysteine mutants in COS cells. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 40-44.
- Kennedy, M.E. & Limbird, L.E. (1993) Mutations of the α_{2A} -adrenergic receptor that eliminate detectable palmitoylation do not perturb receptor-G-protein coupling. *J. Biol. Chem.* **268**, 8003-8011.
- Kihara, A., Anada, Y. & Igarashi, Y. (2006) Mouse sphingosine kinase isoforms SPHK1a and SPHK1b differ in enzymatic traits including stability, localization, modification, and oligomerization. *J. Biol. Chem.* **281**, 4532-4539.
- Kihara, A. & Igarashi, Y. (2008) Production and release of sphingosine 1-phosphate and the phosphorylated form of the immunomodulator FTY720. *Biochim. Biophys. Acta* **1781**, 496-502.
- Kihara, A., Ikeda, M., Kariya, Y., Lee, E. Y., Lee, Y.M. & Igarashi, Y. (2003) Sphingosine-1-phosphate lyase is involved in the differentiation of F9 embryonal carcinoma cells to primitive endoderm. *J. Biol. Chem.* **278**, 14578-14585.
- Kihara, A., Mitsutake, S., Mizutani, Y. & Igarashi, Y. (2007) Metabolism and biological functions of two phosphorylated sphingolipids, sphingosine 1-phosphate and ceramide 1-phosphate. *Prog. Lipid Res.* **46**, 126-144.
- Kohno, T., Wada, A. & Igarashi, Y. (2002) N-glycans of sphingosine 1-phosphate receptor Edg-1 regulate ligand-induced receptor internalization. *FASEB J.* **16**, 983-992.
- Kraft, K., Olbrich, H., Majoul, I., Mack, M., Proudfoot, A. & Oppermann, M. (2001) Characterization

- of sequence determinants within the carboxyl-terminal domain of chemokine receptor CCR5 that regulate signaling and receptor internalization. *J. Biol. Chem.* **276**, 34408-34418.
- Liu, C.H., Thangada, S., Lee, M.J., Van Brocklyn, J.R., Spiegel, S. & Hla, T. (1999) Ligand-induced trafficking of the sphingosine-1-phosphate receptor EDG-1. *Mol. Biol. Cell* **10**, 1179-1190.
- Lobo, S., Greentree, W.K., Linder, M.E. & Deschenes, R.J. (2002) Identification of a Ras palmitoyltransferase in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **277**, 41268-41273.
- Mandala, S., Hajdu, R., Bergstrom, J. *et al.* (2002) Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* **296**, 346-349.
- Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkmann, V., Allende, M.L., Proia, R.L. & Cyster, J.G. (2004) Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* **427**, 355-360.
- Moffett, S., Mouillac, B., Bonin, H. & Bouvier, M. (1993) Altered phosphorylation and desensitization patterns of a human β_2 -adrenergic receptor lacking the palmitoylated Cys341. *EMBO J.* **12**, 349-356.
- O'Dowd, B.F., Hnatowich, M., Caron, M.G., Lefkowitz, R.J. & Bouvier, M. (1989) Palmitoylation of the human β_2 -adrenergic receptor. Mutation of Cys341 in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. *J. Biol. Chem.* **264**, 7564-7569.
- Ogawa, C., Kihara, A., Gokoh, M. & Igarashi, Y. (2003) Identification and characterization of a novel human sphingosine-1-phosphate phosphohydrolase, hSPP2. *J. Biol. Chem.* **278**, 1268-1272.
- Okamoto, H., Takuwa, N., Yokomizo, T., Sugimoto, N., Sakurada, S., Shigematsu, H. & Takuwa, Y. (2000) Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. *Mol. Cell. Biol.* **20**, 9247-9261.
- Oo, M.L., Thangada, S., Wu, M.T., Liu, C.H., Macdonald, T.L., Lynch, K.R., Lin, C.Y. & Hla, T.

- (2007) Immunosuppressive and anti-angiogenic sphingosine 1-phosphate receptor-1 agonists induce ubiquitinylation and proteasomal degradation of the receptor. *J. Biol. Chem.* **282**, 9082-9089.
- Qanbar, R. & Bouvier, M. (2003) Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function. *Pharmacol. Ther.* **97**, 1-33.
- Resh, M.D. (1999) Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim. Biophys. Acta.* **1451**, 1-16.
- Roth, A.F., Feng, Y., Chen, L. & Davis, N.G. (2002) The yeast DHHC cysteine-rich domain protein Akr1p is a palmitoyl transferase. *J. Cell Biol.* **159**, 23-28.
- Roth, A.F., Wan, J., Bailey, A.O., Sun, B., Kuchar, J.A., Green, W.N., Phinney, B.S., Yates, J.R., 3rd & Davis, N.G. (2006) Global analysis of protein palmitoylation in yeast. *Cell* **125**, 1003-1013.
- Sanchez, T. & Hla, T. (2004) Structural and functional characteristics of S1P receptors. *J. Cell. Biochem.* **92**, 913-922.
- Smotrys, J.E. & Linder, M.E. (2004) Palmitoylation of intracellular signaling proteins: regulation and function. *Annu. Rev. Biochem.* **73**, 559-587.
- Spiegel, S. & Milstien, S. (2003) Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat. Rev. Mol. Cell Biol.* **4**, 397-407.
- Watterson, K.R., Johnston, E., Chalmers, C., Pronin, A., Cook, S.J., Benovic, J.L. & Palmer, T.M. (2002) Dual regulation of EDG1/S1P₁ receptor phosphorylation and internalization by protein kinase C and G-protein-coupled receptor kinase 2. *J. Biol. Chem.* **277**, 5767-5777.
- Zhu, H., Wang, H. & Ascoli, M. (1995) The lutropin/choriogonadotropin receptor is palmitoylated at intracellular cysteine residues. *Mol. Endocrinol.* **9**, 141-150.

Figure legends

Figure 1 All S1P receptors are palmitoylated. (A) Amino acid sequences of the C-terminal hydrophilic regions of human S1P receptors are shown. Cys residues that may be potential sites for palmitoylation are highlighted in boxes. TM7, transmembrane 7. (B-D) HEK 293T cells were transfected with plasmids encoding S1P₁ through S1P₅, each C-terminally tagged with 3xFLAG. (B) Twenty-four hours after transfection, total cell lysates were prepared, treated with nothing (-), Endo H (E), or PNGase F (P), and separated by SDS-PAGE, followed by immunoblotting with anti-FLAG antibodies. (C) Twenty-four hours after transfection, the culture medium was changed to serum-free DMEM, and after a 1 h incubation at 37 °C, cells were labeled with 0.2 mCi [³H]palmitic acid for 2 h at 37 °C. Total cell lysates were prepared, and S1P receptor proteins were immunoprecipitated with anti-FLAG M2 agarose beads. The precipitates were separated by SDS-PAGE and detected by autoradiography (upper panel) or by immunoblotting with anti-FLAG antibodies (lower panel). (D) Twenty-four hours after transfection, total cell lysates were prepared and analyzed by ABE assay. Lysates were treated with NEM to block non-palmitoylated Cys residues, then were subjected to immunoprecipitation with anti-FLAG M2 agarose. The precipitates were incubated in the presence or absence of the thioester linkage-cleaving reagent hydroxylamine (NH₂OH), which removes palmitic acid, and with EZ-link™ Biotin-HPDP, to biotinylate free thiol groups. Aliquots of samples were subjected to immunoblotting with anti-FLAG antibodies (lower panel), while the remaining proteins were precipitated with avidin beads then detected by immunoblotting with anti-FLAG antibodies (upper panel). IB, immunoblotting; AvP, avidin-precipitation.

Figure 2 S1P₁ is palmitoylated on three Cys residues in the C-terminal tail. (A) HEK 293T cells transfected with pCE-puro 3xFLAG-4 (vector; Vec), pCE-puro S1P₁-3xFLAG (wild type; WT), or pCE-puro S1P₁(3CA)-3xFLAG (S1P₁(3CA); 3CA) were labeled with 0.2 mCi of [³H]palmitic acid for

3 h. The S1P₁ receptors were immunoprecipitated from total cell lysates with anti-FLAG M2 agarose and separated by SDS-PAGE, followed by autoradiography (upper panel) or by immunoblotting with anti-FLAG antibodies (lower panel). (B) HEK 293T cells were transfected with pCE-puro 3xFLAG-4 or its derivatives encoding wild type or the indicated Cys-to-Ala substituted mutant of S1P₁-3xFLAG. Twenty-four hours after transfection, total cell lysates were prepared and analyzed by ABE assay as in Figure 1. Lysates were treated with NEM, and subjected to immunoprecipitation with anti-FLAG M2 agarose. The immunoprecipitates were incubated in the presence or absence of hydroxylamine (NH₂OH), and biotinylated with EZ-link™ Biotin-HPDP. Aliquots of samples were subjected to immunoblotting with anti-FLAG antibodies (lower panel), while the remaining proteins were precipitated with the avidin beads then detected by immunoblotting with anti-FLAG antibodies (upper panel). IB, immunoblotting; AvP, avidin-precipitation.

Figure 3 Palmitoylation is not required for cell surface expression of S1P₁. (A) S1P₁-CHO and S1P₁(3CA)-CHO cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and immunostained with anti-FLAG antibodies. The stained cells were analyzed using a fluorescence microscope Axioskop 2 PLUS (lens x63/1.40 OIL; ocular x10). Calibration bar, 10 μm. (B) Total lysates were prepared from Vector-CHO, S1P₁-CHO, and S1P₁(3CA)-CHO cells. Proteins (3 μg) were separated by SDS-PAGE and subjected to immunoblotting with anti-FLAG antibodies. Uniform protein loading was demonstrated by immunoblotting with anti-GAPDH antibodies. (C) Vector-CHO (gray peaks in both left and right panels), S1P₁-CHO (left panel), and S1P₁(3CA)-CHO (right panel) cells were incubated for 12 h with F-12 medium containing charcoal-treated FBS, then for another 12 h with F-12 medium. Cells were then stained with anti-S1P₁ antibodies and subjected to flow cytometry. Vec, vector; WT, wild type S1P₁; 3CA, S1P₁(3CA).

Figure 4 Palmitoylation of S1P₁ is required for efficient activation of the S1P signaling pathways.

(A-F) Vector-CHO, S1P₁-CHO, and S1P₁(3CA)-CHO cells were incubated for 12 h with F-12 medium containing charcoal-treated FBS, and for another 12 h with F-12 medium. The cells were then stimulated with the indicated concentrations of S1P (A, B) or FTY720-P (C, D) for 5 min, or with 200 nM S1P for the indicated time (E, F). Total cell lysates were prepared, and proteins (5 µg) were separated by SDS-PAGE, followed by immunoblotting with anti-p-ERK (upper panel) and anti-ERK (lower panel) antibodies. The intensities of the bands in (A), (C), and (E) were quantified using ImageJ software (<http://rsb.info.nih.gov/ij/>) and are expressed as a ratio of p-ERK levels to ERK levels in (B), (D), and (F), respectively. Values presented in (B) and (D) represent a mean ± S.D. from three independent experiments. Statistically significant differences are indicated (**p<0.01 and *p<0.05; Student's t-test). (G) Vector-CHO, S1P₁-CHO, and S1P₁(3CA)-CHO cells suspended in F-12 medium containing 0.1% fatty acid-free BSA were added to the upper chambers of a Transwell filter plate, and 200 nM S1P was placed into each lower chamber. The cells were allowed to transmigrate for 4 h at 37 °C, and the migrated cells were counted. Values presented are the number of migrated cells relative to that observed for Vector-CHO cells, and represent a mean ± S.D. from three independent experiments. Statistically significant differences are indicated (p<0.05; Student's t-test). Vec, vector; WT, wild type S1P₁; 3CA, S1P₁(3CA).

Figure 5 Palmitoylation affects the internalization of S1P₁ stimulated by FTY720-P. S1P₁-CHO and S1P₁(3CA)-CHO cells were incubated for 12 h with F-12 medium containing charcoal-treated FBS, then for another 12 h with F-12 medium. After a 1 h treatment with 10 µg/mL cycloheximide to inhibit protein synthesis, the cells were stimulated for 10 min (arrows) with 200 nM S1P (A) or FTY720-P (B). Cells were washed with F-12 medium containing 4 mg/mL fatty acid-free BSA, suspended in the same medium (t=0), and incubated for 15, 30, and 60 min at 37 °C. Cells were then stained with anti-S1P₁

antibodies and subjected to flow cytometry. Values presented are a percent of the geometric mean values of the fluorescence compared to that at the time ligands were added and represent a mean \pm S.D. from three independent experiments. Statistically significant differences are indicated (* $p < 0.05$, ** $p < 0.01$; Student's t-test).

Figure 6 Palmitoylation affects the phosphorylation levels of S1P₁ in cells stimulated with FTY720-P. S1P₁-CHO and S1P₁(3CA)-CHO cells were incubated for 12 h with F-12 medium containing charcoal-treated FBS, and for another 12 h with F-12 medium. The culture medium was then changed to phosphate-free DMEM, and the cells were labeled with 75 μ Ci [³²P]orthophosphate for 4 h at 37 °C. Cells were then treated with the indicated concentrations of S1P or FTY720-P for 5 min (A, B) or with 200 nM S1P or 200 nM FTY720-P for the indicated times (C, D). S1P₁ proteins were immunoprecipitated from total cell lysates with anti-FLAG M2 agarose and separated by SDS-PAGE, followed by autoradiography (A, C; upper panels) or by immunoblotting with anti-FLAG antibodies (A, C; lower panels). The amount of phosphorylated S1P₁ in (A) and (C) (upper panels) was quantified using a bioimaging analyzer BAS-2500 to measure the radioactivity of the bands, and the total immunoprecipitated S1P₁ in (A) and (C) (lower panels) was quantified using ImageJ software to analyze scanned images of the bands. The values are presented as a ratio of phosphorylated S1P₁ to S1P₁ (B, D). WT, wild type S1P₁; 3CA, S1P₁(3CA); IB, immunoblotting; pS1P₁, phosphorylated S1P₁.

Figure 7 Palmitoylation is increased upon ligand stimulation. (A) S1P₁-CHO cells were incubated for 12 h with F-12 medium containing charcoal-treated FBS, and for another 12 h with F-12 medium. Cells were labeled for 2 h with 0.2 mCi [³H]palmitic acid, during which time the cells were treated with 0 or 200 nM S1P for the indicated times. S1P₁ was immunoprecipitated from total cell lysates with anti-FLAG M2 agarose, separated by SDS-PAGE, and detected by autoradiography (upper panel) or by

immunoblotting with anti-FLAG antibodies (lower panel). (B) S1P₁-CHO cells were incubated for 12 h with F-12 medium containing charcoal-treated FBS, and for another 12 h with F-12 medium in the presence or absence of 100 ng/mL pertussis toxin (PTX). Cells were labeled with [³H]palmitic acid as in (A). S1P₁ was immunoprecipitated from total cell lysates with anti-FLAG M2 agarose, separated by SDS-PAGE, and detected by autoradiography (upper panel) or by immunoblotting with anti-FLAG antibodies (lower panel). IB, immunoblotting.

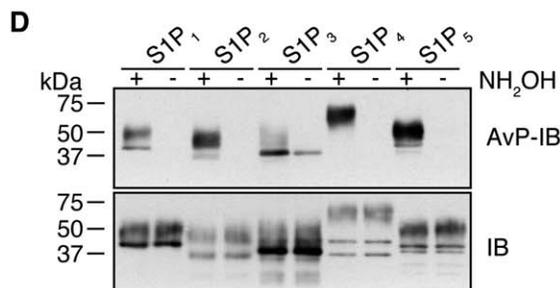
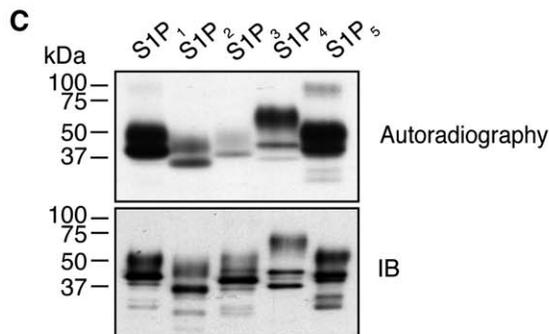
A

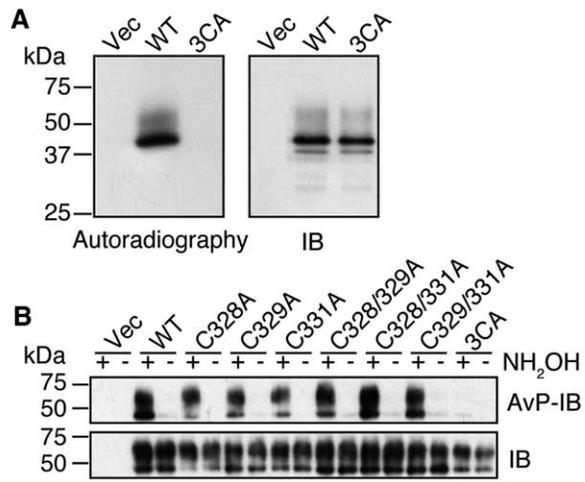
S1P₁ 298 VLAVLNSGTNP¹IYTLTNKEMRRAFIRIMS²CKK³PSGDS 336
 S1P₂ 275 AVSTLNSLLNPVIYTWRSRDLRREVLRLP⁴QWRPVG⁵VQ 313
 S1P₃ 285 VLAVLNSAMNPVIYTLASKEMRRAFFRLV⁶EN⁷LVRGRGA 323
 S1P₄ 294 ALAVLNSAVNP¹IYSFRSREVCRAVLSFL²CG³LRLGMR 332
 S1P₅ 293 GLAMANSLLN¹P²IYTLN³RDLRHALLRLV⁴CG⁵GRHS⁶GRD 331

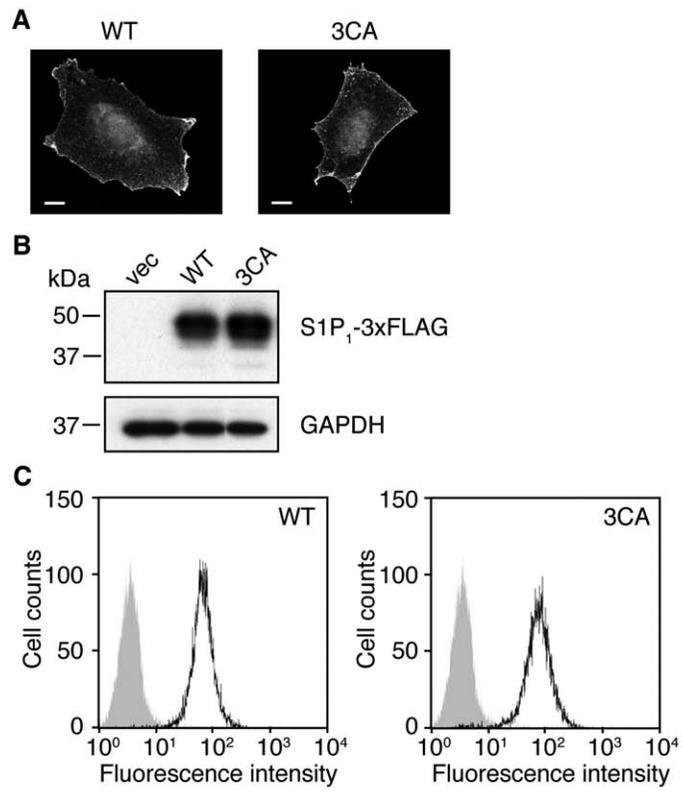
TM7

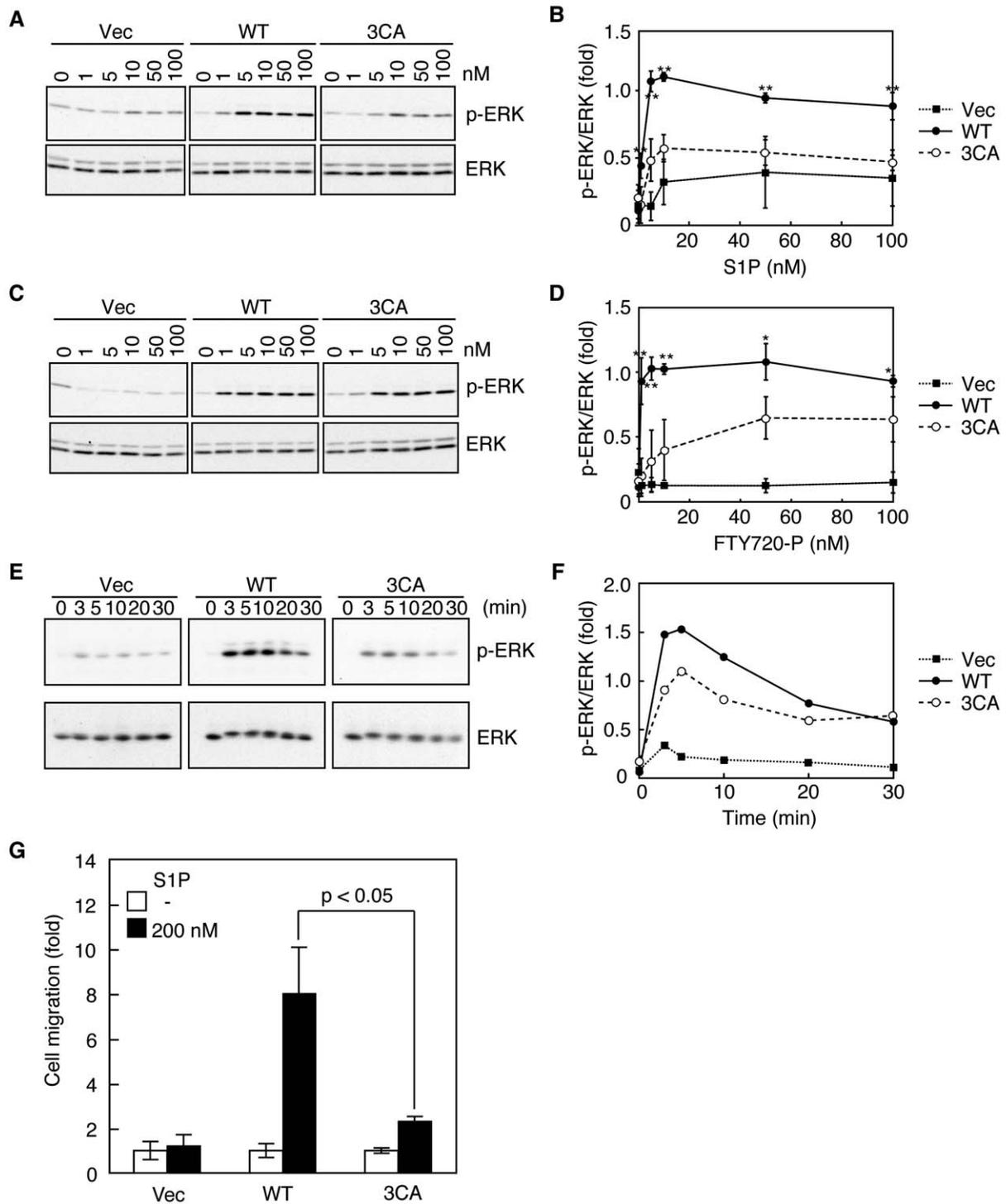
S1P₁ 337 AGKFKRP¹IAGMEFSRSKSDNSSHPQKDEGDNPETIMSS 375
 S1P₂ 314 GRRRG²TPGHLLPLR³SSSLERGMHMTSPTFLEGNTV 352
 S1P₃ 324 RASPIQ⁴PALDPSRSKSSSSN⁵SSHSPKVKEDLPHTAPSS 362
 S1P₄ 333 GPGDCLARAVEAHSGASTT⁶DSSLRPRDSFRGSRSL⁷SFRM 371
 S1P₅ 332 PSGSQQASAAEASGGLRRCLPPGLDGSFSGSERSSPQR 370

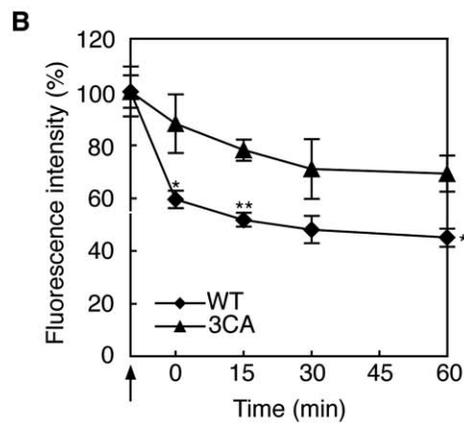
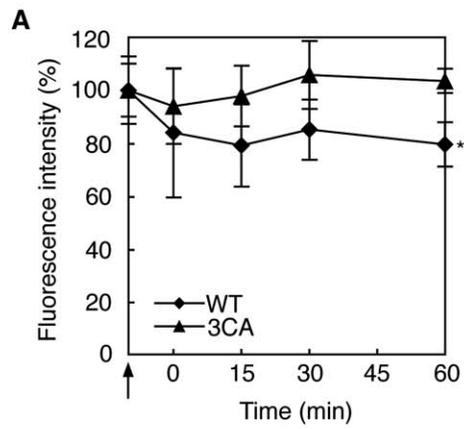
S1P₁ 376 GNVNSSS 382
 S1P₂ 353 V 353
 S1P₃ 363 CIMDKNAALQNGIFCN 378
 S1P₄ 372 REPLSSISSVRSI 384
 S1P₅ 371 DGLDTSGSTGSPGAPTAARTLVSEPAAD 398

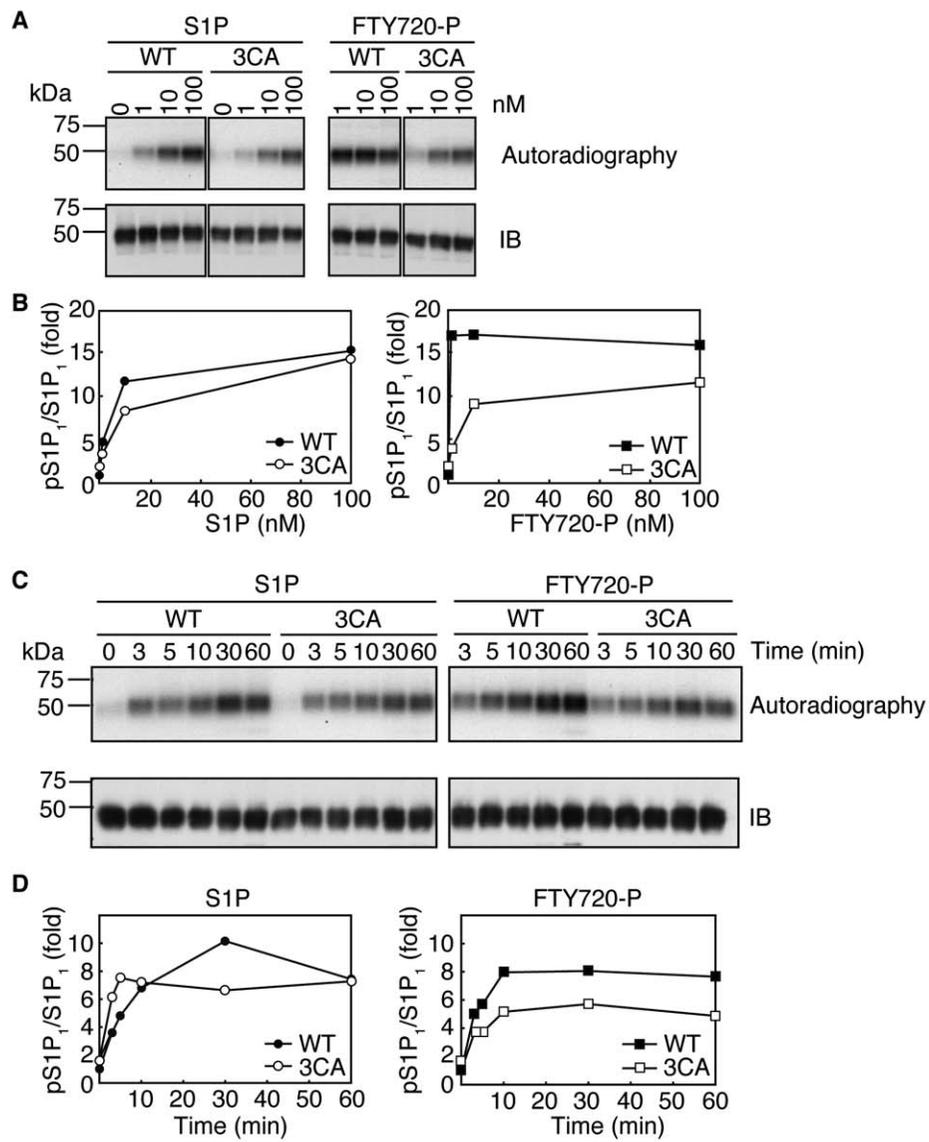


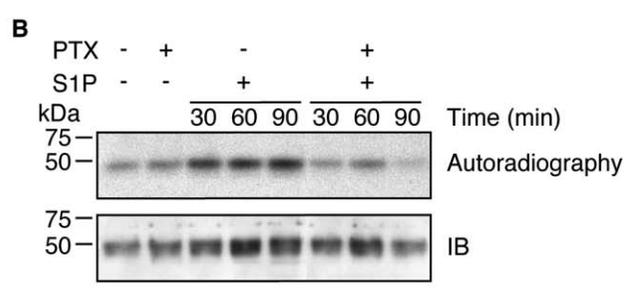
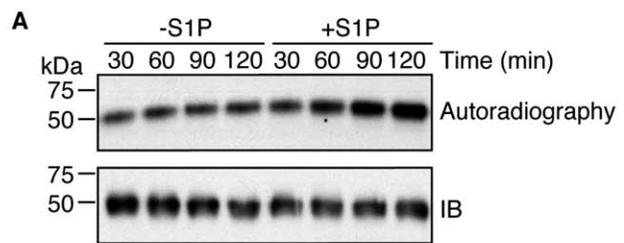












Ohno *et al.*, Fig. 7