Synergistic computational and experimental studies provided previously unforeseen details concerning the structural basis of S1P (sphingosine 1-phosphate) recognition by the S1P4 G-protein-coupled receptor. Similarly to reports on the S1P1 receptor, cationic and anionic residues in the third transmembrane domain (R3.28 and E3.29 at positions 124 and 125) form ion pairs with the phosphate and ammonium of S1P, and alanine mutations at these positions abolished specific S1P binding, S1P-induced receptor activation and cell migration. Unlike findings on the S1P1 receptor, no cationic residue in the seventh transmembrane domain interacts with the phosphate. Additionally, two previously undiscovered interactions with the S1P polar headgroup have been identified. Trp186 at position 4.64 in the fourth transmembrane domain interacts by a cation-π interaction with the ammonium group of S1P. Lys204 at position 5.38 forms an ion pair with the ammonium group of S1P. The S1P4 and S1P1 receptors show differences in binding-pocket shape and electrostatic distributions that correlate with the published structure–activity relationships. In particular, the binding pocket of mS1P4 (mouse S1P4) has recognition sites for the anionic phosphate and cationic ammonium groups that are equidistant from the end of the non-polar tail. In contrast, the binding pocket of hS1P1 (human S1P1) places the ammonium recognition site 2 Å (1 Å = 0.1 nm) closer to the end of the non-polar tail than the phosphate recognition site.

Key words: computational model, endothelial differentiation gene 6 (EDG6), G-protein-coupled receptor (GPCR), mutagenesis, sphingosine 1-phosphate (S1P), sphingosine 1-phosphate receptor 4 (S1P4 receptor).

INTRODUCTION

S1P (sphingosine 1-phosphate) (Figure 1A) is a phospholipid mediator whose importance in immune function, cardiovascular development and cancer invasion is becoming increasingly evident [1–6]. S1P inhibits T-cell proliferation [7] and its endogenous presence in serum is required for optimal CD4+ regulatory T-cell activity [8]. S1P elicits biological responses both through its action on GPCRs (G-protein-coupled receptors) embedded in the cell membrane as well as through activation of inappropriately characterized intracellular targets [3,9–12].

The GPCRs responsive to S1P include five members of the endothelial differentiation gene (EDG) family: S1P1/Edg1, S1P2/Edg3, S1P3/Edg5, S1P4/Edg6 and S1P5/Edg8, as well as another family of receptors, GPR3, GPR6 and GPR12 [6]. The S1P1 receptor, with its restricted expression in the haematopoietic/immunopoietic cell lineage [13], shows approx. 60–200-fold reduced responsiveness to and affinity for S1P compared with other members of the EDG family [14,15]. Two natural S1P analogues, PHS1P (phytosphingosine 1-phosphate) [16,17] and DHS1P (dihydrosphingosine 1-phosphate) [17], have both been reported to bind S1P1, with higher affinity than S1P. The 14-fold lower Kᵢ of DHS1P than S1P at S1P1, compared with the 8–10-fold lower concentration of DHS1P in serum from various species [18] suggests that DHS1P might be a biologically relevant agonist of S1P1. S1P1 activates calcium responses when overexpressed in CHO (Chinese-hamster ovary) cells through phospholipase C in a pertussis-toxin-sensitive fashion [19]. S1P4 promotes cell migration [20,21], influences cell morphology [20] and may contribute to the fine balance of migration-stimulating [21–24] and migration-inhibiting [23,25,26] S1P receptors. S1P recognition by select S1P receptors has been examined previously using agonist structure–activity relationships [14,27–31], computational modelling [31–35] and site-directed mutagenesis [33–35]. Integrated computational modelling and mutagenesis studies of the S1P1 receptor have identified that charged residues in the third and seventh TMs (transmembrane domains) are crucial for S1P binding and S1P-induced receptor activation [33]. A residue in the third TM of S1P1 and LPA1 (lysoosphatidic acid receptor 1) serves as a selectivity switch for S1P against LPA recognition [35]. The present study examines recognition of S1P, PHS1P and DHS1P by the S1P4 receptor, expanding on recognition of the polar headgroup of S1P and emphasizing differences in binding-pocket shape and electrostatic distribution that explain the preference of the S1P4 receptor for saturated S1P analogues.

EXPERIMENTAL

Materials

S1P and PHS1P were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Sphingosine was from Matreya (Pleasant Gap, PA, U.S.A.). Phytosphingosine and DHS1P were from...
Figure 1  S1P, DHS1P, PHS1P and the S1P complex with mS1P4

(A) Chemical structures of S1P, DHS1P and PHS1P. (B) The amino acid sequence of mS1P4 was aligned against the hS1P1 model, and a homology model was developed. The position of S1P was transferred from the hS1P1 receptor model to the mS1P4 receptor model. The model is shown with extracellular loops at the top. Ribbons coloured from red at the N-terminus to blue at the C-terminus represent the backbone of mS1P4. The space-filling atoms represent S1P (red, oxygen; blue, nitrogen; grey, carbon; white, hydrogen; magenta, phosphorus). (C) View of the mS1P4 complex with S1P from the extracellular space. Selected residues are shown as stick models and are labelled. Ribbons representing short segments of each TM are shown; the remainder are omitted for clarity. The colouring is as described for (B).

Biomer (Plymouth Meeting, PA, U.S.A.). BSA (fraction V), dihydrosphingosine and anti-γ-tubulin monoclonal antibody were from Sigma (St. Louis, MO, U.S.A.). Alexa Fluor® conjugated goat anti-mouse IgG conjugate was from Molecular Probes (Eugene, OR, U.S.A.). Anti-FLAG M2 monoclonal antibody was from Stratagene (La Jolla, CA, U.S.A.) and anti-HA (haemagglutinin) antibody (Y-11) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Development of an S1P1-based homology model for mS1P4 (mouse S1P4)
The mS1P4 model was developed based on the validated S1P1 model [34] using the MOE software package from the Computational Chemistry Group (Montréal, Québec, Canada). The target sequence was aligned against the sequences of the EDG receptor family and bovine rhodopsin utilizing the MOE-Align facility. This function implements a modified version of the alignment methodology by Needleman and Wunsch [37]. Except for the target sequence and the template, all of the other sequences were removed before the mS1P4 structure was generated by MOE-Homology. Owing to the lack of structural information for the N- and C-termini, the preliminary model included residues 33–328. The position of S1P was obtained by docking as described below. S1P was modelled with +1 ionization of the ammonium group and −1 ionization of the phosphate group.

Model optimization
In the present study, we employed both energy minimization and MD (molecular dynamics) to optimize the homology model of the mS1P4 receptor. Both steps were performed with the MMFF94 force field [38]. Before the model was subjected to MD, we manually converted all cis-amide bonds into the trans conformation, with the exception of those at proline, and added S1P to the system by superimposition on the validated S1P1 complex with S1P. The presence of the ligand prevents the binding pocket from collapsing during MD simulations. The mS1P4 model was initially minimized for 1000 iterations of steepest descent, followed by conjugate gradient and Truncated Newton minimizations to a RMSG (root mean square gradient) of 0.01 kcal/mol·Å (1 cal ≡ 4.184 J; 1 Å = 0.1 nm). MD simulations were performed using a constant volume and temperature ensemble at an equilibrium temperature of 300 K using 1 fs time steps for 0.5 ns after a thermal equilibration period of 60 ps and dielectric constants of both 1 and 80. Snapshots were recorded every 1000 iterations for further structural evaluation. The minimized lowest energy complex from the dielectric constant of 1 ensemble was used to guide experimental mutagenesis. Mutation sites were selected from the model of the mS1P4 complex with S1P either to validate apparent interactions between the receptor and agonist or as controls that were not expected to alter WT (wild-type) behaviour.

Experimentally driven model refinement
Discrepancies between model-based predictions and experimental observations clustered in TM5 necessitated revision of the hS1P1 (human S1P1) [33,35] and mS1P4 models. New models of the S1P receptors were constructed using the alternative alignment of TM5, shown in Table 1. This alternative alignment was
used to generate models of human S1P1 and subsequently mS1P4. This model was optimized by geometry optimization and MD as described above. Additionally, the polar headgroups of S1P and nearby residues were partially solvated utilizing the solvation function on MOE. A total of 31 water molecules were included in the simulation, all starting within 11 Å of the polar headgroup of S1P. These waters were included to explore a possible indirect water-mediated interaction between S1P and neighbouring residues. The lowest energy structure from the MD simulation at a dielectric constant of 1 was minimized to generate refined mS1P4 and hS1P1 models which were used to generate mutant models and in docking studies with S1P.

Ligand docking

All docking studies were performed with Autodock version 3.0 [39]. This software allows exploration of ligand conformations, and configurations through torsion angle rotations, molecular translations and rotations in a rigid protein until an optimized site is found. Three Autodock parameters were modified from default values: (i) the number of energy evaluations in the genetic algorithm search was increased to 9 × 10^4, (ii) the optimization continued for 3 × 10^4 generations, and (iii) 3000 iterations were used in the local search. A docking box of 30.375 × 24.375 × 22.875 Å^3 was used for docking S1P, PHS1P and DHS1P into mS1P4 and hS1P1. This box contained most of the transmembrane domains and all residues that were computationally and experimentally mutated to alanine.

DNA constructs

Construction and expression of the mS1P4 plasmids was performed as described previously [21]. The mS1P4 receptor was N-terminally tagged with the FLAG-epitope and C-terminally tagged with the HA-epitope and inserted into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, U.S.A.).

Mutagenesis

Point mutations were introduced via pcDNA3-FLAG-S1P4-HA as described previously [40]. PCR was performed using 0.4 ng of template DNA and mutagenic primers as follows (residues underlined represent the mutated codon): R2.67A (112) sense, 5'-CTGCTGGGCGCAACTGTGTTGCGCC-3' and W4.64A antisense, 5'-GGGCGACGAGCAGCGCAGGATAGTCG- CAG-3'; S5.37A sense, 5'-CCCTCTACTGTAAGGGCTTGT- GCTC-3', K5.38A sense, 5'-CCCTCTACTGGCTGTACG- TGTC-3', and S5.37A and K5.38A antisense, 5'-GAGGCAGCCGACGAGCAGGATAGTCG- CAG-3' and R7.33A sense, 5'-GAGGCAGCCGACGAGCAGGATAGTCG- CAG-3' and R7.33A antisense, 5'-GCCAGCCGAGCTCTGCGGGCC-3'. The antisense primers were treated with T4 polynucleotide kinase (TaKaRa, Shiga, Japan). PCR was carried out in the presence of 0.2 mM dNTPs, 1 mM MgCl2, 0.3 mM sense primer and polynucleotide-kinase-treated antisense primer for 30 cycles. The PCR products were ligated with the Ligation High kit (Toyobo, Tokyo, Japan). All mutations were confirmed by sequencing of the complete construct.

Cell culture and transfections

HEK-293T (human embryonic kidney) cells (A.T.C.C., Manassas, VA, U.S.A.) were grown in Dulbecco's modified minimal essential medium with high glucose supplemented with 10% (v/v) foetal calf serum. CHO cells were cultured in Ham's F-12 medium (Sigma) containing 10% (v/v) foetal calf serum, 100 units/ml penicillin and 10 μg/ml streptomycin. Cells were transfected using the Lipofectamine™ Plus kit (Invitrogen), according to the manufacturer's instructions. Stable clones expressing WT mS1P4 and mS1P4 mutants were selected in the presence of 600 μg/ml Geniticin (Sigma).

Western blot analysis

Cells were lysed with Laemmli sample buffer [62.5 mM Tris/HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol and 5% (w/v) 2-mercaptoethanol]. The proteins were separated by SDS/PAGE and transferred on to a PVDF membrane (Millipore, Bedford, MA, U.S.A.). After blocking with 5% (w/v) non-fat dried milk in TBST (Tris-buffered saline with Tween 20), 20 mM Tris/HCl (pH 7.5), 137 mM NaCl and 0.05% (v/v) Tween 20), the blot was incubated with the anti-HA antibody, diluted 1:1000 in TBST, for 1 h at room temperature (25°C). After four washes in TBST, the blot was incubated for 30 min at room temperature with horseradish-peroxidase-conjugated anti-rabbit IgG-F(ab')2 fragment (Amersham Biosciences), diluted 1:10 000 in TBST, and washed as above. Immunoreactive bands were visualized by the ECL® (enhanced chemiluminescence) plus detection kits (Amersham Biosciences).

Flow-cytometric analysis

Expression of mS1P4 mutants at the cell surface was examined by flow cytometry using indirect immunofluorescence staining with anti-FLAG M2 monoclonal antibody. CHO cells were harvested by trypsinization and washed with ice-cold FACS buffer (PBS and 0.1% BSA). After washing twice with FACS buffer, the cells were incubated at 4°C for 60 min with the anti-FLAG M2 monoclonal antibody in FACS buffer (1:200 dilution). Subsequently, the cells were washed twice with FACS buffer, and then incubated with Alexa Fluor® 488-conjugated goat anti-mouse IgG secondary antibody (1:400 dilution) at 4°C for 60 min. After washing the cells twice, samples were analysed using a FACSsort flow cytometer (Beckton Dickinson, Palo Alto, CA, U.S.A.). Data were analysed with the Cell Quest software (Beckton Dickinson).

S1P, PHS1P and DHS1P radioligand binding assay

Preparation of radiolabelled ligands and binding assays were performed essentially as described previously [21]. Purified
MBP (maltose-binding protein)—mSPHK1 (murine sphingosine kinase 1) fusion protein was used for preparation of \[^{35}S\]GTP[S] and \[^{32}P\]DHS1P. For preparation of \[^{32}P\]PHS1P, we used purified 3 × FLAG LCB4p. CHO cells (2 × 10^5) were incubated at 4°C in binding buffer [20 mM Tris/HCl (pH 7.4), 100 mM NaCl, 15 mM NaF and 0.4% (w/v) BSA] containing 60 nM (specific radioactivity 10 mCi/µmol) \[^{35}S\]GTP[S], \[^{32}P\]PHS1P or \[^{32}P\]DHS1P for 30 min. Non-specific binding was determined in the presence of 6 µM unlabelled competitor. Cells were washed three times with ice-cold binding buffer for 5 min and bound radioactivity was measured by liquid-scintillation counting.

**S1P-activated \[^{35}S\]GTP[S] (guanosine 5'-32P) binding assay**

HEK-293T cells transiently transfected with the different receptor constructs were homogenized on day 2 in 20 mM Hepes (pH 7.5), 50 mM NaCl and 2 mM EDTA. Nuclei and cell debris were removed by centrifugation at 2000 g for 5 min at 4°C, and the supernatant was centrifuged at 100,000 g for 1 h at 4°C. The pellet containing the membrane fraction was rinsed with and then resuspended in 50 mM Hepes (pH 7.5), 100 mM NaCl, 1 mM MgCl\(_2\) and 2 mM EDTA. A 10 µg sample of freshly prepared membrane protein was incubated in 100 µl of binding buffer containing 50 mM Hepes (pH 7.5), 100 mM NaCl, 5 mM MgCl\(_2\), 10 µM GDP, 2 mM dithiothreitol, 50 µg/ml saponin, and 0.1 nM \[^{35}S\]GTP[S] (1000 Ci/mmol; Amersham Biosciences) and different concentrations of S1P for 30 min at 30°C. Membrane-bound radioactivity was separated by filtration using a 96-well Brandel Cell Harvester (Gaithersburg, MD, U.S.A.) through a Whatman GF/B filter, rinsed with a total of 1 litre of 20 mM Tris/HCl (pH 7.4), 120 mM NaCl and 25 mM MgCl\(_2\) wash buffer and quantified by liquid-scintillation counting. Samples were run in triplicate and their means ± S.D. were plotted as a function of S1P concentration.

**Cell migration assay**

The cell migration assay was performed as described previously [21]. Briefly, the number of cells migrating from the upper chamber to the lower surface of the Transwell filter (6.5 mm diameter, 8 µm pores; Corning Costar, Cambridge, MA, U.S.A.) was determined by counting the number of cells in five random fields.

**Statistical methods**

The significance of differences was determined by Student’s t test. Values were considered significantly different at \(P < 0.05\).

**RESULTS**

**S1P\(_1\)-derived model of the mS1P\(_4\) receptor**

A model of the mS1P\(_4\) receptor was developed by homology with our experimentally validated published hS1P\(_1\) receptor model [33] with the TM5 alignment shown in Table 1. The mS1P\(_4\) model was used in docking studies with S1P in order to identify amino acids with important roles in S1P recognition. The initial complex is shown in Figures 1(B) and 1(C). The model predicts, similar to S1P interactions with S1P\(_1\) ion-pairing interactions between R3.28 (124) and the phosphate, as well as E3.29 (125) and the ammonium. W4.64 (186) makes a cation–π interaction with the ammonium of S1P, and interaction between the cationic residue in TM7, R7.33, and the phosphate is unclear. Three of the five S1P receptors have a cationic amino acid at position 7.34, which was demonstrated by mutagenesis to be required for S1P binding to and activation of S1P\(_1\) [33]. The cationic residue in mS1P\(_4\) differs by one position in the sequence alignment, and that in S1P\(_2\) by more than a helical turn. Thus the importance of a cationic residue in TM7 of S1P\(_1\) does not necessarily generalize to all S1P receptors. Although R7.33 faces away from the phosphate in the model, the side chain is flexible and sufficiently long to interact with the phosphate, given a less favourable side chain conformation. Thus this initial model leads to the hypothesis that mutations R3.28A, E3.29A and W4.64A would have a detrimental impact on S1P binding and activation. Additionally, mutations R2.67A (112A), H3.24A (120A), S5.37A (203A) and K5.38A (204A) would have little or no effect on binding and activation. The R7.33A (289A) mutation might have significant impact on binding.

**Expression and plasma membrane targeting of mS1P\(_4\) mutants**

Comparable levels of expression of epitope-tagged WT mS1P\(_4\) and eight point mutants in CHO cells were evident from the intensity of the ~40 kDa band relative to γ-tubulin in the Western blot performed using membrane fractions of transiently transfected cells (Figure 2A). The FACS data shown in Figure 2(B) demonstrate comparable cell-surface localization of all constructs studied.

![Figure 2](image-url)
Figure 3 [32P]S1P, [32P]DHS1P and [32P]PHS1P binding to mS1P4 and mS1P4 mutants

(A) CHO cells stably expressing empty vector, FLAG–S1P 4–HA or FLAG–S1P 4–HA mutants were incubated with 60 nM [32P]S1P, [32P]DHS1P or [32P]PHS1P in the absence (total binding) or presence (non-specific binding) of 6 µM unlabelled competitor for 30 min at 4°C. Cells were lysed, and bound radioligand was quantified by liquid-scintillation counting. Data show specific binding, which is binding in the absence of unlabelled competitor minus binding in the presence of 6 µM unlabelled competitor. Results are means ± S.E.M. for three independent experiments; *P < 0.05 compared with vector control. (B) CHO cells stably expressing empty vector, FLAG–S1P4–HA or FLAG–S1P4–HA mutants were incubated with indicated concentrations of [32P]S1P. Data show specific binding, which is binding in the absence of unlabelled competitor minus binding in the presence of unlabelled competitor. Results are means ± S.D. for three independent experiments.

Pharmacological characterization of mS1P4 mutants

The roles of eight individual amino acids in the mS1P4 receptor were tested experimentally using [32P]S1P, [32P]PHS1P and [32P]DHS1P radioligand binding (Figure 3) and S1P-induced [35S]GTP[S] binding assays (Figure 4). mS1P4 or mutant mS1P4 receptors were overexpressed in CHO and HEK-293 cells respectively for these assays. Northern blotting analysis demonstrated very low expression of only S1P2 in CHO cells [41]. HEK-293 cells express S1P1, S1P2 and S1P3 [42] at levels unlikely to be relevant in the presence of overexpressed constructs. As predicted by the model, the R3.28A, E3.29A and W4.64A mutations abolished specific S1P binding and S1P-induced receptor activation, thus confirming their importance for S1P binding and subsequent receptor activation. Control mutations R2.67A, H3.20A and S5.37A exhibited specific S1P binding comparable with WT, confirming that these positions do not interact with S1P.

Saturation binding analysis (Figure 3B) of R2.67A confirmed S1P binding potency similar to WT, with a Kd value (± S.D.) of 25.4 (± 10.3) nM. S1P-induced receptor activation for these mutants, with EC50 values of 63, 49 and 110 nM for R2.67A, H3.20A and S5.37A was likewise comparable with WT, with an EC50 of 24 nM. The R7.33A mutant showed similar S1P binding to the WT receptor in these assays with a Kd value (± S.D.) of 33.6 (± 19.4) nM, indicating that the side chain does not adopt the relatively high-energy conformation that would be necessary for it to interact with the phosphate group of S1P. The R7.33A mutant dose–response in the S1P-induced [35S]GTP[S]-binding assay gave an EC50 of 94 nM, relative to the WT response of 24 nM. In contrast with the model-derived hypotheses, K5.38A failed to specifically bind S1P or activate in response to S1P. An indirect role of this residue is possible. However, the location of the residue in TM5, and the absence of the conserved proline residue found in TM5 of most GPCRs, suggests that the modelled positions of TM5 residues in the S1P4-based mS1P4 model might be incorrect.

mS1P4 model refinement

Site-directed mutagenesis results revealed one discrepancy relative to the S1P4-based mS1P4 model. The inconsistency between the model and the experimental data occurs in TM5 of the mS1P4 receptor, which lacks the conserved proline residue found in most GPCRs and which therefore had the least reliable alignment in the initial homology model development. The hS1P1 and mS1P4 models were refined by shifting each amino acid in the TM5 by one level as shown in Table 1. This effectively moves K5.38 of mS1P4 to the position occupied by S5.37. The refined mS1P4 model was optimized using MD with five layers of hydrating waters around the polar headgroup of S1P and the charged residues surrounding that headgroup, in order to explore potential

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A refined mS1P4 model was generated using the TM5 sequence alignment 2 from Table 1, which differs from the alignment initially used by one position only in TM5. A refined hS1P1 model was generated in the same fashion. The models were generated using standard homology modelling methods in MOE followed by optimization by MD. Complexes with S1P, DHS1P and PHS1P generated by docking and refined by MD. Views of complexes are coloured as described for Figure 1(B) and include ribbons representing short segments of each TM, selected residues are labelled and shown as stick models and are shown from the extracellular space. (A) S1P4 complex with S1P. (B) S1P4 complex with DHS1P. (C) S1P4 complex with PHS1P. (D) S1P1 complex with S1P. (E) S1P1 complex with DHS1P. (F) S1P1 complex with PHS1P.

Water-bridged interactions. The complex resulting from the hydrated molecular dynamics simulations is shown in Figure 5(A). No water-bridged interactions between mS1P4 and S1P were observed. The refined model instead shows a clear ionic interaction between K5.38 and the phosphate group of S1P (Figure 5B). Thus the refined model is consistent with all experimental S1P binding and S1P-induced receptor activation results obtained with mS1P4 and mS1P1K5.38A.

**S1P-induced cell migration**

In order to evaluate whether the pharmacological impact of mutations was consistent with the cell-biological responses elicited by S1P-elicited activation, stably transfected cell lines expressing mS1P4 and the eight mutants were characterized by assaying S1P-induced cell migration in a Transwell assay (Figure 6). S1P induced significant increases in cell migration when applied to cells expressing mS1P4, R2.67A, H3.20A, S5.37A or R7.33A. In contrast, no increases in cell migration were observed in response to S1P application to R3.28A, E3.29A, W4.64A or K5.38A. These findings are consistent with the observed specific S1P binding and S1P-induced [35S]GTP[S] binding for only the mS1P4-, R2.67A-, H3.20A-, S5.37A- and R7.33A-expressing cells.

**Ligand selectivity**

The binding selectivities of mS1P4 and mS1P1 for S1P, PHS1P and DHS1P were determined using radioligand binding assays.
DISCUSSION

The synergistic application of computational homology modelling and experimental site-directed mutagenesis has defined important features of S1P recognition in the mS1P4 receptor. Some of these features are analogous to those identified previously in our previous studies on the S1P1 receptor, namely the cationic residue R3.28, which ion pairs with the phosphate of S1P, and the anionic residue E3.29, which ion pairs with the ammonium group of S1P [33–35,44]. The present study also identifies two new conserved interactions. The first is between the cationic K5.38 residue and the phosphate of S1P in both mS1P4 (Figure 5A) and hS1P (Figure 5D). A second newly discovered interaction involves a cation–π interaction between the ammonium group of S1P and W4.64 in mS1P4 (Figure 5A) and hS1P (Figure 5D). One characteristic unique to the mS1P4 receptor is the lack of interaction between a cationic residue in TM7 and the phosphate of S1P. These interactions were validated by experimental assay of S1P binding (Figure 3), S1P-induced [35S]GTP[S] binding (Figure 4) and S1P-induced migration (Figure 6) in S1P4-expressing cells, as well as S1P binding in S1P4-transfected cells (see supplementary Figure S3 at http://www.BiochemJ.org/bj/389/bj3890187add.htm, and previous studies [33,35]). S1P responses in these assays were observed for cells or membranes from cells expressing mS1P4, or its R2.67A, H3.20A, S5.37A and R7.33A, but not R3.28A, E3.29A, W4.64A and K5.38A mutants.

Models of both S1P1 [31] and S1P4 [32] have been published by other researchers. The S1P1 model reported by Lim et al. [31] includes several features observed in our current models, as well as interactions that do not occur in our model. Consistent with our current model and our previous reports [33,35], Lim et al. [31] observe ion-pairing interactions between R3.28, R7.34 and the phosphate of S1P, as well as an ionic interaction between E3.29 and the ammonium group of S1P. The positions of W4.64 and K5.38 are not described. In contrast with our model, Lim et al. [31] suggest hydrogen bonds from the side chain hydroxy group of Y1.57 (98) and the backbone carbonyl group of F7.38 (296) to the hydroxy group of S1P. Figure 5(D) shows that, in our model, TM1 (red ribbon) is too distant from S1P for any residue to make direct contact with S1P. This difference between the models can be tested in future studies by site-directed mutagenesis. The S1P4 model reported by Vaidhehi et al. [32] shows little similarity to our mS1P4 model or our previously published hS1P1 model [44]. Vaidhehi et al. [32] note interactions between S1P and human S1P4 residues T3.34 (127), E7.30 (284) and W7.37 (291), all displaced towards the extracellular loops compared with the residues mutated in the present study. The consistency between the mS1P4-model-derived hypotheses and the experimental mutant characterization in the present study, as well as the observed receptor selectivity change to short-chain LPA species in the E3.29Q mutant [44], suggest that S1P is unlikely to interact with residues in the extracellular loops.

Tryptophan 4.64 is conserved in S1P1–5, LPA1–3 and the cannabinoid receptors, CB1 and CB2, although it is not found in other reported phospholipid receptors, GPR3/6/12 or LPA4/P2Y9 [45]. Of these receptors, W4.64 (172) mutations to alanine, leucine, phenylalanine and tyrosine have been reported for the CB2 receptor [46]. Mutations W4.64F and W4.64Y were found to retain binding to an aromatic, uncharged agonist. In contrast, mutations W4.64A and W4.64L resulted in loss of agonist binding. These results suggest that W4.64 is commonly located near the agonist-binding pocket in the EDG and cannabinoid families of lipid receptors, and can contribute to binding of endogenous and synthetic agonists.
Relative affinities in the order DHS1P > PHS1P > S1P (Figure 1A) have been reported recently for human S1P4, with a 10–20 fold preference for DHS1P based on $K_d$ values determined from competition binding experiments [17]. Less than 2-fold differences were observed experimentally in the mouse S1P1 and S1P4 receptors studied here based on $K_d$ values derived from saturation binding experiments (see supplementary Figure S1 at http://www.BiochemJ.org/bj/389/bj3890187add.htm). The relatively small differences in ligand preference observed may be due to the different experiments used to measure ligand binding, or to species differences between human and mouse S1P1. Whether this is the case is currently unknown. 

In particular, these studies highlight the previously unknown importance of cation–π interactions between W4.64 and the ammonium of S1P as well as ion-pairing interactions between the phosphate of S1P and K5.38 of both S1P1 and S1P4.

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Sphingosine 1-phosphate analogue recognition by S1P₄


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