The immunosuppressant FTY720 is phosphorylated by sphingosine kinase type 2

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Received 25 August 2003; revised 22 September 2003; accepted 6 October 2003

First published online 16 October 2003

Edited By Judit Ovádi

Abstract The potent immunosuppressive drug FTY720, a sphingosine analog, induces redistribution of lymphocytes from circulation to secondary lymphoid tissues. FTY720 is phosphorylated in vivo and functions as an agonist for four G-protein-coupled sphingosine-1-phosphate receptors. The identity of the kinase that phosphorylates FTY720 is still not known. Here we report that although both sphingosine kinase type 1 (SphK1) and type 2 (SphK2) can phosphorylate FTY720 with low efficiency, SphK2 is much more effective than SphK1. FTY720 inhibited phosphorylation of sphingosine catalyzed by SphK2 to a greater extent than it inhibits SphK1. Thus, SphK2 may be the relevant enzyme that is responsible for in vivo phosphorylation of FTY720.

Key words: FTY720; Sphingosine kinase; Sphingosine; Sphingosine-1-phosphate; Phosphorylation

1. Introduction

The novel immunosuppressant FTY720 (2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol) prevents transplant graft rejections and inhibits a variety of experimental autoimmune disorders, including type I diabetes mellitus [1], systemic lupus erythematosus [2], autoimmune encephalomyelitis [3], and arthritis [4]. FTY720 is even effective in preventing rejection of renal transplants in man [5,6]. FTY720 has recently received much attention since in contrast to other immunosuppressive agents, it acts to specifically induce sequestration of lymphocytes without causing cytotoxicity or suppressing growth potential [5]. Thus, FTY720 may provide an advantage over current immunosuppressive therapies.

FTY720 is derived from ISP-1 (myriocin), a fungal sphingosine-like metabolite used in Chinese herbal medicine [7]. Unlike ISP-1, FTY720 does not cause gastrointestinal toxicity and does not inhibit serine palmitoyl transferase [8], the rate limiting enzyme in de novo sphingolipid biosynthesis. It has recently been reported that FTY720 is phosphorylated to form a potent sphingosine-1-phosphate (S1P) analog that interacts with four of the five S1P receptors and thus could activate many signaling pathways [9,10]. Treatment with FTY720 or its phosphorylated form results in transient lymphopenia due to reversible redistribution of lymphocytes from the circulation to secondary lymphoid tissues by still not well-defined mechanisms [8–10]. The redirection of T lymphocytes away from tissue grafts and sites of inflammation is thought to underlie the effectiveness of FTY720 in transplantation and autoimmunity.

As FTY720 is phosphorylated in vivo and is a sphingosine analog, it might be expected to be phosphorylated by a sphingosine kinase (SphK), the enzyme that catalyzes the formation of S1P [11–13]. Because recombinant SphK1 catalyzed the phosphorylation of FTY720 and the active R enantiomer of the FTY720 analog AAL, but not the inactive S enantiomer, it was suggested SphK1 is the kinase that converts FTY720 to its active form [10]. However, FTY720 was phosphorylated by SphK1 around 500-fold less efficiently than sphingosine [10], raising the possibility that SphK1 may not phosphorylate FTY720 in vivo. We have cloned and characterized another isoform of SphK [13]. Although it contains the five conserved domains found in all SphKs, SphK2 is much larger than SphK1, has a different tissue distribution and different pattern of developmental expression, and slightly different kinetic properties and substrate specificity [13] (Table 1). Whereas SphK1 stimulates growth and survival, we recently found that SphK2 enhanced apoptosis in diverse cell types and also suppressed cellular proliferation [14]. The apoptotic effect of SphK2 might be due to its putative BH3 domain [14], a motif present in the BH3-only pro-apoptotic subgroup of the Bcl-2 family. Because of the clinical importance of FTY720 and its phosphorylated product, it is important to identify the kinase that phosphorylates it. Here we report that SphK2 catalyzes the phosphorylation of FTY720 much more efficiently than SphK1, suggesting that the therapeutic efficacy of FTY720 might be dependent on in vivo activity of SphK2.

2. Materials and methods

2.1. Materials

S1P was from Biomol Research Laboratory Inc. (Plymouth Meeting, PA, USA). All other lipids were purchased from Avanti Polar Lipids (Birmingham, AL, USA). FTY720 was kindly provided by Dr. Volker Brinkmann, Novartis Pharma AG. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Perkin Elmer (Boston, MA, USA). Lipofectamine was from Life Technologies (Gaithersburg, MD, USA).

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Abbreviations: BSA, bovine serum albumin; S1P, sphingosine-1-phosphate; SphK1, sphingosine kinase type 1; SphK2, sphingosine kinase type 2; TLC, thin layer chromatography

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doi:10.1016/S0014-5793(03)01168-2

Table 1
Distinct characteristics of SphK1 and SphK2

	SphK1	SphK2
Conserved domains	5	5
BH3 motif	None	1
Tissue expression (high)	Lung, spleen	Liver, heart
Developmental expression	E7	E11
Localization	Cytosol (~70%)	Cytosol (~70%)
Cell growth	Stimulated	Inhibited
Apoptosis	Suppressed	Stimulated
Substrate specificity	D-*erythro*-sphingosine	D-*erythro*-DH-sphingosine > D-*erythro*-sphingosine
Inhibitors	DMS[a] (competitive)	DMS[a] (non-competitive)
KCl	Inhibits	Stimulates
Triton X-100	Stimulates	Inhibits
Acidic phospholipids	Stimulate	Stimulate
Chromosome location	17q25.2	19q13.2

[a]DMS, *N*,*N*-dimethylsphingosine.

2.2. Cell culture and transfection

Human embryonic kidney (HEK 293) cells (ATCC CRL-1573) cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine supplemented with 10% heat inactivated fetal bovine serum were seeded (2×10^6 cells) in poly-D-lysine-coated 100 mm dishes. After 24 h, cells were transfected for 3 h with 4 μg of V5-His-pcDNA3.1 vector (Invitrogen), C-terminal V5-His-tagged murine SphK1, or C-terminal V5-His-tagged murine SphK2 and 20 μl of Lipofectamine PLUS reagent plus 30 μl of Lipofectamine reagent as recommended by the manufacturer. Cells were then cultured for 2 days in the same DMEM medium and lysed by freeze–thawing as described previously [12]. Proteins were measured by the Bradford method (Bio-Rad).

2.3. SphK activity

SphK activity was measured using 10 μg cell lysates with 50 μM sphingosine, prepared as a complex with 4 mg/ml bovine serum albumin (BSA), and [γ-^{32}P]ATP (10 μCi, 1 mM) containing $MgCl_2$ (10 mM) in buffer containing 20 mM Tris (pH 7.4), 10% glycerol, 1 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM sodium orthovanadate, 40 mM β-glycerophosphate, 15 mM NaF, 10 μg/ml leupeptin, aprotinin and soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine in a final volume of 200 μl, as described previously [12], unless otherwise indicated. Phosphorylation was also determined in the presence of 200 mM KCl, conditions in which SphK2 activity is optimal [13]. SphK1 activity was also measured with FTY720 or sphingosine in 0.25% Triton X-100, as described previously [12]. In preliminary experiments, phosphorylation of FTY720 or sphingosine was found to be linear with time for at least 60 min. Thus, phosphorylation reactions were usually carried out for 30 min at 37°C and less than 10% of the substrate was converted to product. Labeled FTY720 and S1P were separated by thin layer chromatography (TLC) on silica gel G60 with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v) as solvent and the radioactive spots corresponding to phosphorylated FTY720 and S1P were quantified with a FX Molecular Imager (Bio-Rad, Hercules, CA, USA). Background was determined in the absence of substrate. Activity is expressed as pmol phosphorylated product formed per min per mg protein.

Inhibition of sphingosine phosphorylation by FTY720 was examined with D-*erythro*-[3-^{3}H]sphingosine (10 μM, 1 μCi) from Perkin Elmer (Boston, MA, USA) as substrate. [^{3}H]S1P produced was measured by a differential solvent extraction procedure [15]. Briefly, kinase reactions were stopped by the addition of 1 ml methanol and 100 μl 3 N NaOH. After adding 1 ml $CHCl_3$ and 0.8 ml 1 N NaCl, samples were vigorously vortexed, phases separated by centrifugation, and an aliquot of the aqueous phase containing >95% of the [^{3}H]S1P counted by liquid scintillation. Essentially all (>95%) of the [^{3}H]sphingosine was recovered in the organic phase.

2.4. Western blotting

HEK 293 cells were scraped in kinase buffer, equal amounts of proteins (10 μg) separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and then transblotted to nitrocellulose. Anti-V5 (1:5000, Life Technologies) or anti-tubulin (1:2000, Santa Cruz Biotechnology) were used as primary antibodies followed by anti-rabbit HRP-conjugated IgG (1:10 000, Jackson Immunoresearch Laboratories). Immunocomplexes were visualized by enhanced chemiluminescence (Pierce) as described previously [14].

3. Results and discussion

3.1. Expression of SphK1 and SphK2

To examine which of the SphKs is the kinase that phosphorylates FTY720, C-terminal V5 epitope-tagged mSphK1 and mSphK2 were overexpressed in HEK 293 cells. These cells were selected because they are efficiently transfected and control HEK 293 cells have low levels of SphK activity (Fig. 1A). Forty-eight hours after transfection with SphK1, sphingosine phosphorylating activity in cell lysates was increased by more than 200-fold, whereas transfection with SphK2 resulted in only a 20-fold increase of sphingosine phosphorylating activity (Fig. 1A). The relative in vitro activities also correlated somewhat with expression of the recombinant proteins as determined by Western blotting (Fig. 1B).

SphK1 and SphK2 expressed in HEK 293 cells can be distinguished on the basis of differential activity measured when the substrate sphingosine is added as a BSA complex in the presence of 200 mM KCl, conditions which are optimal for SphK2 and decrease SphK1 activity [13]. Whereas, when sphingosine is presented to the enzymes in Triton X-100, SphK2 activity is strongly inhibited and SphK1 activity is increased [13] (Fig. 1A).

3.2. FTY720 is a poor substrate for SphK1

Initial kinase reactions were carried out in the presence of Triton X-100, a detergent that stimulates SphK1 activity with sphingosine as substrate. Although vector-transfected cells did not significantly phosphorylate FTY720, recombinant SphK1 was able to catalyze concentration-dependent phosphorylation of FTY70 (Fig. 2B,C). Typical Michaelis–Menten kinetics were observed for recombinant SphK1 with either sphingosine or FTY720 as substrate. The K_m for D-*erythro*-sphingosine as substrate was similar to previously reported values for SphK1 [11,12] and was not significantly different than that of FTY720. However, V_{max} for FTY720 was more than 100-fold lower. Although FTY720 is much more water soluble than sphingosine, similar results were obtained when FTY720 was added as a BSA complex and there were no significant changes in phosphorylation of FTY720 (V_{max} was

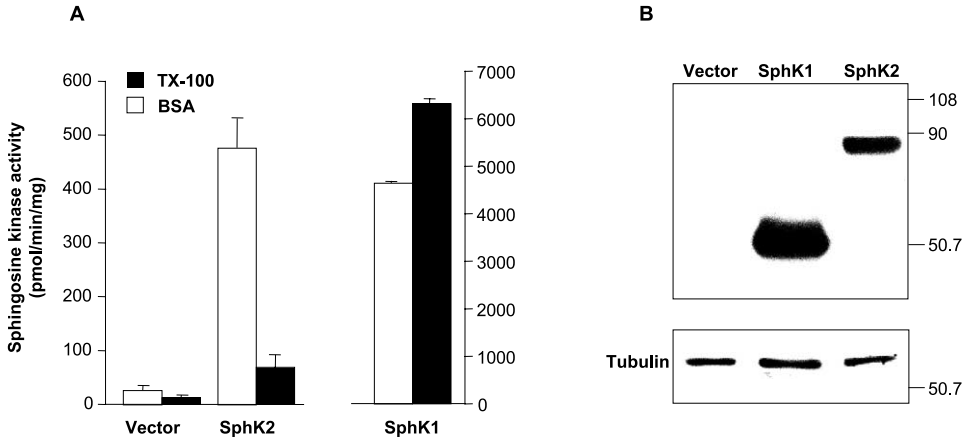

Fig. 1. Expression and enzymatic activities of SphK1 and SphK2. A: HEK 293 cells were transiently transfected with vector, V5-tagged mSphK1, or V5-tagged mSphK2. Cell lysates were prepared and SphK activity measured with sphingosine (50 μM) added in Triton X-100 (filled bars) or as a BSA complex in the presence of 200 mM KCl (open bars). Data are means ± S.D. of triplicate determinations. Similar results were obtained in three independent experiments. B: In duplicate cultures, lysate proteins were resolved by SDS–PAGE and analyzed by Western blotting with anti-V5 antibody. The blots were stripped and probed with anti-β-tubulin as a loading control.

28 ± 1 in BSA conditions compared to 29 ± 1 pmol/min/mg in Triton X-100).

3.3. Phosphorylation of FTY720 by SphK2

Because FTY720 was phosphorylated by SphK1 at less than 1% of the rate of sphingosine, we next examined the ability of SphK2 to phosphorylate FTY720. In contrast to SphK1, which has pro-survival effects and can be highly overexpressed, SphK2 overexpression is detrimental to cells [14] and it cannot be expressed to the same level as SphK1 (Fig. 1B). Nevertheless, the sphingosine phosphorylating activity of HEK 293 cells transiently transfected with SphK2 was markedly increased compared to empty vector-transfected cells.

Although SphK2 expression at the protein level was much lower than SphK1 (Fig. 1B) and the total sphingosine phosphorylating activity in SphK1 expressing cells was much

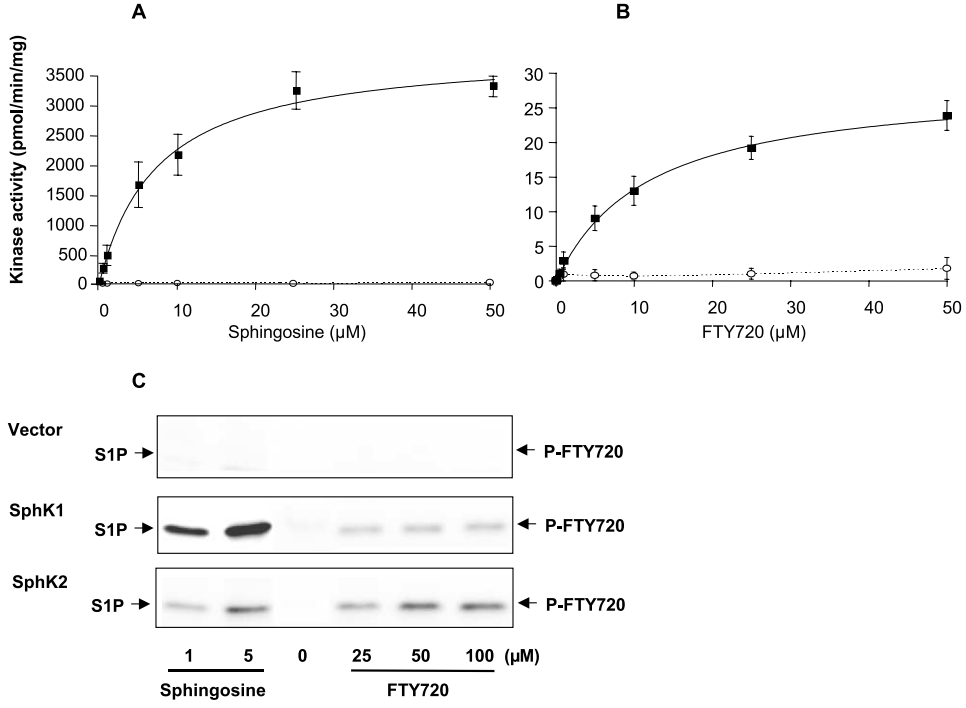

Fig. 2. FTY720 is a poor substrate for SphK1. Phosphorylation of sphingosine (A) and FTY720 (B), added as Triton X-100 mixed micelles, was measured in lysates prepared from HEK 293 cells transiently transfected with vector (open circles) or mSphK1 (filled squares). Data are means ± S.D. of triplicate determinations. Similar results were obtained in three independent experiments. Data were fit by non-linear regression to the Michaelis–Menten equation with Sigma Plot ($R^2 = 0.99$). The apparent K_m values for D-*erythro*-sphingosine and FTY720 were 7 ± 1 and 11 ± 1 μM, respectively. V_{max} values for D-*erythro*-sphingosine and FTY720 were 3850 ± 170 and 29 ± 1 pmol/min/mg, respectively. C: TLC analysis of phosphorylated sphingosine and FTY720. The indicated concentrations of sphingosine and FTY720 were phosphorylated by lysates from HEK 293 cells transfected with vector, mSphK1, or mSphK2. Phosphorylated products were extracted and separated by TLC on silica gel G60 plates and radioactive spots were visualized by a phosphoimager.

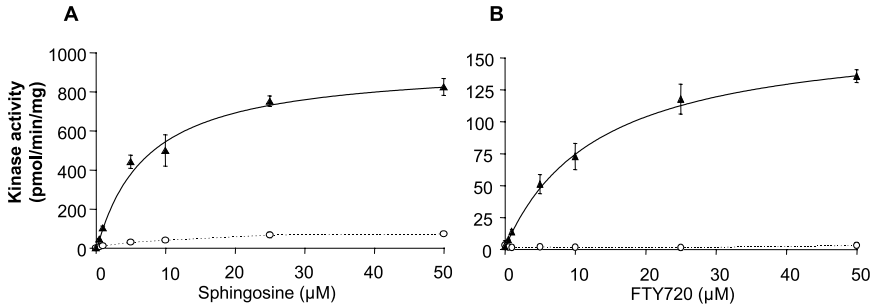

Fig. 3. SphK2 phosphorylates FTY720. Phosphorylation of sphingosine (A) and FTY720 (B) added as BSA complexes was measured in the presence of 200 mM KCl using lysates prepared from HEK 293 cells transiently transfected with vector (open circles) or mSphK2 (filled triangles). Data are means ± S.D. of triplicate determinations. Similar results were obtained in three independent experiments. Data were fit by nonlinear regression to the Michaelis–Menten equation with Sigma Plot ($R^2 = 0.99$). The apparent K_m values for D-*erythro*-sphingosine and FTY720 were 7 ± 1 and 13 ± 1 μM, respectively. V_{max} values for D-*erythro*-sphingosine and FTY720 were 940 ± 50 and 170 ± 5 pmol/min/mg, respectively.

greater, lysates from SphK2-transfected cells more effectively phosphorylated FTY720 (Figs. 2C and 3). It should be noted that phosphorylation of FTY720 by vector-transfected cells was below detection limits, suggesting that high levels of SphK2 must be present to significantly phosphorylate FTY720. As with SphK1, saturation kinetics were also observed with both FTY720 (Fig. 3B) and sphingosine as substrates for SphK2 (Fig. 3A). While the apparent K_m values for both substrates were similar, SphK2 had much higher activity with FTY720 as substrate than SphK1 and phosphorylated it at 15% of the rate of phosphorylation of its natural substrate.

3.4. FTY720 inhibits phosphorylation of sphingosine

FTY720 is a substrate for both SphK1 and SphK2 and thus might inhibit S1P formation by competing with sphingosine. Because S1P and phosphorylated FTY720 cannot be separated by TLC (Fig. 2C), the effect of FTY720 on inhibition of sphingosine phosphorylation was examined using [^{3}H]sphingosine as substrate. [^{3}H]S1P produced was measured by a differential solvent extraction procedure by which S1P partitions into the aqueous phase at alkaline pH with high recovery while sphingosine remains in the organic phase (Fig. 4A). When SphK1 activity was measured in the presence of Triton X-100, FTY720 did not inhibit phosphorylation of [^{3}H]sphingosine (Fig. 4B). However, when both were presented as a complex with BSA, high concentrations of FTY720 reduced [^{3}H]S1P formation (Fig. 4B). It is not clear why FTY720 only inhibits phosphorylation of sphingosine in the absence of Triton X-100 but it is possible that the mixed micelles might enhance the presentation of sphingosine to the enzyme. FTY720 also inhibited [^{3}H]sphingosine phosphorylation catalyzed by SphK2 (Fig. 4C). A concentration of 100 μM FTY720 inhibited phosphorylation of sphingosine mediated by SphK2 by more than 80%.

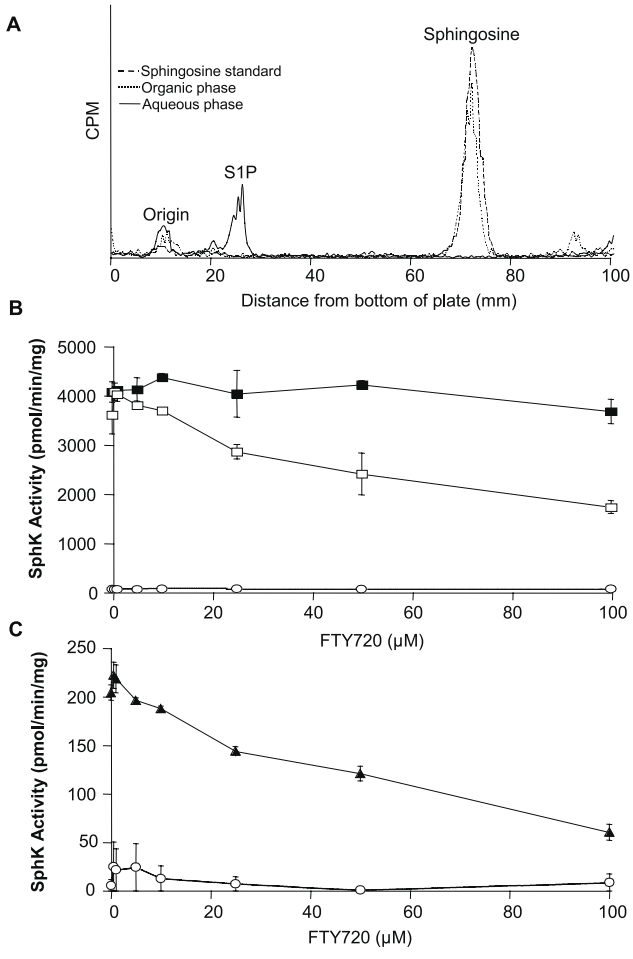

Fig. 4. FTY720 inhibits phosphorylation of sphingosine by SphK1 and SphK2. A: Separation of [^{3}H]S1P from [^{3}H]sphingosine by differential extraction. [^{3}H]sphingosine phosphorylation was measured in lysates from HEK 293 cells expressing SphK1 by the alkaline extraction procedure. [^{3}H]-labeled lipids in both aqueous and solvent phases were separated by TLC and radioactivity determined with a Bioscan AR-2000 scanner (Washington, DC, USA). B: Effect of FTY720 on [^{3}H]sphingosine phosphorylation catalyzed by SphK1. SphK1 activity was measured with lysates from vector-transfected (circles) or SphK1-transfected (squares) cells with 10 μM [^{3}H]sphingosine added as a complex with BSA (open squares) or in Triton X-100 (filled squares) and the indicated concentrations of FTY720. C: Dose-dependent inhibition of SphK2 by FTY720. SphK activity in lysates from vector (open circles) or SphK2 (filled triangles) transfectants was measured with 10 μM [^{3}H]sphingosine added as a BSA complex in the presence 200 mM KCl and increasing concentrations of FTY720. Data are means ± S.D. of triplicate determinations. Similar results were obtained in two independent experiments.

4. Conclusions

In this study, we have shown that FTY720 can be phosphorylated in vitro by both SphK1 and SphK2. Neither SphK isozyme efficiently utilizes FTY720 as substrate compared to the natural sphingosine substrate, although SphK2 seems more likely to be the relevant biological catalyst since it catalyzes the phosphorylation of FTY720 at about 15% of the rate of sphingosine phosphorylation and has similar affinities for both substrates, while SphK1 has less than 1% activity with FTY720 compared to sphingosine. It remains to be determined how FTY720 is apparently so readily phosphorylated in vivo and how the phosphorylated drug is transported out of the cell. It is tempting to speculate that, in contrast to S1P, phosphorylated FTY720 is inefficiently dephosphorylated and metabolized in vivo, thus remaining more available to activate S1P receptors. Moreover, while SphK1 and more likely SphK2 may indeed convert FTY720 to a bioactive S1P mimetic in vivo, it should be recognized that it is possible that some FTY720 actions might result from decreased S1P formation rather than direct effects of phosphorylated FTY720. Finally, it is still not clear how phosphorylated FTY720 is recognized by S1P receptors which seem to have much more exquisite requirements for ligand binding than the active sites of SphKs for which FTY720 is a rather poor fitting substrate.

Acknowledgements: This work was supported by National Institutes of Health grants CA61774 and AI50094 (to S.S.). We thank Heidi Sankala and Lin Mel for excellent technical assistance.

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