Characterization of the Human and Mouse Sphingosine 1-Phosphate Receptor, $S1P_5$ (Edg-8): Structure−Activity Relationship of Sphingosine1-Phosphate Receptors†

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ABSTRACT: Five G protein-coupled receptors ($S1P_1$/Edg-1, $S1P_3$/Edg-3, $S1P_2$/Edg-5, $S1P_4$/Edg-6, and $S1P_5$/Edg-8) for the intercellular lipid mediator sphingosine 1-phosphate have been cloned and characterized. We found human and mouse sequences closely related to rat $S1P_5$ (97% identical amino acids) and report now the characterization of the human and mouse $S1P_5$ gene products as encoding sphingosine 1-phosphate receptors. When HEK293T cells were cotransfected with $S1P_5$ and G protein DNAs, prepared membranes showed sphingosine 1-phosphate concentration-dependent increases in [γ-^{35}S]GTP binding (EC_{50} = 12.7 nM). The lipid mediator inhibited forskolin-driven rises in cAMP by greater than 80% after introduction of the mouse or human $S1P_5$ DNAs into rat hepatoma RH7777 cells (IC_{50} = 0.22 nM). This response is blocked fully by prior treatment of cultures with pertussis toxin, thus implicating signaling through $G_{i/o}\alpha$ proteins. Northern blot analysis showed high expression of human $S1P_5$ mRNA in spleen, corpus collosum, peripheral blood leukocytes, placenta, lung, aorta, and fetal tissues. Mouse $S1P_5$ mRNA is also expressed in spleen and brain. Finally, we found that one enantiomer of a sphingosine 1-phosphate analogue wherein the 3-hydroxyl and 4,5-olefin are replaced by an amide functionality shows some selectivity as an agonist $S1P_1$ and $S1P_3$ vs $S1P_2$ and $S1P_5$.

Sphingosine 1-phosphate (S1P)[1] is a potent, intercellular lysophospholipid mediator that is released, for example, during platelet activation (*1*). S1P elicits a wide variety of responses by cells, including angiogenesis (*2*−*4*) and increased glucose metabolism (*5*). S1P and the structurally related lysophospholipid mediator lysophosphatidic acid (LPA) are recognized now to signal cells through a set of G protein-coupled receptors known colloquially as the Edg receptors (*6*). Discovered initially as 'orphan' receptors (*7*), five members of the group [$S1P_1$ (formerly Edg-1), $S1P_2$ (Edg-5), $S1P_3$ (Edg-3), $S1P_4$ (Edg-6), and $S1P_5$ (Edg-8)][2] have been shown to be S1P receptors (*8*−*14*). Their signaling properties have been characterized regarding G protein coupling, second messenger activation, and cell growth (*15*−*17*). The three remaining Edg family proteins [LPA_1 (formerly Edg-2), LPA_2 (Edg-4), and LPA_3 (Edg-7)] are the LPA-preferring members of this family (*18*−*21*).

We recently characterized a fifth S1P receptor, $S1P_5$, from rat brain (*9*). To understand the $S1P_5$ gene product more fully, we undertook the molecular characterization of human and mouse $S1P_5$ receptors. In the course of these studies, we identified an S1P structural analogue that is a full agonist and shows receptor type selectivity.

MATERIALS AND METHODS

Cloning of Human and Mouse $S1P_5$ cDNA. We found a human nucleotide sequence in the HTGS (High Throughput Genome Sequence) division of GenBank (accession no. AC011461) using the FAST_PAN program (*22*). We used this DNA sequence to design oligonucleotide primers that were in turn used to amplify a fragment from the human chromosome 19 BAC (Bacterial Artificial Chromosome) harboring the human $S1P_5$ gene. The amplified DNA contained an intron-less translational open reading frame encoding the human ortholog of rat $S1P_5$. This DNA fragment was subcloned into the plasmid vector pCR3.1 (Invitrogen). Likewise, the mouse $S1P_5$ gene was also found in the public database (GenBank accession no. AC073749) amplified from the corresponding mouse BAC and cloned into the pCR3.1 plasmid vector.

Transient Expression in HEK293T Cells. Human or mouse $S1P_5$ DNA was mixed with an equal amount of DNA encoding a rat $G_{i2}\alpha$ protein as well as DNAs encoding cow β_1 and γ_2 proteins and used to transfect monolayers of HEK293T cells using the calcium phosphate precipitate

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[1] Abbreviations: S1P, sphingosine 1-phosphate; dihydro S1P, sphinganine 1-phosphate; LPA, lysophosphatidic acid; Edg, endothelial differentiation gene; SPC, sphingosylphosphorylcholine; PCR, polymerase chain reaction; GPCR, G protein-coupled receptor; HTGS, high through-put genome sequence; BAC, bacterial artificial chromosome; EC_{50}, effective concentration yielding half-maximal response; IC_{50}, inhibitory concentration giving half-maximal response.

[2] The IUPHAR subcommittee on lysophospholipid nomenclature has recommended that henceforth the colloquial 'Edg' nomenclature be replaced by agonist name subscript number, where the number represents the order of molecular cloning. Thus, Edg-1 becomes $S1P_1$, Edg-3 $S1P_3$, Edg-5 $S1P_2$, Edg-6 $S1P_4$, and Edg-8 $S1P_5$.

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method (*23*). After 60 h, cells were harvested, and microsomes were prepared, aliquoted, and stored at −70 °C until use.

[γ-^{35}S]GTP Binding. Briefly, 5 μg of membranes from $S1P_5$ expressing HEK293T cells was incubated in 0.1 mL of binding buffer (in mM: HEPES 50, NaCl 100, $MgCl_2$ 5), pH 7.5, containing 5 μg of saponin, 10 μM GDP, 0.1 nM [γ-^{35}S]GTP (1200 Ci/mmol), and test lipid. After incubating for 30 min at 30 °C, bound radionuclide was separated from free by filtration through Whatman GF/C paper using a Brandel Cell Harvester (Gaithersburg, MD).

Stable Expression in RH7777 Cells. Rat hepatoma RH7777 cell monolayers were transfected with human or mouse $S1P_5$/pCR3.1 DNA using the calcium phosphate precipitate method, and clonal populations expressing the neomycin phosphotransferase gene were selected by addition of Geneticin (G418) to the culture medium. The RH7777 cells were grown in monolayers at 37 °C in a 5% CO_2/95% air atmosphere in growth medium consisting of 90% MEM, 10% fetal bovine serum, 2 mM glutamine, and 1 mM sodium pyruvate.

Measurement of cAMP Accumulation. Assay of cAMP accumulation was performed as described previously by us (*9*). Assays were conducted on populations of 5×10^5 cells stimulated with 1 μM forskolin in the presence of the phosphodiesterase inhibitor isomethylbutylxanthine (IBMX, 1 mM) for 15 min. cAMP was measured by automated radioimmunoassay.

RNA Analysis. For hybridization, a phosphorus-32-labeled human or mouse $S1P_5$ cDNA fragment was prepared. The human RNA master blot (Clontech) was hybridized and washed according to the protocol supplied by the manufacturer. For the mouse blot, we performed RT-PCR with mouse $S1P_5$-specific primers from tissue poly A^+ RNA (Clontech). The resultant cDNAs were transferred to a nylon membrane, which was the hybridized with the ^{32}P-labeled mouse $S1P_5$ DNA and washed.

Sources of Materials. RH7777 (CRL 1601) cells were from ATCC (Manassas, VA) while the bacterial artificial chromosome DNAs were from Research Genetics (Huntsville, AL). HEK293T cells were a gift from Dr. Judy White (Department of Cell Biology, University of Virginia), sphingolipids were purchased from Biomol (Plymouth Meeting, PA), [γ-^{35}S]-GTP was from New England Nuclear (Boston, MA), [α-^{32}P]-ATP was from ICN Radiochemicals (Costa Mesa, CA), Geneticin, cell culture media, and sera were from GibcoBRL Life Technologies (Bethesda, MD), oligonucleotides were from Operon Technologies (Alameda, CA), and expression plasmids were from Invitrogen (La Jolla, CA). Chemicals for syntheses were purchased from Aldrich Chemical Co., Inc., Sigma Chemical Co., Inc., Advanced ChemTech Chemical Co., Inc., and/or NovaBiochem Chemical Co., Inc., and were used without further purification. Other chemicals were from Sigma (St. Louis, MO).

Chemical Syntheses (Overview). The syntheses of the S1P analogues [VPC22041, (2*S*)-2-amino-*N*-dodecyl-3-(phosphonooxy)propanamide; VPC22051, tetradecyl (2*S*)-2-amino-3-(phosphonooxy)propanoate; VPC22053, (2*S*)-2-amino-*N*-tetradecyl-3-(phosphonooxy)propanamide; VPC22063, (2*S*)-2-amino-*N*-hexadecyl-3-(phosphonooxy)propanamide; VPC22135, (2*R*)-2-amino-*N*-tetradecyl-3-(phosphonooxy)-propanamide] were accomplished using solvents purified by filtration through alumina (activity I). All reactions were performed under an inert atmosphere, and all products were purified using 230−400 mesh silica gel. Each product was analyzed by thin-layer chromatography (single spot) and spectroscopic methods including ^{1}H NMR, ^{13}C NMR, and mass spectrometry. The assigned structures of the S1P analogues were consistent with all spectral data obtained. A typical synthesis is as follows.

Synthesis of (2S) S1P Analogue VPC22041. (A) Benzyl Protection of N-boc-Serine. To a stirring solution of *N*-boc-L-serine (1 g, 4.87 mmol) in dimethylformamide (DMF, 100 mL) was added cesium carbonate (1.67 g, 5.11 mmol), and stirring was continued 30 min. Benzyl bromide (0.7 mL, 5.84 mmol) was then added, and the resulting solution was stirred 12 h. The reaction mixture was then diluted with ethyl acetate (25 mL) and washed with lithium bromide (3 × 15 mL), sodium bicarbonate (2 × 15 mL), and brine (2 × 15 mL). The organic layer was dried over sodium sulfate. The solvent was then removed under reduced pressure, and the resulting tan oil was purified by flash chromatography, using 1:1 petroleum ether/diethyl ether, to afford the product (1.44 g, 100%) as a white solid. $R_f = 0.26$ (1:1 petroleum ether/diethyl ether).

(B) Phosphorylation of Resulting Alcohol. For phosphorylation, the reaction was performed in the absence of light; work-up and columns were completed with as little light as possible. To a solution of the benzyl-protected serine (0.581 g, 1.98 mmol) in 1:1 CH_2Cl_2/THF (50 mL) was added tetrazole (0.277 g, 3.96 mmol), and the resulting mixture was stirred 30 min. Di-*tert*-butyldiisopropylphosphoramidite (1.25 mL, 3.96 mmol) was then added, and the resulting reaction mixture was stirred for 15 h. Hydrogen peroxide (1.72 mL, 7.92 mmol) was then added, and the resulting mixture was stirred for 3 h, cooled to 0 °C, and quenched by addition of aqueous $Na_2S_2O_5$. The resulting solution was diluted with ethyl acetate (100 mL) and extracted with 50% aqueous $Na_2S_2O_5$ (2 × 20 mL). The organic layer was dried over sodium sulfate, and the solvent was removed under reduced pressure to afford a tan oil. Flash chromatography, using 90:10 $CHCl_3$/acetone, provided the product (0.931 g, 97%) as a clear oil. $R_f = 0.67$ (90:10 $CHCl_3$/acetone).

(C) Debenzylation of Phosphorylated Serine. To a solution of the phosphorylated serine (0.757 g, 1.55 mmol) in 100% ethanol (25 mL) was added a catalytic amount of palladium on activated carbon. To the resulting solution was applied a positive pressure of hydrogen gas, and the reaction mixture was stirred 12 h. The reaction mixture was then filtered through a plug of Celite and eluted with methanol, and the solvent was removed under reduced pressure to yield the product (0.561 g, 91%) as a slightly yellow oil. $R_f = 0$ (90:10 $CHCl_3$/methanol).

(D) Coupling of Long-Chain Amine with Phosphorylated Acid. A solution of the acid (100 mg, 0.252 mmol), a catalytic amount of 4-(dimethylamino)pyridine, 1-hydroxybenzotriazole hydrate (37.4 mg, 0.277 mmol), 1-dodecylamine (46.7 mg, 0.252 mmol), and 15 mL of CH_2Cl_2 were cooled to 0 °C with stirring. To the resulting solution at 0 °C was added dicyclohexylcarbodiimide (57 mg, 0.277 mmol), and the mixture was allowed to return to room termperature with stirring continuing for 12 h. The reaction mixture was then recooled to 0 °C and filtered. The filtrate was washed with sodium bicarbonate (3 × 10 mL) and

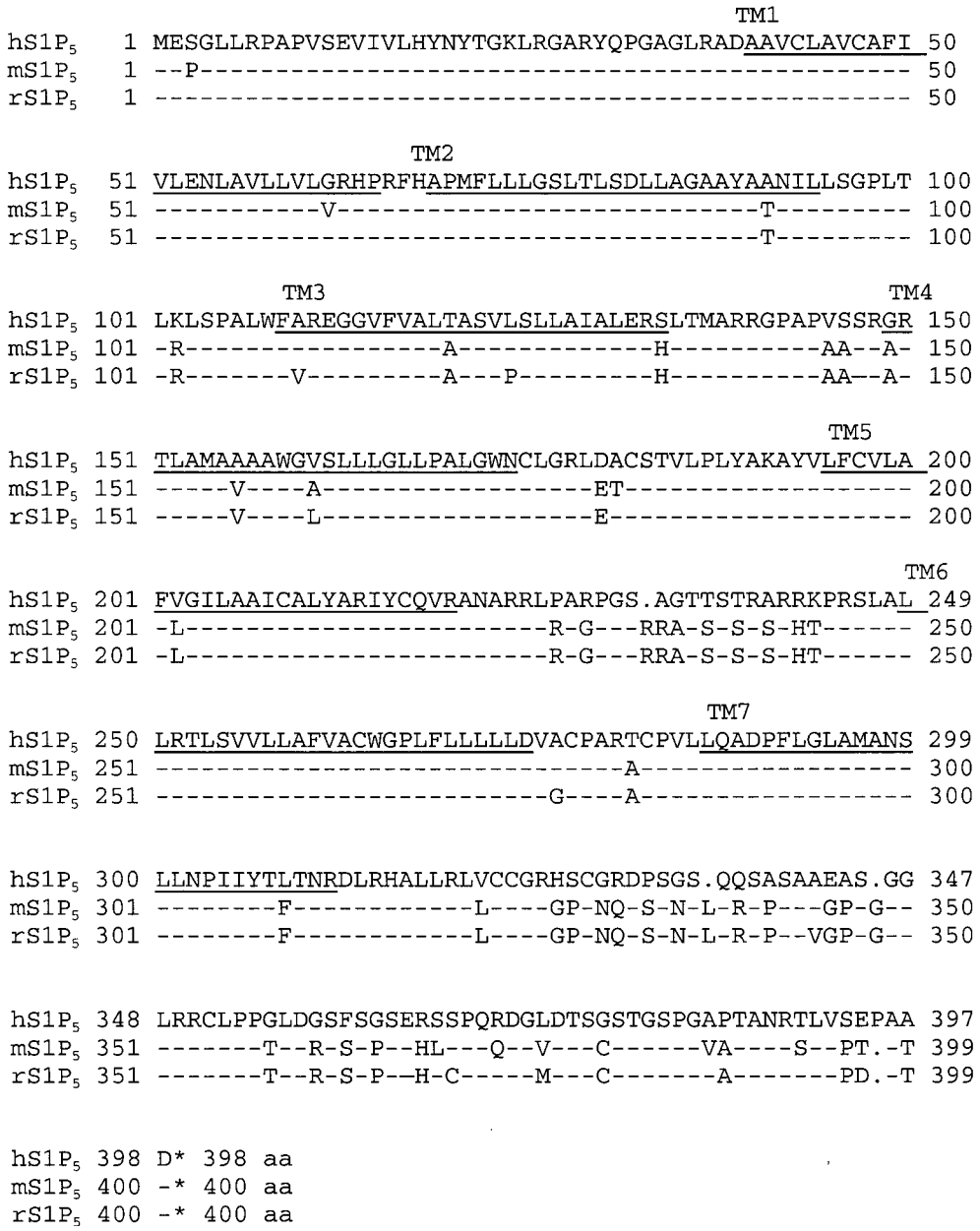


FIGURE 1: Alignment of human, mouse, and rat S1P$_5$ sequences was constructed using FASTA. Amino acid residues that are identical in human S1P$_5$ are indicated by dashes; differences are shown. The putative transmembrane regions, as determined by alignment with the helical regions of bovine rhodopsin, are underscored. The human and mouse S1P$_5$ genomic DNA sequences are available from GenGank (accession numbers AF317676, AF327535).

ammonium chloride (3 × 10 mL), and the organic layers were dried over sodium sulfate. The solvent was then removed under reduced pressure, and the resulting yellow oil was purified by flash chromatography, using 90:10 $CHCl_3$/methanol, to afford the product (48 mg, 33%) as a white solid. $R_f = 0.78$ (90:10 $CHCl_3$/methanol).

(E) Deprotection of N-boc and Phosphate Groups. To a stirring solution of the protected final product (42 mg, 0.072 mmol) in CH_2Cl_2 (1 mL) was added trifluoroacetic acid (1 mL, 12.98 mmol), and stirring was continued 4 h. Under reduced pressure, solvent and excess trifluoroacetic acid were removed, affording a brown oil. The oil was rinsed with ether, and the solvent was removed under vacuum 5 times to afford the product (26 mg, 100%) as a white solid. $R_f = 0$ (90:10 $CHCl_3$/methanol).

Synthesis of (R) S1P Analogue VPC22135. (A) Coupling of Long-Chain Amine with Protected Serine. To a stirring solution of *N*-boc-D-serine-OBn (250 mg, 0.847 mmol) in CH_2Cl_2 (20 mL) was added PyBOP (441 mg, 0.847 mmol) followed by diisopropylethylamine (0.148 mL, 0.847 mmol). After 5 min of stirring, 1-tetradecylamine (181 mg, 0.847 mmol) was added, and stirring was continued for 1 h after which time more 1-tetradecylamine was added (54 g, 0.254 mmol). Stirring was continued for another 3 h, and then the reaction mixture was diluted with ethyl acetate (20 mL) and washed with sodium bicarbonate (3 × 15 mL), ammonium chloride (2 × 15 mL), and the organic layer was dried over sodium sulfate. Solvents were removed under reduced pressure to afford a clear gelatinous solid, which was purified by flash chromatography, using 95:5 $CHCl_3$/methanol, to afford the product (284 mg, 68%) as a white solid. $R_f = 0.78$ (95:5 $CHCl_3$/methanol).

(B) Benzyl Deprotection of Coupled Product. To a solution of the coupled product (0.284 g, 0.579 mmol) in 100%

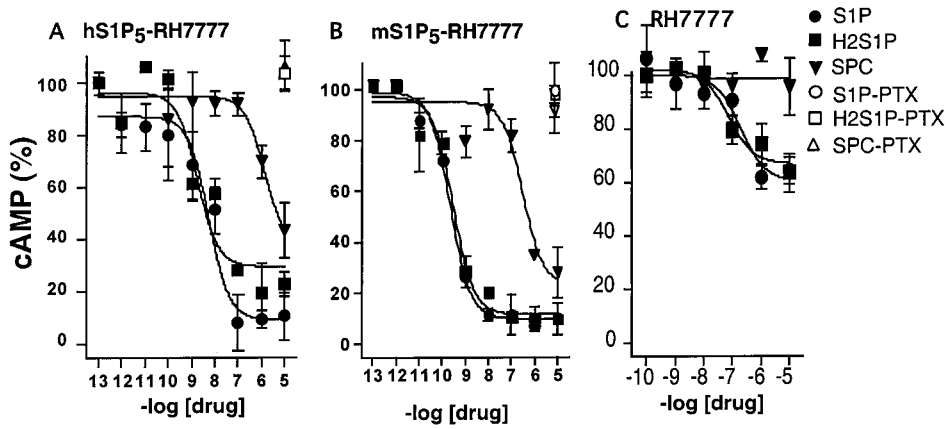


FIGURE 2: Inhibition of forskolin-evoked cAMP accumulation in human or mouse $S1P_5$ DNA transfected RH7777 cells. Clonal populations of RH7777 transfected with human or mouse $S1P_5$ DNA were treated with forskolin and challenged with S1P, dihydro S1P (H2S1P), or SPC. Pertussis toxin (PTX) treatment was for 24 h at a concentration of 100 ng/mL prior to the assay. The absolute values for cAMP accumulation were the following: basal (i.e., IBMX alone) = 56.75 ± 1.06 pmol/well, IBMX + forskolin (100%) = 175.50 ± 4.77 pmol/well. Each data point represents the mean of three determinations ± SEM.

ethanol (15 mL) was added a catalytic amount of palladium on activated carbon. To the resulting solution was applied a positive pressure of hydrogen gas, and the reaction mixture was stirred 12 h. The reaction mixture was then filtered through a plug of Celite eluting with methanol, and then the solvent was removed under reduced pressure to yield the product (0.202 g, 87%) as a clear oil. $R_f = 0.5$ (95:5 $CHCl_3$/methanol).

(C) Phosphorylation of Resulting Alcohol. For phosphorylation, the reaction was performed in the absence of light; work-up and columns are completed with as little light as possible. To a solution of the alcohol (0.190 g, 0.474 mmol) in 1:1 CH_2Cl_2/THF (20 mL) was added tetrazole (0.066 g, 0.948 mmol), and the resulting mixture was stirred 30 min. Di-*tert*-butyldiisopropylphosphoramidite (0.3 mL, 0.948 mmol) was then added, and the resulting reaction mixture was stirred 15 h. Hydrogen peroxide (0.13 mL, 1.896 mmol) was then added, and the resulting mixture was then stirred 24 h, cooled to 0 °C, and quenched by addition of aqueous $Na_2S_2O_5$. The resulting solution was diluted with ethyl acetate (50 mL) and washed with sodium bicarbonate (2 × 15 mL), water (1 × 15 mL), and finally brine (1 × 15 mL). The organic layer was dried over sodium sulfate, and the solvent was removed under reduced pressure to afford a clear oil. Flash chromatography, using 90:10 $CHCl_3$/acetone, provided the product (0.281 g, 100%) as a clear oil. $R_f = 0.23$ (90:10 $CHCl_3$/acetone).

(D) Deprotection of N-boc and Phosphate Groups. To a stirring solution of the protected product (43 mg, 0.071 mmol) in CH_2Cl_2 (1 mL) was added trifluoroacetic acid (1 mL, 12.98 mmol), and stirring was continued 4 h. Under reduced pressure, solvent and excess were removed under vacuum 5 times to afford the product (15 mg, 56%) as a white solid. $R_f = 0$ (90:10 $CHCl_3$/methanol).

For S1P analogue VPC22051, the PyBOP coupling procedure was used in place of DCC coupling. The product was obtained in 15% yield as a clear oil. All final products were obtained as the TFA salts of the compounds.

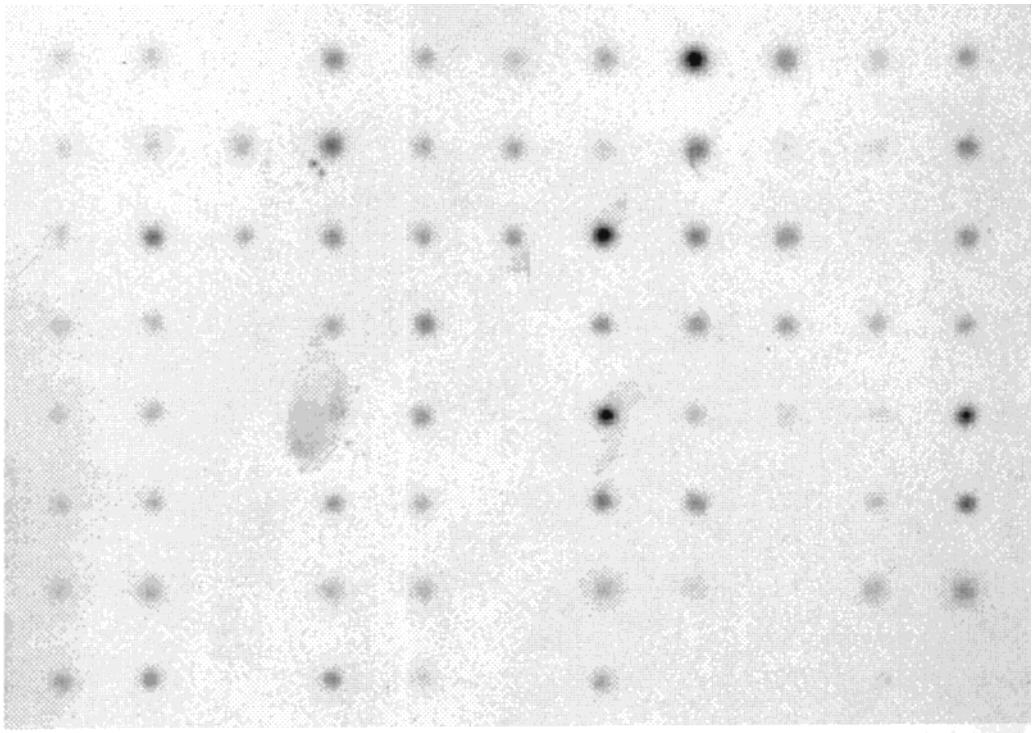
RESULTS

Molecular Cloning of Human and Mouse $S1P_5$ Receptors. Recently, using the FAST_PAN data display tool (*22*) with a G protein-coupled receptor (GPCR) query set that included several S1P receptors, we found human and mouse gene sequence depositions (accession numbers AC011461, AC073749) in the HTGS division of GenBank. Both genes contain intron-less translational open reading frames that are very similar to that of rat $S1P_5$. Figure 1 shows the conceptualized amino acid sequences of human and mouse $S1P_5$ aligned with that of rat $S1P_5$. The human $S1P_5$ protein (398 amino acids) is 97% identical to the rat or mouse $S1P_5$ receptors, 42−49% identical to the other human S1P receptors ($S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$), and 33−36% identical to the human LPA receptors (LPA_1, LPA_2, and LPA_3). Furthermore, human $S1P_5$ is 34−35% identical to the orphan GPCR cluster GPR3/6/12 but less than 28% identical to any other GPCR. Hydropathy analysis of human and mouse $S1P_5$ (not shown) suggests the heptahelix motif assumed to be common to GPCRs. The human and mouse $S1P_5$ proteins have the conserved amino acid motifs expected of a rhodopsin-like (family A) GPCR.

Functional Analysis of Two Mammalian $S1P_5$ Receptors. To test the notion that the two mammalian genes are S1P receptors, we introduced the DNAs into RH7777 rat hepatoma cells by transfection and selected clones expressing the transgenes. This rat hepatoma line was chosen because these cells exhibit only modest endogenous responses to S1P (*9*). After introducing human or mouse $S1P_5$ DNA, S1P treatment of cultures resulted in about 80% inhibition of forskolin-driven rises in cAMP (see Figure 2). The reduced form of S1P, dihydro S1P (sphinganine 1-phosphate, H2S1P), was equipotent to S1P and showed the same efficacy as S1P in this assay. The EC_{50} values for S1P and dihydro S1P in this assay were 0.22 and 0.29 nM, respectively, in mouse $S1P_5$-expressing RH7777 cells. The sensitivity to S1P in human or mouse $S1P_5$-transfected cells increased about 2 log orders as compared to the endogenous response (IC_{50} 0.22 vs 100 nM).

Sphingosylphosphorylcholine (SPC) also functions as an extracellular mediator, and a high-affinity receptor for SPC was reported recently (*24*). Therefore, we tested SPC for activity at the $S1P_5$ receptor and found this ligand to be a low-potency (IC_{50} 1.33 or 0.36 μM) agonist in inhibiting cAMP accumulation in human or mouse $S1P_5$ expressing

A



	1	2	3	4	5	6	7	8	9	10	11
A	whole brain	cerebellum, left		heart	esophagus	colon, transverse	kidney	lung	liver	leukemia, HL-60	fetal brain
B	cerebral cortex	cerebellum, right	accumbens nucleus	aorta	stomach	colon, desending	skeletal muscle	placenta	pancreas	HeLa S3	fetal heart
C	frontal lobe	corpus callosum	thalamus	atrium, left	duodenum	rectum	spleen	bladder	adrenal gland	leukemia, K-562	fetal kidney
D	parietal lobe	amygdala		atrium, right	jejunum		thymus	uterus	thyroid gland	leukemia, MOLT-4	fetal liver
E	occipital lobe	caudate nucleus		ventricle, left	ileum		peripheral blood leukocyte	prostate	salivary gland	Burkitt's lymphoma, Raji	fetal spleen
F	temporal lobe	hippo-campus		ventricle, right	ilocecum		lymph node	testis		Burkitt's lymphoma, Daudi	fetal thymus
G	p. g.* of cerebral cortex	medulla oblongata		inter-ventricular septum	appendix		bone morrow	ovary		colorectal adeno-carcinoma, SW480	fetal lung
H	pons	putamen		apex of the heart	colon, ascending		trachea			lung carcinoma, A549	

B

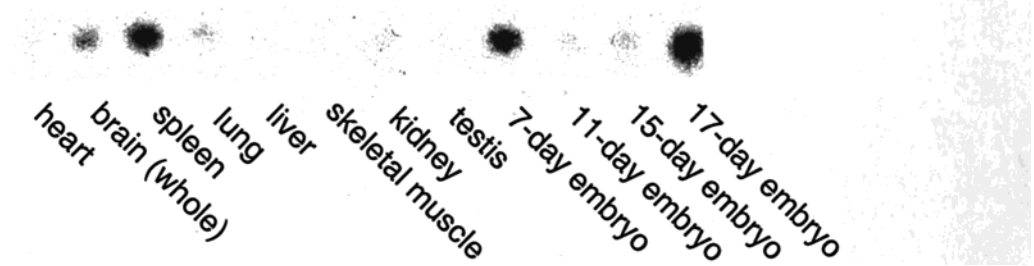


FIGURE 3: Northern blot analyses of human and mouse $S1P_5$. (A) Human Clontech RNA blot probed with ^{32}P-labeled human $S1P_5$. (B) Mouse northern blot probed with ^{32}P-labeled mouse $S1P_5$. Details are described under Materials and Methods.

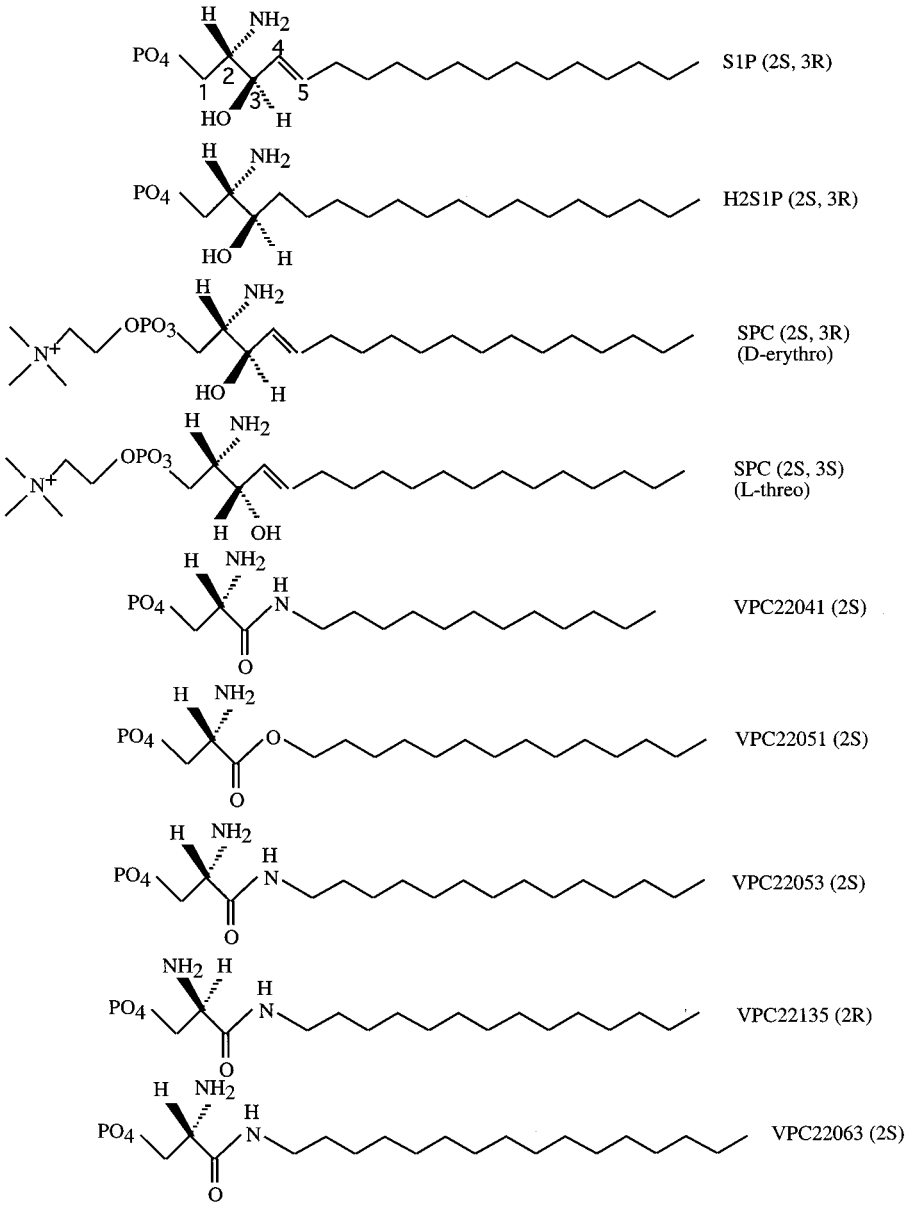


FIGURE 4: Structures of S1P, dihydro S1P, SPC, and dihydro S1P analogues.

RH7777 cell cultures (Figure 2). These inhibitory responses were blocked by prior treatment of cultures with pertussis toxin, suggesting the involvement of $G_{i/o}\alpha$ proteins. In a previous study, we showed that rat $S1P_5$ also markedly sensitized RH7777 cells to S1P (*9*). Like rat $S1P_5$ and human $S1P_1$, but in contrast with human $S1P_3$ and $S1P_2$ (*16*), human $S1P_5$ failed to confer on RH7777 cells a calcium-mobilizing response to S1P or dihydro S1P (data not shown).

$S1P_5$ RNA Expression in Human and Mouse Tissues. Our previous study showed that rat $S1P_5$ RNA was detected only in spleen and brain extracts, and the $S1P_5$ expression in the latter tissue was concentrated in white matter (*9*). For comparison purposes, we investigated the expression pattern of the human $S1P_5$ gene in human tissues by probing a Clontech multiple tissue array RNA blot. This analysis revealed that human $S1P_5$ RNA is widely expressed in the brain, most prominently in the corpus callosum, which is predominantly white matter. Among peripheral tissues, this RNA was detected in spleen, peripheral blood leukocytes, placenta, lung, aorta, and fetal spleen (Figure 3). A low-level signal was detected in many tissue extracts; we do not know how to apportion this signal among tissue parenchyma, leukocytes, or background. Furthermore, mouse RNA analysis showed that mouse $S1P_5$ is expressed in spleen and brain and in 7-day and 17-day embryos, but not in 11-day and 15-day embryos, implying its role in mammalian development (Figure 3).

Activity of S1P Analogues. The medicinal chemistry of S1P is poorly developed; for example, there are no selective agonists or antagonists reported to date. However, the availability of all five human S1P receptors in recombinant form provides a platform to test novel chemical entities for the desired activities. As part of our ongoing efforts to synthesize S1P analogues to use as receptor type-specific tools, we have made and tested a small series wherein the 3-hydroxyl and 4,5-*trans*-olefin are replaced by an amide or ester group. The synthesis of these compounds is described under Materials and Methods, and their structures are shown in Figure 4. We compared the potency and efficacy of S1P, dihydro S1P, and SPC as well as five synthetic compounds on six S1P receptors (zebrafish $S1P_1$, human $S1P_1$, human $S1P_2$, human $S1P_3$, human $S1P_5$, and mouse $S1P_5$) using a

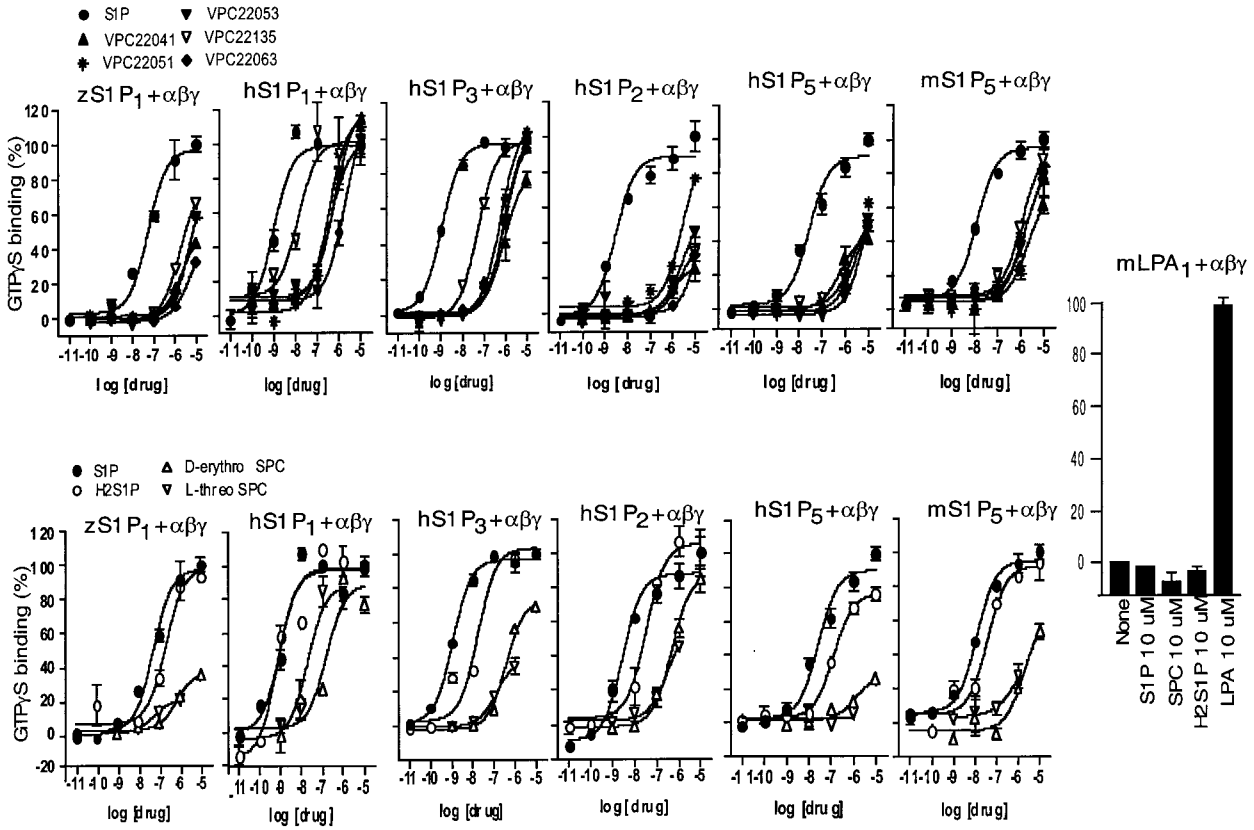


FIGURE 5: [γ-^{35}S]GTP binding to HEK293T cell membranes in response to S1P and S1P analogues. Each data point represents the mean of three determinations ± SEM. The actual minimum and maximum values (dpm) for [γ-^{35}S]GTP binding were as follows: $zS1P_1$, 663 ± 69 (no drug), 2982 ± 177 (10 μM S1P); $hS1P_1$, 455 ± 62 (no drug), 906 ± 32 (10 μM S1P); $hS1P_3$, 959 ± 67 (no drug), 3587 ± 106 (10 μM S1P); $hS1P_2$, 545 ± 17 (no drug), 1185 ± 100 (10 μM S1P); $hS1P_5$, 616 ± 50 (no drug), 1609 ± 59 (10 μM S1P); $mS1P_5$, 432 ± 47 (no drug), 1003 ± 44 (10 μM S1P).

Table 1: EC_{50} Values (nM) for S1P and S1P Analogues at Recombinant S1P Receptors

	$zS1P_1$	$hS1P_1$	$hS1P_3$	$hS1P_2$	$hS1P_5$	$mS1P_5$
S1P	54.6	0.9	1.1	2.9	43.9	12.7
H2S1P	213.6	0.7	16.8	33.3	124.4	37.3
SPC D-*erythro*	nd	167.7	368.1	482.6	nd	nd
SPC L-*threo*	nd	19.3	131.8	313.3	nd	nd
VPC22041	2053.0	598.4	845.4	973.2	645.5	>5000
VPC22051	>5000	322.1	601.9	2760.0	>5000	>5000
VPC22053	>5000	397.0	862.4	2685.0	1606.0	2006.0
VPC22063	>5000	1805.0	878.6	>5000	1220.0	1326.0
VPC22135	1625.0	12.7	50.8	2107.0	>5000	1821.0

[γ-^{35}S]GTP binding assay. We have found that the GTPγS binding assay using HEK293T cell membranes expressing S1P receptors and G protein DNAs (α_{i2}, β_1, γ_2) is very useful in assaying compounds for relative potency and efficacy. The assay is dependent on exogenous S1P receptors (*9*), and all of the S1P receptors (except $S1P_4$) are functional.

Figure 5 shows concentration−response curves of these compounds at each S1P receptor, and the corresponding EC_{50} values are summarized in Table 1. S1P increases GTPγS binding significantly (2−5-fold) at each receptor with EC_{50} values from 1 to 55 nM. Dihydro S1P was equipotent to S1P at all five receptors while SPC was a less potent agonist at all the S1P receptors. We also tested a diastereoisomer of SPC (L-*threo*- or 2*S*,3*S*-SPC) and found there was no difference in potency between the naturally occurring D-*erythro*- and L-*threo*-SPC at the $S1P_2$, $S1P_3$, or $S1P_5$ receptors. However, at the human $S1P_1$ receptor, L-*threo*-SPC was somewhat more potent than the D-isomer. However, the toxicity of 10 μM L-*threo*-SPC in this assay limited our ability to assess this compound.

Our synthetic series consisted of five dihydro S1P analogues where the alcohol at the third position (CHOH) is replaced by a carbonyl group (C═O) and the fourth carbon (−CH═) is replaced by an amide nitrogen (−NH−) or, in one case, an ester oxygen (−O−). The amide-containing compounds contained alkyl chains of 12 (VPC22041), 14 (VPC22053), or 16 (VPC22063) carbons, and the 2′-amino group was in the natural configuration (*S*), except for VPC22135, wherein the 2′-amino was in the (*R*) configuration. VPC22053 and VPC22135 are an enantiomeric pair, while VPC22051 is the ester-containing equivalent of VPC22053 (Figure 4).

All of these compounds had significant agonist activity at each of the six S1P receptors, although none were as potent as S1P itself (Figure 5 and Table 1). However, one compound, VPC22135, approached the potency of S1P at both the human $S1P_1$ and human $S1P_3$ receptors. Curiously, this compound has the amino group in the unnatural (*R*) configuration. Its enantiomer, VPC22053, was more than 1 log order less potent at both the $S1P_1$ and $S1P_3$ receptors.

DISCUSSION

The molecular cloning and characterization of four S1P receptors ($S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$) have been achieved by several groups (*8, 10*−*14*). The $S1P_1$ and $S1P_2$ receptors from zebrafish have been shown also to be functional S1P receptors (*25, 26*). We recently characterized a fifth S1P

receptor $S1P_5$ from rat (*9*). Here we report a characterization of the human and mouse $S1P_5$ receptors regarding molecular identity, functional properties, and expression profiles. Human and mouse $S1P_5$ share >95% amino acids with rat $S1P_5$ and, like rat $S1P_5$, are highly expressed in brain, specifically in white matter, and in spleen.

The high-throughput GTPγS binding assay system is useful for S1P receptor drug discovery, because S1P receptors are ubiquitous in mammalian cells, but our assay only detects recombinant receptors. Using this assay system, we found that the human and mouse $S1P_5$ receptors, like the $S1P_1$ receptor, did not discriminate well between H2S1P and S1P. Among the S1P receptors, the $S1P_2$ and $S1P_3$ types are most selective for S1P (see Table 1). The activity of SPC at some S1P receptors was surprising to us since addition of a choline headgroup to LPA eliminates all activity (*27*). This activity at the ubiquitous $S1P_1$ receptor might complicate analysis of the SPC-preferring receptor, OGR-1 (*24*), as well as the lysophosphatidylcholine receptor, G2A (*28*).

Our effort to simplify the dihydro S1P structure by replacement of the chiral 3-hydroxy with a carbonyl functionality resulted mostly in compounds with at least 2 log orders less potency than S1P or H2S1P. However, a single compound (VPC22135) approached the potency of S1P at both the $S1P_1$ and $S1P_3$ receptors. Interestingly, VPC22135 has the 2-amino group in the unnatural stereo-configuration (*R*); its enantiomer (VPC22053, *S*) was about 1 log order less potent. Insofar as we are aware, VPC22135 is the first reported selective agonist for a subset of S1P receptors. The rudimentary structure−activity study that we report herein also highlights the importance of S1P receptor ligands, in retaining potency, of (1) the overall length of S1P mimetics (e.g., VPC22135 has the same overall length as S1P), (2) the relative insignificance of the 4,5-*trans*-olefin, and (3) the deleterious effect of the addition of a choline headgroup.

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