Identification of the Hydrophobic Ligand Binding Pocket of the S1P₁ Receptor*5

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Sphingosine 1-phosphate (S1P), a naturally occurring sphingolipid mediator and also a second messenger with growth factor-like actions in almost every cell type, is an endogenous ligand of five G protein-coupled receptors (GPCRs) in the endothelial differentiation gene family. The lack of GPCR crystal structures sets serious limitations to rational drug design and in silico searches for subtype-selective ligands. Here we report on the experimental validation of a computational model of the ligand binding pocket of the S1P₁ GPCR surrounding the aliphatic portion of S1P. The extensive mutagenesis-based validation confirmed 18 residues lining the hydrophobic ligand binding pocket, which, combined with the previously validated three head group-interacting residues, now complete the mapping of the S1P₁ ligand recognition site. We identified six mutants (L3.43G/L3.44G, L3.43E/L3.44E, L5.52A, F5.48G, V6.40L, and F6.44G) that maintained wild type [²³S]S1P binding with abolished ligand-dependent activation by S1P. These data suggest a role for these amino acids in the conformational transition of S1P₁ to its activated state. Three aromatic mutations (F5.48Y, F6.44G, and W6.48A) result in differential activation, by S1P or SEW2871, indicating that structural differences between the two agonists can partially compensate for differences in the amino acid side chain. The now validated ligand binding pocket provided us with a pharmacophore model, which was used for in silico screening of the NCI, National Institutes of Health, Developmental Therapeutics chemical library, leading to the identification of two novel nonlipid agonists of S1P₁.

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Sphingosine 1-phosphate (S1P)³ (see Fig. 1) is a naturally occurring sphingolipid mediator and also a second messenger with growth factor-like actions in almost every cell type (1–3). S1P plays fundamental physiological roles in vascular stabilization (4), heart development (5), lymphocyte homing (6), and cancer angiogenesis (7). S1P elicits its biological effects through the activation of G protein-coupled receptors (GPCR) (8–10) and through yet undefined intracellular targets (11–15). The endothelial differentiation gene (EDG) family of GPCR encodes eight highly homologous receptors. Five of these receptors, designated S1P₁–S1P₅, are specific for S1P, and the other three, LPA₁–LPA₃, are specific for the related lysophospholipid mediator lysosphosphatidic acid (LPA) (16).

FTY-720, an immunosuppressive prodrug presently in phase 3 clinical trials, has attracted a lot of interest due to its effective inhibition of kidney transplant rejection and attenuation of autoimmune diseases, including multiple sclerosis (6, 17, 18). In vivo, FTY-720 becomes phosphorylated by sphingosine kinase type 2, and FTY-720-P is a high affinity ligand of all EDG family S1P receptors with the exception of S1P₂ (19). In atrial myocytes, FTY-720-P, similarly to S1P (20, 21), activates an inwardly rectifying K⁺ conductance through the activation of the S1P₃ receptor, which in turn elicits unwanted bradycardia (22). The immunosuppressive effects of FTY-720-P are mediated by the S1P₁ receptor (23). FTY-720-P is an agonist of S1P₁ and causes a long lasting desensitization of this receptor subtype, which appears to be the mechanism responsible for the inhibition of lymphocyte egress from the secondary lymphoid organs and the lymphopenia and the sequestration and eventual death of T lymphocytes (24, 25). Therefore, S1P₁-selective antagonists or functional antagonists would probably be devoid of cardiac side effects and retain the immunosuppressive effect. Given the simple and highly flexible structure of S1P and its high similarity to LPA combined with the high degree of homology in the EDG receptor family, identification of novel nonlipid drug candidates with FTY-720-P-like effects is a fundamental challenge in the field.

Development of receptor subtype-selective pharmacophores could aid rational drug design and lead optimization as well as.

The abbreviations used are: S1P, D-erythro-sphingosine-1-phosphate; FACS, fluorescence-activated cell sorting; GPCR, G protein-coupled receptor; GTPγS, guanine-γ-thiotriphosphate; TM, transmembrane domain; WT, wild type; EDG, endothelial differentiation gene; LPA, lysosphosphatidic acid; BSA, bovine serum albumin.
identification of novel molecular scaffolds through in silico searches of large chemical libraries. However, the lack of crystal structures of GPCR sets serious limitations on this effort. Our groups have embarked on a computational modeling-driven mutagenesis approach to delineate agonist recognition by S1P1 at the atomic level. This effort has enabled us to identify S1P receptor residues that make essential interactions with the charged phosphate and amino moieties of the S1P pharmacophore. We have identified three basic amino acids, Arg-3.28, Lys-5.38, and Arg-7.34 in S1P1 and S1P4, that form salt bridges with the phosphate group of S1P and are essential for ligand binding in one or both receptors (26, 27). Furthermore, we have pinpointed position 3.29, conserved as glutamine in LPA- and LPA-binding protein via required ion pairing between glutamate and the ammonium moiety of S1P (28). The Gln/Asn-3.29 residue also plays an essential role in ligand binding, because substitution to alanine results in a loss of S1P and LPA binding and receptor activation. We also succeeded in elucidating differences between S1P1 and S1P4, as in the latter subtype Lys-5.38 and Trp-4.64 together compensate for the lack of a cationic residue at position 7.34 as in S1P1 (27). These polar head group interactions are essential for ligand binding, activation, and specificity. However, the hydrophobic tail constituting the bulk of S1P has not been assigned a function, and its interaction with the ligand binding pocket has not been elucidated.

In the present study, we set out to identify the residues of S1P1 that interact with the aliphatic part of S1P, which we designate the hydrophobic binding pocket of the receptor. In this context, we examined the role of these hydrophobic interactions in ligand binding and receptor activation. Starting with our previously published S1P1-S1P complex, we hypothesized that 15 residues line the hydrophobic binding pocket. Combined characterization of mutations at these 15 positions and work done in parallel on S1P4 revealed that the orientation of transmembrane helix 5 (TM5) was incorrect in the original model. We developed a new S1P1 model that predicted an additional six residues in TM5 constituting the hydrophobic binding pocket and resolved the apparent inconsistencies between our experimental findings and the original S1P1 model (supplementary Fig. 1). The new theoretical model predicts that the hydrophobic tail of S1P interacts with 20 residues in TM3, TM5, and TM6. We evaluated the impact of mutations that changed the size and/or electrostatic properties of these residues on ligand activation and binding. Among these mutants, we found that six mutations that showed no or greatly reduced ligand-dependent activation yet maintained [32P]S1P binding similar to the wild type (WT) S1P1. The lack of activation in the presence of ligand binding suggests that these residues play an important role in the conformational transition of S1P1 to its activated state. The theoretical model was used as the basis for in silico screening of the NCI, National Institutes of Health, Developmental Therapeutics Database library for novel scaffolds that might produce selective ligands of the S1P1 receptor using the Enhanced NCI Database Browser. Two novel non-lipid lead compounds were experimentally confirmed as partial agonists of S1P1. Additionally, comparison of residues at analogous positions in other S1P receptors suggests modifications that will lead to selective agonists of each receptor.

**EXPERIMENTAL PROCEDURES**

**Reagents**

All reagents were of analytical purity obtained from Sigma unless specified otherwise. S1P was purchased from Avanti Polar Lipids (Alabaster, AL). SEW2871 (Fig. 1) was a generous gift from Dr. Hugh Rosen (Scripps Research Institute, San Diego, CA).

**Computational Homology Modeling**

**Residue Nomenclature**

Amino acids in the TM domains of S1P1 can be assigned index positions to facilitate comparison between GPCRs with different numbers of amino acids, as described by Ballesteros and Weinstein (29). An index position is in the format x.xx. The first number denotes the TM domain in which the residue appears. The second number indicates the position of that residue relative to the most highly conserved residue in that TM domain, which is arbitrarily assigned position 50. E3.29, then, indicates the relative position of this glutamate in TM3 relative to the highly conserved arginine 3.50 in the (E/D)RY motif (29).

**Receptor Model Development**

S1P1—A model of human S1P1 (GenBank™ accession number AF23365) was developed by homology to a model of rhodopsin (Protein Data Bank entry 1boj) in a manner described in our previous publications (26, 30). Briefly, the rhodopsin model used to generate TM1–TM6, whereas the structure for the seventh TM was based on TM7 of the dopamine D2 receptor model (31). The preliminary model was further refined by converting all cis amide bonds to the trans configuration and by manually rotating side chains at polarity-conserved positions to optimize hydrogen bonding between TMs. The AMBER94 force field (32) was utilized to optimize the receptor to a 0.1 kcal/mol Å root mean square gradient. A corrected model was constructed using the previous model as the template with a manual realignment of TM5 to move each residue back one position in the alignment. The corrected model was refined and minimized using the same protocol.

**S1P1 Single/Double Point Mutants**—Mutant models of S1P1 were developed by homology to the corrected S1P1 model.
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Using the MOE software package, the appropriate mutation was constructed by side chain replacement. Nonpolar hydrogen atoms were added to the mutated amino acid side chain, and the model was subsequently geometry-optimized. The AMBER94 force field (32) was utilized again to optimize each mutant receptor to a 0.1 kcal/mol-Å root mean square gradient.

**Ligand Model Development**

A computational model of S1P was built using the MOE software package. The phosphate group was modeled with a net −1 charge, and the amine moiety was modeled with a net +1 charge. S1P was geometry-optimized using the MMFF94 force field (33).

**Docking**

Using the AUTODOCK 3.0 software package (34), S1P was docked into S1P\textsubscript{1} and the S1P\textsubscript{1} mutant receptor models, and these complexes were evaluated based on final docked energy as well as visual analysis of electrostatic and other nonbonded interactions between the ligand and receptor. Docking parameters were set to default values with the exception of the number of energy evaluations (2.5 \times 10^{5}), number of generations (30,000), local search iterations (3000), and number of runs (15). The complexes exhibiting the best interactions based on either final docked energy or visual analysis were geometry-optimized using the MMFF94 force field (33) and were subjected to critical qualitative analysis. SEW2871 was docked into the S1P\textsubscript{1} receptor model using the same parameters and evaluation criteria.

**New Lead Identification**

The docked positions of S1P and SEW2871 in the S1P\textsubscript{1} receptor model were superposed and used to derive pharmacophore features sharing common location in both structures. Distances between these common pharmacophore features comprise the pharmacophore. The pharmacophore was used to search the Enhanced NCI Database Browser (available on the World Wide Web at 129.43.27.140/ncidb2/) for novel lead compounds. A trifluoromethylphenyl group was used for the anionic biosostere, and carbon atoms were used to represent the hydrophobic functionality at other pharmacophore points. Hits from the search were evaluated based on their superposition onto the S1P and SEW2871 conformations from the S1P\textsubscript{1} complexes. Hits were categorized as good, marginal, or negative based on these superpositions. Hits were considered negative if they exceeded the volume occupied by S1P or SEW2871 due to likely steric interactions with receptor atoms.

**Experimental Validation**

**Site-directed Mutagenesis**

The N-terminal FLAG epitope-tagged S1P\textsubscript{1} receptor construct (GenBank\textsuperscript{TM} accession number AF233365) was provided by Dr. Timothy Hla. Site-specific mutations were generated using the ExSite\textsuperscript{TM} mutagenesis kit (Stratagene, La Jolla, CA) as described previously (26, 28). S1P\textsubscript{1} and the generated mutants were subcloned into pcDNA3.1 vector (Invitrogen). The sequence information of the mutants is listed in supplemental Table 1. Clones were verified by complete sequencing of the inserts.

**Cell Culture and Transfection**

RH7777 and HEK293 cells (ATCC, Manassas, VA) were maintained in Dulbecco’s modified minimal essential medium containing 10% fetal bovine serum (Hyclone, Logan, UT). Cells (2 \times 10^{5}) were transfected with 2 μg of plasmid DNA with Effectene (Qiagen, Valencia, CA) according to the manufacturer’s instructions for 24 h. Before ligand binding and receptor activation assays, the cells were washed twice with serum-free Dulbecco’s modified minimal essential medium and serum-starved for at least 6 h.

**Western Blotting**

Western blot analysis of the FLAG epitope-tagged receptor construct was performed in transiently transfected RH7777 cells using a protocol described earlier (27). Anti-FLAG M2 and anti-β-actin antibody were purchased from Sigma. Goat antimouse antibody conjugated with horseradish peroxidase was purchased from Promega (Madison, WI).

**Flow Cytometry Analysis**

Cell surface expression of the FLAG-tagged S1P\textsubscript{1} and its mutants was determined by flow cytometry (FACS) as described previously (27). Transfected RH7777 cells were harvested by trypsinization, and upon harvesting, the cells were maintained at 4 °C for the subsequent steps. The cells were washed with ice-cold FACS buffer (phosphate-buffered saline, pH 7.4, and 3% bovine serum albumin (BSA)). After washing once with FACS buffer, the cells were incubated for 30 min in blocking solution (5% BSA and 5% donkey serum (Sigma) in phosphate-buffered saline, pH 7.4). The cells were washed once with FACS buffer, and the cells were subsequently incubated for 60 min in FACS buffer with the anti-FLAG M2 monoclonal antibody (Sigma) (1:200). After washing the cells twice with FACS buffer, the cells were incubated for 30 min in FACS buffer with the Alexa Fluor 488-labeled donkey anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) (1:1600). After washing the cells twice, samples were resuspended in 1% BSA in phosphate-buffered saline, pH 7.4, and analyzed using an LSR II flow cytometer (BD Biosciences). Data were analyzed with the Cell Quest software (BD Biosciences).

**[32P]S1P Radioligand Binding Assays**

The S1P binding assays were done essentially as previously described (28). Transfected HEK293 cells (5 \times 10^{5}) were incubated at 4 °C in 20 mM Tris-HCl (pH 7.5) binding buffer containing 100 mM NaCl, 15 mM NaF, protease inhibitor mixture (Sigma), and 0.2 mg/ml essentially fatty acid-free BSA with 1 nM [32P]S1P in 50 nM S1P for 40 min. Cells were centrifuged and washed twice in binding buffer. The final pellet was resuspended in 2:1 CHCl\textsubscript{3}/MeOH, and the suspension was equilibrated in scintillation fluid overnight. Cell-bound radioactivity was measured by liquid scintillation counting using a Beckman LS5000 TA counter (Beckman Coulter, Irvine, CA). Specific binding was defined as the difference between total binding and nonspecific binding (in the presence of 2–5 μM cold S1P). S.E. values were computed on the basis of triplicate samples from two transfections.
For the competition assays, HEK293 cells were used. Briefly, 4 × 10^5 cells were plated in 24-well dishes and allowed to adhere overnight. The cells were then transfected with 0.4 μg of the cDNA using Lipofectamine 2000 (Invitrogen), and the transfection proceeded for 48 h. After washing the cells twice with ice-cold binding buffer (20 mM Tris–HCl, pH 7.4, and 150 mM NaCl), 0.1 nM [32P]S1P and competing concentrations of cold S1P (1 nM–10 mM NaCl), the cells were applied to the cells and incubated on ice for 30 min. After washing the cells twice with ice-cold binding buffer plus 0.4% BSA, the cells were lysed with 0.5% SDS and equilibrated in scintillation fluid. The triplicate samples were measured. The K_D and B_max values were determined using GraphPad Prism software (San Diego, CA).

**S1P1 Receptor Activation Assays**

Receptor functional assays were performed as previously described (28) in transiently transfected RH7777 cells by measuring either S1P- or SEW-activated [35S]GTPγS binding.

**Statistical Analysis**

The significance of differences was determined by one-way analysis of variance, Bonferroni post hoc test, using Prism statistical software (GraphPad, San Diego, CA). Values were considered significantly different at p < 0.05.

**RESULTS**

**Mutagenesis Strategy**—The previously reported computationally modeled complex of S1P in S1P1 features 15 previously unexplored amino acid residues in TM3, TM4, and TM6 with atoms within 4.5 Å of S1P. In the present study, we pursued a three-pronged replacement strategy of these residues. First, we introduced property-conserving mutations of these residues that either reduced or increased size. The logic behind this approach was to probe the impact of increased or relaxed steric constraints in the hydrophobic binding pocket on ligand-induced activation. In addition, we replaced many of these residues with charged amino acids of similar size to probe whether disruption to hydrophobicity in the putative binding pocket would have an impact on receptor function. Third, in a few cases, we replaced charged residues with noncharged residues of similar size to test the effect of polar interactions between the ligand and the receptor.

**Theoretical Model of the S1P1–S1P Complex; Revisions of the Previous Model**—Discrepancies between model-derived predictions and experimental observations in this and a previous study on S1P4 (27) involved residues localized at the extracellular end of TM5. The differences we found were not consistent with proposed structural differences between active and inactive GPCR conformations; thus, they were probably an error in our previous S1P1 model (26, 28). A corrected model of S1P1 was built based on an alternative alignment of TM5 derived from the recently validated S1P4 model (27). As depicted in supplemental Fig. 1, the corrected model demonstrates that eight residues in TM5 have atoms within 4.5 Å of S1P. One of these residues, Lys-5.38, forms an ion pair with the phosphate group of S1P. This polar interaction was not identified in previous validation of the S1P1 model due to the incorrect positioning of amino acid residues at the top of TM5.

**Cell Surface Expression of S1P1 Mutants**—In order to verify that the WT and mutant constructs were expressed at comparable levels, membrane fractions were prepared and analyzed for expression by Western blot analysis using the N-terminal FLAG epitope present in the constructs (supplemental Fig. 2). The levels of expression on the membrane fractions were comparable with that of the WT receptor with the exception of L3.43E/L3.44E, L6.41G, F5.48Y, F6.44G, and W6.48E. FACS analysis was used to determine if cell surface expression of the N-terminal FLAG epitope was similar for the mutant constructs to that of the WT (Table 1). The WT and the mutant constructs, with the exception of V5.47T and L6.41G, were expressed at the cell surface in similar levels based on immunolabeling for the FLAG epitope. However, the V5.47T and L6.41G mutants were expressed and included in the pharmacological testing. The cell surface expression of L3.43E/L3.44E, F5.48Y, F6.44G, and W6.48E were comparable with that of the WT receptor.

**The Effects of Mutations of Residues Lining Predicted Hydrophobic Binding Pocket on Ligand-induced Activation of S1P1**—In the first round of pharmacological testing, we evaluated the impact of all amino acid replacements on the EC_50 and maximal activation (E_max) elicited by S1P. The summary of the pharmacological properties caused by these replacements is presented in Table 2. We arranged the findings into four categories that correspond to different colors in Fig. 2, A–E. After the first round of GTPγS activation experiments was completed, it became apparent that of 15 residues mutated on the basis of the previously published S1P1 model, 11 produced changes in receptor activation with the exception of two residues in TM6, Val-6.37 and Leu-6.41, and two residues in TM5, Phe-5.43 and Thr-5.49. In addition, parallel studies carried out on S1P4 (27) revealed that our model needed revisions with regard to the orientation of the top half of TM5. The position of Phe-5.43 was

| Construct | Anti-FLAG-stained cells % |
|-----------|---------------------------|
| Vector    | 2.5                       |
| WT        | 23.0                      |
| L3.32K    | 19.3                      |
| L3.36E    | 15.2                      |
| L3.43E/L3.44E | 10.3                   |
| L3.43G/L3.44G | 14.7                   |
| F6.44G    | 22.1                      |
| F6.44C    | 22.1                      |
| V5.47T    | 19.6                      |
| V5.47T    | 19.6                      |
| L5.51E    | 17.4                      |
| L5.52A    | 14.1                      |
| V6.40L    | 19.6                      |
| L6.41G    | 5.6                       |
| F6.44G    | 17.2                      |
| W6.48A    | 12.6                      |
| W6.48E    | 23.4                      |

For the competition assays, HEK293 cells were used. Briefly, 4 × 10^5 cells were plated in 24-well dishes and allowed to adhere overnight. The cells were then transfected with 0.4 μg of the cDNA using Lipofectamine 2000 (Invitrogen), and the transfection proceeded for 48 h. After washing the cells twice with ice-cold binding buffer (20 mM Tris–HCl, pH 7.4, and 150 mM NaCl), 0.1 nM [32P]S1P and competing concentrations of cold S1P (1 nM–10 mM NaCl), the cells were applied to the cells and incubated on ice for 30 min. After washing the cells twice with ice-cold binding buffer plus 0.4% BSA, the cells were lysed with 0.5% SDS and equilibrated in scintillation fluid. The triplicate samples were measured. The K_D and B_max values were determined using GraphPad Prism software (San Diego, CA).
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TABLE 2
Ligand-induced activation properties of S1P₁ mutants
Ligand-induced [35S]GTPγS binding was determined using 5–10 μg of crude RH7777 membranes in the presence and absence of ligand. EC₅₀ values for S1P-induced activation (0.1 nM to 3 μM) are shown. E₅₀ values were normalized to the maximal response of the WT in the same transfection. At least two independent transfections were performed, and data points represent the mean ± S.D. (n = 3). AA, amino acid; NA, no activation up to 3 μM; NS, no saturation up to 3 μM.

| Constructs | EC₅₀ ± S.D. | Percentage of WT E₅₀ ± S.D. | Nature of AA replacement |
|------------|------------|-----------------------------|--------------------------|
| S1P₁ mutants with retained WT S1P₁-like S1P-induced activation | | | |
| WT-S1P₁ | 6.0 ± 1.6 | 100 ± 4.3 | |
| Vector | NA | NA | |
| K5.38A | 32.4 ± 8 | 97 ± 15 | Decrease in electron density and size of AA |
| F5.43Y | 23 ± 4.7 | 90 ± 7 | Introduction of polar functional group |
| F5.43G | 11.8 ± 3.9 | 70 ± 6 | Decrease in electron density and size |
| V5.47T | 13 ± 3.1 | 77 ± 3.3 | Change from a nonpolar to a polar AA |
| V5.47L | 22 ± 5.3 | 147 ± 11 | Increase in size |
| T5.49G | 5.4 ± 0.56 | 107 ± 1.4 | Removal of polar side chain |
| T5.49S | 6.6 ± 1.2 | 168 ± 15 | Reduction in size |
| V6.37T | 13 ± 2.4 | 181 ± 26 | Change from a nonpolar to a polar AA |
| V6.37L | 37.6 ± 8.4 | 247 ± 11.3 | Increase in size of AA |
| L6.41E | 4.1 ± 0.63 | 59 ± 1.8 | Change from a nonpolar to a charged AA |

S1P₁ mutants with either increased EC₅₀ or decreased E₅₀ depending on the properties of the amino acid replacement

| Constructs | EC₅₀ ± S.D. | Percentage of WT E₅₀ ± S.D. | Nature of AA replacement |
|------------|------------|-----------------------------|--------------------------|
| L3.36E | 0.62 ± 0.46 | 39 ± 2.8 | Change from a nonpolar to a charged AA |
| L3.36G | 23 ± 8 | 75 ± 12 | Reduction in size |
| V3.40T | 13.8 ± 1.8 | 137 ± 10 | Change from a nonpolar to a polar AA |
| V3.40L | 7.0 ± 4.5 | 55 ± 6.3 | Increase in size |
| I5.41A | NS | 75 ± 3.7 | Reduction in size |
| I5.41F | 18.9 ± 5 | 27 ± 3.4 | Increase in size and electron density |
| C5.44D | 7.9 ± 1.5 | 137 ± 4.5 | Increase in polarity |
| C5.44H | 20.4 ± 2.1 | 37 ± 2.3 | Removal of polar side chain |
| T5.45G | 30.9 ± 8.5 | 96 ± 18 | Removal of polar side chain |
| T5.45S | 7.9 ± 3 | 28 ± 4 | Reduction in size |
| L5.51G | 2.6 ± 2.1 | 46 ± 8 | Reduction in size |
| L5.51E | 6.3 ± 3.6 | 41 ± 3.7 | Change from a nonpolar to a charged AA |

S1P₁ mutants with abolished or impaired ligand-induced activation depending on the properties of the amino acid replacement

| Constructs | EC₅₀ ± S.D. | Percentage of WT E₅₀ ± S.D. | Nature of AA replacement |
|------------|------------|-----------------------------|--------------------------|
| M3.32G | 35 ± 5 | 77 ± 5 | Reduction in size |
| M3.32K | NS | 12 ± 3.2 | Change from a nonpolar to a charged AA |
| F5.48Y | 7.8 ± 2.1 | 80 ± 3.5 | Increase in polarity |
| F5.48G | NA | NA | Decrease in electron density and size |
| V6.40A | 14.8 ± 3.8 | 69 ± 4.4 | Reduction in size |
| V6.40T | 57.3 ± 25 | 61 ± 9.4 | Change from a nonpolar to a polar AA |
| V6.40L | NS | 37 ± 2.6 | Introduction of polar functional AA |
| F6.44Y | 3.7 ± 3.3 | 37 ± 2.6 | Introduction of polar functional AA |
| F6.44G | NS | 28 ± 13 | Decrease in electron density and size of AA |
| W6.48A | 23 ± 13 | 38 ± 4.0 | Decrease in electron density and size of AA |
| W6.48E | 20 ± 9.9 | 84 ± 11 | Decrease in electron density, size, and introduction of a charged AA |

S1P₁ mutants with abolished ligand-induced activation

| Constructs | EC₅₀ ± S.D. | Percentage of WT E₅₀ ± S.D. | Nature of AA replacement |
|------------|------------|-----------------------------|--------------------------|
| L3.43E/L3.44E | NA | NA | Change from a nonpolar to a charged AA |
| L3.43G/L3.44G | NA | NA | Reduction in size |
| L5.52A | NA | NA | Reduction in size |

shifted by one position in the helix compared with the old model, causing a 100° difference in its orientation (supplemental Fig. 1). Mutations of Lys-5.38, Phe-5.43, Val-5.47, Thr-5.49, Val-6.37, and Leu-6.41 (depicted in green in Fig. 2A) had the least impact on S1P activation (Table 2A). Replacement of Thr-5.49 with Gly or Ser, which removed the polar side chain and reduced the residue size, respectively, had no effect on E₅₀ and increased EC₅₀ by 3-fold. The Tyr and Gly replacement of Phe-5.43, introducing a polar residue or one with decreased electron density and size, respectively, caused a modest reduction in E₅₀ and an ~10-fold increase in EC₅₀. Replacement of Lys-5.38 with Ala, which removed the polar side chain and reduced the residue size, had no effect on E₅₀ and increased EC₅₀ by a factor of 5. Replacement of Val-5.47 or Val-6.37 by Thr or Leu, which introduced a polar side chain and increased hydrocarbon length, respectively, resulted in a 2–6-fold increase in EC₅₀. The L6.41E and V5.47T replacements resulted in a lowered E₅₀ (59 and 77% of WT, respectively), suggesting that hydrophobicity at these two positions is necessary for the conformational change to the fully activated state. These residues, therefore, point away from the ligand, consistent with the modest impact replacements to these residues caused and the role they play in the hydrophobic binding pocket (Table 2 (second section) (yellow in Fig. 2B) caused either marked decreases in E₅₀ or increased EC₅₀ substantially. With the exception of Leu-5.51, these findings corroborate the predic-
tions of the model and appear to be consistent with the hypothesis that even conservative mutational replacements of these amino acids in close proximity of the aliphatic part of S1P have a marked impact on the function of the receptor. The isobutyl functionality of Leu-5.51 is positioned such that it does not interact with S1P in the hydrophobic binding pocket, and in this regard the model is in excellent agreement with the WT-like EC50 values for L5.51G and L5.51E. The E_{\text{max}} values, however, are significantly lower than WT (46 and 41% of WT, respectively), and this observation may indicate that mutations to Leu-5.51 that shorten its side chain or introduce charge may interfere with S1P-induced activation of S1P\(_1\) to the fully activated conformation.

Mutants at five residues showed significant variability in S1P-induced response, depending on the type of substitution. However, for these residues, one of the replacement mutants remained responsive to S1P, sometimes with little or more pronounced decrease in activation (Table 2 (third section), orange in Fig. 2C). Substitution of a charged moiety for Met-3.32 led to a receptor that showed no dose-dependent activation by S1P. Introduction of charge to Met-3.32 (M3.32K), reduction of size

FIGURE 2. **Residues in the vicinity of S1P aliphatic chain in the new and refined S1P\(_1\) receptor model.** S1P is shown as a space-filling model. Residues mutated are shown as stick models and colored according to the experimentally observed activation properties. **A**, green stick models highlight residues at which mutations caused little or no change from S1P-induced activation compared with WT. **B**, yellow stick models highlight residues at which mutations caused significant changes in EC50, E_{\text{max}}, or both. **C**, orange stick models highlight residues at which mutations showed variable influence on S1P-induced activation, depending on the properties of the amino acid replacement. **D**, red stick models highlight residues at which mutation greatly diminished or completely abolished S1P-induced activation. **E**, this panel highlights residues at which mutations showed a profound decrease in S1P-induced activation without concomitant decrease in S1P radioligand binding. Compare E with Table 2 for further details.
of Phe-5.48 (F5.48G), or introduction of a longer chain functionality for Val-6.40 (V6.40L) led to the loss of S1P-induced activation. In contrast, decreases in size at Met-3.32 (M3.32G) and Val-6.40 (V6.40A) or changes in polarity at Phe-5.48 (F5.48Y), Phe-6.44 (F6.44Y), and Val-6.40 (V6.40T) resulted in less drastic effects on ligand-induced receptor activation. Moreover, amino acid replacements to Trp-6.48 had little effect on the EC50 but had rather substantive effects on the Emax, indicating a role for this residue in the transition from the fully inactive to fully active conformation of S1P1.

The computational model placed leucine 3.43 at the bottom of the hydrophobic binding pocket (Fig. 2D). Positions 3.43 and 3.44 are a conserved LL motif in all S1P receptors; therefore, their combined importance was tested by simultaneously replacing both residues with either a charged glutamic acid or smaller glycine. Either replacement caused a complete loss of ligand-induced activation (Table 2 (bottom section), red in Fig. 2D). These mutants, similar to those that were functionally dead listed in Table 2 (third section), always showed less than 20% of the basal GTPγS binding, indicating that they were not constitutively active. Additionally, L5.52A resulted in abolished activation of the S1P1 receptor by S1P.

This unique behavior of mutations that abolished ligand-induced activation of S1P, led us to hypothesize an important role for these residues in receptor function. We posited that much impaired activation could be due to a loss of ligand binding. Alternatively, if these mutants maintained significant ligand binding, it could be indicative of their critical role in receptor activation.

[32P]S1P Binding to S1P1 Mutants without Ligand-induced Activation—We performed [32P]S1P radioligand binding studies with the mutants M3.32K, L3.43E/L3.44E, L3.43G/L3.44G, I5.41A, F5.48G, L5.52A, V6.40L, F6.44G, and W6.48A, which showed much impaired dose-dependent activation by S1P in the GTPγS activation experiments. In our system, the apparent KD for S1P binding at the WT receptor was 38.7 ± 1.2 nM. Hence, we chose a radioligand concentration of 50 nM to test whether those mutants that lacked activation would maintain some degree of S1P binding. Compared with the vector-transfected cells, six mutants, L3.43G/L3.44G, L3.43E/L3.44E, F5.48G, L5.52A, V6.40L, and F6.44G, showed binding that displayed more than 70% of the specific binding of the WT (Figs. 2E and 3). These mutants were further characterized in S1P competition displacement assays and showed KDs and Bmax essentially the same as WT S1P1 (supplemental Table 2). Thus, these replacements introduced in the putative hydrophobic ligand binding pocket had a pronounced tendency to affect ligand activation while maintaining some degree of ligand binding, suggesting that these residues play a crucial role in receptor activation, rather than solely ligand binding.

To further characterize the ligand-induced activation of these mutants, we also exposed them to SEW2871 (Figs. 1 and 4), a recently identified nonlipid agonist of S1P1, (35). L3.43G/L3.44G, L3.43E/L3.44E, F5.48G, and L5.52A showed similarly impaired activation to SEW2871 as we have seen with S1P (Table 3). In contrast, V6.40L showed a dose-dependent partial activation, and F6.44G showed WT-like activation by SEW2871 (Fig. 4).

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**FIGURE 3. Ligand binding properties of S1P1 mutants.** S1P ligand binding to S1P1, mutants with greatly diminished or completely abolished S1P-induced activation. HEK293 cells were transfected with empty vector, WT S1P1, and mutant receptors, which displayed no significant S1P-induced activation. Total [32P]S1P radioligand binding was determined using 50 nM [32P]S1P, whereas nonspecific binding was determined in the presence of 2 μM cold S1P (nonspecific binding). Specific binding was normalized to that of WT S1P1 (1944 ± 149 fmol/5 × 105 cells).

*Identification of New S1P1, Agonist Scaffolds—Docked complexes of two structurally distinct S1P1 receptor agonists, S1P and SEW2871, were used to derive a pharmacophore describing the important chemical functional moieties and distances between those moieties that produce S1P1 receptor activation. The superposed structures of S1P and SEW2871 from the docked complexes are shown in Fig. 5A, and the distances between pharmacophore elements are shown in Fig. 5B. A subset of these distances was used to identify 13 hits in the Enhanced NCI Database Browser using a trifluoromethylphenyl group to provide the anionic bioisostere. However, no hits were identified using the complete set of pharmacophore points and distances in Fig. 5B. Three hits were eliminated due to poor superposition on the known agonists. The remaining 10 hits were categorized based on the quality of their rigid superposition onto the known agonist structures (Fig. 6). Four hits were considered good matches (NSC146266, NSC145964, NSC59474, and NSC75030). Two hits were considered marginal matches (NSC55879 and NSC68644). Four hits were considered questionable, with additional bulk or incorrect curvatures (NSC147843, NSC53638, NSC55534, and NSC99548). The 10 hits were requested from the NCI Developmental Therapeutics Program.

Samples of seven compounds were available (good, NSC146266 and NSC59474; moderate, NSC55879; poor, NSC147843, NSC53638, NSC55534, and NSC99548) (Fig. 6A) and were screened for S1P1 receptor activation in the GTPγS activation assay. Two of the seven showed GTPγS activation greater than that of the vehicle at 10 μM, NSC59474 and NSC99548. Dose-response curves were then generated for...
these two compounds as shown in Fig. 6B. These two compounds display partial agonism and are less potent than S1P, reflecting the fact that they matched only a subset of the pharmacophore features identified in Fig. 5B.

DISCUSSION

A computational model of S1P docked in the S1P1 receptor was developed, and the hydrophobic region of the ligand binding pocket has been experimentally validated with an accuracy of 70%, in which mutations of 14 of 20 residues predicted to interact with the hydrophobic tail displayed impaired or altered S1P-induced activation. The present results extend our previous analysis of the S1P polar head group interactions with S1P1 (26–28) to additionally include hydrophobic interactions with the receptor. In lieu of a crystal structure, we are limited in the structural analysis of the S1P1 GPCR. Even if a receptor-ligand complex crystal structure is available, mutagenesis remains essential for evaluating the functional role of those residues that are positioned to interact with the ligand. In the present study, we used computational modeling to guide our mutagenesis strategy to gain insight into the structure-function relationship of S1P1. The choice of replacement of residues in the predicted hydrophobic ligand binding pocket determined the type of effect observed in ligand-induced activation. For example, at least one of the two types of replacements we introduced into four residues had little or no impact on $E_{\text{max}}$ and only slightly increased the EC$_{50}$ values relative to WT. At the same time, at least one of the two replacements for four of these same residues had a major impact on $E_{\text{max}}$ and/or EC$_{50}$ providing us with internal controls in a sense that receptor function was not always affected.

The experimentally validated predictions of the theoretical model localize the hydrophobic binding pocket to the TM3, TM5, and TM6 domains. All but one of these residues were property-conserved residues with similar side chain chemical properties in the EDG family of receptors. Of the 20 residues tested, three of five in TM3, three of 10 in TM5, and two of five in TM6 are identical in all EDG family S1P receptors. Interestingly, of the eight identical residues among S1P receptors, five are also identical in the LPA receptors of the EDG family. However, if we relax the identity criterion to include residues that are identical in at least three of the five S1P receptors, then four of five in TM3, three of 10 in TM5, and five of five in TM6 are identical. Eight strictly conserved residues and an additional 10 nearly conserved residues suggest that the hydrophobic binding pocket is highly conserved among these receptors. Furthermore, relative to S1P1, S1P5 deviates most strongly in the hydrophobic binding pocket, with six differences, followed by S1P1 and S1P3, which each differ at four residues, and S1P4, which only differs at three residues. Thus, the hydrophobic binding pocket shows the least diversity between S1P1 and S1P3. This coincides with the similar ligand properties of FTY-720-P, with $K_d$ values of 0.21 ± 0.17 and 5.0 ± 2.7 nM for S1P1 and S1P3, respectively (19). Comparison of the ligand proper-
ties with the S1P₁-specific agonist SEW2871 (22) using four mutants that showed no or greatly reduced activation by S1P indicated that three of the four residues also impaired activation to the synthetic ligand. In contrast, the V6.40L mutant was slightly activated by SEW2871 but not by S1P. These results add further proof of the general importance of the residues identified by the computationally guided mutagenesis in S1P₁ function but also point out that differences do exist between the individual ligands.

The present findings place the hydrophobic tail of S1P pointing down toward the intracellular face in a pocket formed by the transmembrane helices. Yamamura and colleagues (36) found that S1P covalently coupled to glass beads via its hydrophobic tail pulled down several proteins from B16 melanoma cells, including a 41-kDa protein that could correspond to an S1P GPCR. If this protein was indeed an EDG receptor, its ability to bind to tail-immobilized S1P would seemingly contradict our model and mutagenesis data. However, the phosphate head group interaction with Arg-3.28, Arg-7.33, and Glu-3.29 could still take place and anchor the protein to the bead, permitting pull down. However, according to our data, the immobilized S1P should not activate the receptor, since activation requires the interactions occurring between the receptor and the hydrophobic tail.

Several of the mutants that showed no or greatly diminished dose-dependent S1P-induced activation displayed significant ligand binding. This could indicate that these residues play a
critical role in the conformational change required for activation, but their interaction with the ligand is not essential, as indicated by the retained binding.

The model demonstrates that Leu-3.43, Leu-3.44, Leu-5.52, Phe-5.48, Val-6.40, and Phe-6.44 make good van der Waals contact with each other and also with the alkyl chain of S1P (supplemental Fig. 3). The detrimental effect of mutation at any of these sites on receptor activation but not ligand binding suggests that this van der Waals contact is unique to the activated conformation of S1P1. Docked complexes of S1P with these mutants displayed the polar interactions typical of the wild-type complex. This finding is consistent with the observed S1P binding by these mutants. Particularly interesting is the finding that F5.48G binds S1P but does not allow activation of S1P1, whereas F5.48Y results in wild type activation of S1P1 by S1P. The computational model suggests that Phe-5.48 does not significantly interact with the alkyl chain of S1P. Hence, the F5.48G data indicates that this residue is important for activation. The F5.48Y mutation, however, amplifies this finding to indicate that mutation to a residue that occupies significantly less volume than the phenyl moiety results in loss of activation. It seems likely, therefore, that it is the interaction of Phe-5.48 with other hydrophobic residues that confers its importance in the activation process.

Introduction of charged residues to replace M3.32K, L3.43E/L3.44E, and L5.51E severely disrupted activation and either abolished or significantly reduced (L5.51E) ligand binding compared with the WT receptor. Thus, the hydrophobic environment is necessary for ligand binding and consequent activation.

Some of the residues that we identified as part of the hydrophobic binding pocket of S1P1 have also been mutated in other GPCRs, and some were also found to play a role in ligand recognition/activation. Leu-3.36 when mutated to alanine in the human bradykinin B2 receptor subtype did not reduce ligand affinity (37). Our L3.36E mutants showed altered $E_{\text{max}}$ properties but similarly retained ligand affinity. Phe-5.48 when mutated to alanine in the human VIP receptor reduced potency but not efficacy (38). Our F5.48G mutants both failed to show dose-dependent activation by S1P but showed over 50% ligand binding relative to WT, suggesting that this residue is involved in the activation of other receptors as well. There was a striking similarity between our W6.48A mutation with the melancortin MC4R (39), cholecystokinin CCKR (40), and AA3R receptors (41), since in all instances receptor activation was reduced without loss of binding. This unique property of Trp-6.48 is consistent with its putative role in the activation of GPCR by a diverse family of ligands. However, Trp-6.48 does not play an identical role in the receptor most closely related to the EDG family, the cannabinoid receptor. The W6.48A mutation of the CB1 receptor displayed an enhancement of ligand-induced GTP$\gamma$S binding (42). Enhanced efficacy was also observed for some agonists at the corresponding mutant of the CCK-B/gastrin receptor (43). Enhanced efficacy of W6.48A in concert with modeling studies and increased basal activity and lack of ligand-induced response by the CB1 F3.36A mutant led these authors to conclude that CB1 activation involves loss of contact between Phe-3.36 and Trp-6.48 (42). In contrast, our results suggest that S1P1 receptor activation involves formation of contact between these residues.

The computational model of S1P1 has previously predicted that SEW2871 interacts with the same charged residues (Arg-3.28, Glu-3.29, and Arg-7.34) in S1P1 as does S1P. This prediction was validated by site-directed mutagenesis studies that demonstrated the inability of R3.28A, E3.92A, and R7.34A mutants to mediate Akt and extracellular signal-regulated kinase phosphorylation upon treatment with either SEW2871 or S1P. To further characterize the interaction of SEW2871 with S1P1, we probed the hydrophobic interactions between residues found to line the S1P1 binding pocket with SEW2871. The activation data for mutations in the S1P1 binding pocket for the SEW2871 and S1P ligands shown in Table 3 revealed numerous similarities and differences with regard to the relative positions of alkyl and aromatic agonists in the binding pocket. Mutants M3.32K, L3.43G/L3.44G, L3.43E/L3.44E, I5.41A, F5.48G, L5.52A, and V6.40L resulted in either no receptor activation or no saturation at a ligand concentration of 3 $\mu$M for both SEW2871 and S1P. The C5.44D mutation, on the other hand, resulted in WT activity for each ligand tested. Hence, it is reasonable to conclude from these data that there is some overlap in ligand volume of S1P versus SEW2871 in the S1P1 binding pocket. It is clear from the structures of S1P and SEW2871 (Fig. 1) that S1P is much more flexible than SEW2871 and, as such, is more capable of adjusting its conformation to mutations in the binding pocket. The contrasting rotational degrees of freedom of these two S1P1 agonists are reflected in key differences that the data in Table 3 depict. The most significant disparities with regard to ligand-induced activation appear to involve three aromatic amino acid residues, Phe-5.48, Phe-6.44, and Trp-6.48. The conservative F5.48Y mutation in S1P1 has no effect on the S1P-induced activation of the receptor but greatly increases the EC$_{50}$ value for SEW2871-induced activation. The same trend is observed with the W6.48A mutation, which increases the EC$_{50}$ for the S1P-S1P1 complex by a factor of 4 but results in no saturation for the SEW2871-S1P1 complex at a concentration of 3 $\mu$M. However, the F6.44G mutation results in no saturation for the S1P-S1P1 complex at a concentration of 3 $\mu$M yet displays wild type-like SEW2871-induced activation.

The computational model of SEW2871-S1P1 was utilized to lend a chemical rationale to these experimental observations. Fig. 4D illustrates the importance of the positioning of aromatic residues around the periphery of the SEW2871 binding pocket. Specifically, Trp-6.48 is positioned in the binding pocket such that it makes favorable $\pi-\pi$ stacking interactions with the thio- and oxazolidine moieties of the ligand. Mutation of this residue to alanine has two separate effects, both of which are potentially deleterious to ligand binding (1). These $\pi-\pi$ interactions between the indole functionality and the aromatic domains of SEW2871 are lost (2). Favorable van der Waals contacts between Trp-6.48 and the ligand are sacrificed. The less bulky methyl moiety cannot compensate for the loss of contact between Trp-6.48 and SEW2871. In the S1P-S1P1 complex, Trp-6.48 is positioned to make van der Waals contact with the
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ligand, but the orientation of the indole moiety is such that only the edge of the aromatic ring interacts with the alkyl portion of S1P (Fig. 4B). Hence, the computational model of S1P₁ is consistent with the experimental observation that the loss of the Trp-6.48-ligand interaction has a greater impact on the binding and subsequent activation of SEW2871 relative to S1P. Trp-6.48 is positioned to interact with Phe-6.44 via a favorable edge-to-face π-stacking interaction. The edge-to-face orientation of these two aromatic amino acid side chains is likely to lock both residues in the conformation observed in the computational model of S1P₁. In the S1P-S1P₁ complex, the center of the Phe-6.44 benzene functionality makes excellent contact with the terminal alkyl chain of S1P. The model places the aromatic centroid of Phe-6.44 4.51 Å away from the C-12 alkyl carbon, and the aromatic ring appears to be nearly perfectly centered around the C-12 carbon of S1P. Hence, F6.44G results in the loss of a favorable van der Waals contact. However, in the SEW2871-S1P complex, the aromatic center of Phe-6.44 is positioned directly above one of the fluorine atoms of the thiophene trifluoromethyl moiety, which is predicted to result in repulsion between the fluorine and Phe-6.44. Mutation of F6.44G in the SEW2871-S1P complex should allow Trp-6.48 to adopt a more favorable π-stacking interaction with the previously described aromatic domains of the ligand. Accordingly, the model is consistent with the experimental observation that F6.44G impairs S1P-induced S1P₁ activation but should have no effect (or perhaps improve) SEW2871-induced S1P₁ activation.

According to the validated computational model of S1P₁, Phe-5.48 is positioned to make van der Waals contacts with SEW2871 (4.01 Å) and S1P (3.89 Å). In contrast to the S1P-S1P₁ complex, in which Phe-5.48 makes van der Waals contact with a methylene functionality of the ligand, the SEW2871-S1P₁ activated complex results in the aromatic moiety of 5.48 being positioned near a fluorine atom of the thiophene trifluoromethyl functionality. It is likely that the F5.48Y replacement results in electronic repulsion between the phenolic hydroxyl and the fluorine atom. Hence, F5.48Y is predicted to have no effect on ligand-induced activation in the S1P-S1P₁ complex but to significantly impair ligand-induced activation in the SEW2871-S1P₁ complex. Surprisingly, the F5.48G mutation abolishes ligand-induced activation of both complexes. Moreover, this lack of activation in the S1P-S1P₁ complex is not due to impaired ligand binding, as shown in Fig. 4. Hence, it is plausible that Phe-5.48 serves as an important amino acid in the activation process, and amino acid replacements to residues that occupy significantly less volume than phenylalanine contribute to loss of S1P₁ activation.

The now extensively validated model not only serves as a good template for the modeling of the other EDG receptors but also defines the specific conformation of S1P relevant to S1P₁ agonism. This structure in combination with our recently published S1P₁ complex of the S1P₁-selective agonist, SEW2871 (35), defines the pharmacophore for S1P₁ agonism. Fig. 5 shows the structures of S1P and SEW2871 obtained by superposition of their S1P₁ complexes. The phosphate group of S1P occupies the same geometric position as a trifluoromethyl group of SEW2871. Similarly, the ammonium group of S1P occupies the same space as a weakly electron-poor hydrogen atom. The remainder of each structure occupies a common volume, and the superposed structures have quite similar lengths. These superposed structures define a geometric pharmacophore with distance ranges between pharmacophore elements shown in Fig. 5B. This pharmacophore was used to identify novel lead compounds from the Enhanced NCI Database Browser. Successful identification of NCI 59474 and NCI 99548 compounds as partial agonists of S1P₁ provide proof that in silico screening of large chemical libraries to identify novel molecular scaffolds that interact with the S1P₁ receptor is now possible.

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