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(57) Abstract: A *Canis* sphingosine-1-phosphate (S1P) receptor isoform 1 (cS1P₁), the nucleic acid encoding the cS1P₁ receptor, and methods for using the cS1P₁ receptor and the nucleic acid encoding the cS1P₁ receptor in assays for identifying analytes which modulate activity of the cS1P₁ receptor.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

TITLE OF THE INVENTION

CANIS SPHINGOSINE 1-PHOSPHATE RECEPTOR ISOFORM 1

CROSS-REFERENCE TO RELATED APPLICATIONS

- 5 This application claims the benefit of U.S. Provisional Application No. 60/591,333, filed July 27, 2004, the contents of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

10 (1) Field of the Invention

The present invention relates to a *Canis* sphingosine-1-phosphate (S1P) receptor isoform 1 (cS1P₁), the nucleic acid encoding the cS1P₁ receptor, and methods for using the cS1P₁ receptor and the nucleic acid encoding the cS1P₁ receptor in assays for identifying analytes which modulate activity of the cS1P₁ receptor. The assays are useful for identifying analytes with immunosuppressive activities.

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(2) Description of Related Art

Sphingosine-1-phosphate is a bioactive lysolipid that mediates a variety of diverse cellular functions such as cell adhesion, motility, differentiation, proliferation, and survival (Pyne and Pyne, *Biochem. J.* 349: 385-402 (2000); Hla, *Pharmacol. Res.* 47: 401-7(2003); Spiegel and Milstien, *Nat. Rev. Mol. Cell. Biol.* 4(5): 397-407 (2003)). It is a metabolic product of sphingolipids which are ubiquitous phospholipids found in all eukaryotic cells. It is also an abundant blood lipid secreted by hematopoietic cells and released from activated platelets (Yatomi *et al.*, *J. Biochem.* 121: 969-73 (1997)). Many of its cell signaling functions occur through activation of a family of G protein coupled receptors (GPCRs). Five sphingosine 1-phosphate (S1P) activated GPCRs have been identified, S1P₁, S1P₂, S1P₃, 20 S1P₄, and S1P₅ (previously known as endothelial differentiation genes Edg1, Edg5, Edg3, Edg6, and Edg8, respectively). These S1P receptors have a widespread cellular and tissue distribution and are well conserved in human and rodent species (Spiegel and Milstien, *Biochim. Biophys. Acta* 1484: 107-16 (2000); Fukushima *et al.*, *Annu. Rev. Pharmacol. Toxicol.* 41: 507-34 (2001); Hla, *Prostaglandins Other Lipid Mediat.* 64(1-4): 135-42 (2001)).

30 Each S1P receptor has a unique tissue expression pattern and couples to a distinct set of heterotrimeric G proteins (G α , G β , and G γ) each of which leads to activation of an isoform-specific panel of multiple intracellular signaling pathways. Each S1P receptor is a transmembrane protein comprising a ligand binding domain, seven transmembrane domains, and a cytoplasmic domain which interacts with G α of the set of heterotrimeric G proteins. In the inactive state, G α is bound to GDP. When 35 sphingosine-1-phosphate binds to the ligand binding domain, a signal is transduced through the S1P

receptor which results in the GDP bound to $G\alpha$ to be replaced by GTP and the $G\alpha$ to dissociate from $G\beta$ and $G\gamma$ (which remain as a $G\beta G\gamma$ dimer). $G\alpha$ and the $G\beta G\gamma$ dimer activate effectors which in turn activate distinct intracellular pathways specific to the receptor and G protein. At present, five different $G\alpha$ proteins subtypes are known; they are G_s , $G_{i/o}$, G_q , G_{12} , and G_{13} . G_s activates adenylyl cyclase, $G_{i/o}$ inhibits adenylyl cyclase, and G_q activates phospholipase C beta (PLC) which cleaves phosphoinositol-4,5 biphosphate (PIP₂) in the cell membrane to release second messengers diacylglycerol (DAG) and inositol-(1,4,5)-triphosphate (IP₃). G_{12} and G_{13} interact with Rho-specific guanine nucleotide exchange factors and regulate the actin cytoskeleton. The S1P₁ receptor is coupled primarily via $G_{i/o}$ to inhibit adenylyl cyclase and stimulate mitogen-activated protein kinase (MAPK). It is also coupled to stimulate PLC via a PTX-sensitive $G_{i/o}$. The S1P₂ receptor is coupled via $G_{i/o}$ to Ras/MAPK like S1P₁ but unlike S1P₁, S1P₂ is also coupled to stimulation of PLC via a PTX-insensitive G_q . It is also coupled to Rho stimulation. S1P₃ also interacts with multiple $G\alpha$ subtypes including $G_{i/o}$, G_q , and $G_{12/13}$, whereas S1P₄ primarily activates $G_{i/o}$ and the MAPK pathway. The S1P₅ receptor is coupled via $G_{i/o}$ and G_{12} to inhibit adenylyl cyclase in a PTX-sensitive manner but unlike S1P₁, it does not stimulate MAPK.

Many of the physiological functions of sphingosine 1-phosphate and its receptors have now been elucidated. The S1P₁ receptor is required for vascular maturation in mice (Liu *et al.*, J. Clin. Invest. 106: 951-61 (2000)). Ligand-induced activation of S1P₁ and S1P₃ on endothelial cells has been shown to promote angiogenesis, chemotaxis, and adherens junction assembly through Rac and Rho (Lee *et al.*, Cell 99: 301-12(1999)). Lung endothelial barrier function is enhanced by sphingosine 1-phosphate activation of the S1P₁ receptor (Schaphorst *et al.*, Am. J. Physiol. Lung Cell Mol. Physiol. 285: L258-67 (2003)) whereas endothelial cell permeability is increased by activation of the S1P₂ receptor (Schaphorst *et al.*, Am. J. Physiol. Lung Cell Mol. Physiol. 285: L258-67 (2003)). S1P activation of the S1P₂ receptor also inhibits chemotaxis by blocking Rac activation (Sugimoto *et al.*, Mol. Cell. Biol. 23(5): 1534-45 (2003)) and promotes neurite retraction (Van Brocklyn, *et al.*, J. Biol. Chem. 274(8): 4626-32 (1999)). Cardiovascular effects have been measured for sphingosine 1-phosphate in rats and in dog hearts (Sugiyama *et al.*, Jpn. J. Pharmacol. 82: 338-42 (2000); Sugiyama *et al.*, Cardiovasc. Res. 46: 119-25 (2000); Yatomi *et al.*, J. Biochem. 121: 969-73 (1997); Forrest *et al.*, J. Pharm. Exp. Therap. 309: 758-768 (2004)); the S1P₂ receptor has been implicated in contraction of coronary arteries (Ohmori *et al.*, Cardiovasc. Res. 58: 170-7 (2003)); and, the S1P₃ receptor has been found to mediate vasoconstriction of cerebral arteries (Salomone *et al.*, Eur. J. Pharmacol. 469: 125-34 (2003)) and induce bradycardia and hypertension in rodents (Forrest *et al.*, J. Pharm. Exp. Therap. 309: 758-768 (2004)). Pharmacological agonists of the S1P receptors are immunosuppressive; they regulate leukocyte trafficking by sequestering lymphocytes in secondary lymphoid organs (Brinkmann *et al.*, J Biol. Chem. 277: 21453-7 (2002); Mandala *et al.*, Science 296: 346-9 (2002). While the functions of the S1P₄ and S1P₅ receptors are less well understood, the S1P₄ receptor has been shown to be localized to hematopoietic cells and tissues

(Graeler *et al.*, Curr. Top. Microbiol. Immunol. 246: 131-6 (1999)) and the S1P₅ receptor has been shown to be primarily a neuronal receptor with some expression in lymphoid tissue in rodents but with a broader expression pattern in human tissues (Im *et al.*, J. Biol. Chem. 275(19): 14281-6 (2000); Niedernberg *et al.*, Biochem. Pharmacol. 64: 1243-50 (2002)).

5 In light of the above, there is a need for methods for identifying analytes which modulate the activity of the S1P₁ receptor.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a *Canis* sphingosine-1-phosphate (S1P) receptor isoform 1 (cS1P₁), the nucleic acid encoding the cS1P₁ receptor, and methods for using the cS1P₁ receptor and the nucleic acid encoding the cS1P₁ receptor in assays for identifying analytes which modulate activity of the cS1P₁ receptor. The assays are useful for identifying analytes with immunosuppressive activities and as such can be useful for treating a wide variety of autoimmune and chronic inflammatory diseases, including systemic lupus erythematosus, chronic rheumatoid arthritis, type I diabetes mellitus, inflammatory bowel disease, biliary cirrhosis, uveitis, multiple sclerosis and other disorders such as Crohn's disease, ulcerative colitis, bullous pemphigoid, sarcoidosis, psoriasis, autoimmune myositis, Wegener's granulomatosis, ichthyosis, Graves ophthalmopathy, atopic dermatitis, and asthma; and, as part of chemotherapeutic regimens for the treatment of cancers, lymphomas, and leukemias, and regimens for inhibiting tissue rejection in organ transplants.

20 The present invention provides an isolated nucleic acid molecule comprising a nucleotide sequence encoding a cS1P₁ receptor or fragment thereof, preferably a cS1P₁ receptor or fragment thereof, which comprises an amino acid sequence of SEQ ID NO:2. In various embodiments, the isolated nucleic acid is a DNA, an RNA, or a cDNA. In a further embodiment of the nucleic acid, the nucleotide sequence of the nucleic acid comprises a nucleotide sequence of SEQ ID NO:1.

25 The present invention further provides an isolated protein or fragment thereof comprising the amino acid sequence or part thereof of SEQ ID NO:2.

The present invention further provides an antibody that binds a protein comprising the amino acid sequence or part thereof of SEQ ID NO:2. In particular embodiments, the antibody is selected from the group consisting of polyclonal antibodies, monoclonal antibodies, recombinant scFv polypeptides, recombinant V_H polypeptides, and variants thereof.

30 The present invention further provides a vector comprising a nucleic acid encoding a cS1P₁ receptor or fragment thereof. Preferably, the cS1P₁ receptor or fragment thereof comprises an amino acid sequence of SEQ ID NO:2.

The present invention further provides a gene expression cassette comprising a nucleic acid encoding a cS1P₁ receptor or fragment thereof. Preferably, the cS1P₁ receptor or fragment thereof

comprises an amino acid sequence of SEQ ID NO:2. In further embodiments of the gene expression cassette, the nucleic acid encoding the cS1P₁ receptor is operably linked to a heterologous promoter that can either be constitutive or inducible

The present invention further provides a cell comprising a nucleic acid encoding a cS1P₁ receptor or fragment thereof which preferably comprises an amino acid sequence as set forth of SEQ ID NO:2 wherein the nucleic acid is operably linked to a heterologous promoter which can either be constitutive or inducible. In a further embodiment of the cell, the nucleic acid is integrated into the genome of the cell.

The present invention further provides a method for producing a cS1P₁ receptor comprising providing a nucleic acid encoding the cS1P₁ receptor operably linked to a heterologous promoter; introducing the nucleic acid into a cell to produce a recombinant cell; and culturing the recombinant cell under conditions which allows expression of the cS1P₁ receptor encoded by the nucleic acid to produce the cS1P₁ receptor.

In a further embodiment of the method, the nucleic acid is integrated into the genome of the recombinant cell. In a further still embodiment of the method, the cS1P₁ receptor comprises the amino acid sequence of SEQ ID NO:2.

The present invention is particularly useful for identifying analytes that have immunosuppressive activity. Therefore, the present invention further provides a method for screening for analytes with an immunosuppressive activity useful for treating autoimmune and inflammatory diseases, as a component of a chemotherapeutic regimen for treating cancers, or a regimen for inhibiting tissue rejection of transplants in a mammal, which comprises in one aspect determining the activity of a cS1P₁ receptor in the presence of a particular concentration of the analyte or in the absence of the analyte, and determining the activity of the cS1P₁ receptor at a different concentration of the analyte. The screening method can be cell-based or cell-free and can comprise one or more embodiments of the functional or binding assays set forth below.

Functional assays include a method for identifying an analyte that modulates activity of a cS1P₁ receptor, which comprises providing a recombinant cell which produces the cS1P₁ receptor; incubating the recombinant cell in a medium with the analyte; and determining the activity of the cS1P₁ receptor wherein a change in the activity of the cS1P₁ receptor indicates the analyte modulates activity of the cS1P₁ receptor.

The activity of the cS1P₁ is determined by one or more means for measuring S1P₁ activity selected from the group consisting of measuring a change in the intracellular concentration of Ca²⁺ in the presence of the analyte; measuring a change in the intracellular concentration of a metabolite selected from the group consisting of inositol triphosphate (IP₃) and diacylglycerol (DAG) in the presence of the analyte; measuring a change in the activity of phospholipase C beta (PLC β) or protein

kinase C (PKC) in the presence of the analyte; and measuring a change in the synthesis of cyclic AMP (cAMP) in the presence of the analyte. In assays that measure the change in the synthesis of the cAMP, Ca^{2+} , or other signaling molecules, an embodiment is further provided wherein measuring the change in signaling molecule is accomplished by including in the recombinant cell a gene expression cassette comprising a reporter gene which encodes an assayable product (e.g., a reporter gene encoding luciferase, β -lactamase, secreted alkaline phosphatase (SEAP), or the like) operably linked to a promoter which is responsive to the signaling molecule.

In further still embodiments of the above method, the recombinant cell further produces a chimeric protein G or a promiscuous G protein. The chimeric G protein can be selected from the group consisting of $\text{G}\alpha_{\text{q}05}$ and $\text{G}\alpha_{\text{q}55}$ and the promiscuous G protein can be selected from the group consisting of $\text{G}\alpha_{15}$ or $\text{G}\alpha_{16}$. In a further embodiment of the method, the cS1P₁ comprises the amino acid sequence of SEQ ID NO:2. In a further still embodiment of the method, the cS1P₁ and/or the chimeric or promiscuous G protein are encoded by gene expression cassettes, which in particular aspects, are integrated into the genome of the recombinant cell. Therefore, the recombinant cell can be transiently or stably transfected with one or more gene cassettes selected from the group consisting of gene cassettes encoding the cS1P₁ receptor, a chimeric or promiscuous G protein, and a reporter gene expression cassette.

The present invention further provides a method for identifying an analyte that binds to a cS1P₁ receptor, which comprises providing a recombinant cell which produces the cS1P₁ receptor; incubating the recombinant cell in a medium with the analyte; and, determining the amount of the analyte bound to the recombinant cell. Analytes which have been identified to bind to the cS1P₁ receptor using the aforementioned assays can be further analyzed using one of the functional assays above to determine whether the analyte is an agonist or an antagonist.

In a further embodiment of the method, the cS1P₁ comprises the amino acid sequence of SEQ ID NO:2. In a further still aspect of the method, the cS1P₁ is encoded by a nucleic acid which in particular embodiments is integrated into the genome of the recombinant cell. In a further embodiment of the above method, a competition assay is provided wherein the recombinant cell is incubated in a medium comprising the analyte and labeled S1P and the amount of analyte bound to the cS1P₁ receptor on the surface of the recombinant cell is determined by measuring the amount of labeled S1P bound to the recombinant cell. A decrease in the amount of label bound to the recombinant cell indicates that the analyte is a competitor of the labeled S1P for binding to the cS1P₁ receptor. In a further still embodiment, the analyte is labeled and the amount of analyte bound to the recombinant cell is determined either alone or in competition with differing concentrations of unlabeled S1P.

The present invention further provides a method for determining whether an analyte is a cS1P₁ receptor agonist or antagonist, which comprises providing a membrane which has the cS1P₁

receptor integrated therein and a G protein heterotrimer associated therewith; incubating the membrane in the presence of the analyte and labeled GTP for a time sufficient for the labeled GTP to be associated with the membrane when an agonist is present; and separating the membrane from unbound labeled GTP and determining the amount of labeled GTP associated with the membrane wherein an increase in the labeled GTP associated with the membrane indicates that the analyte is an agonist, a decrease in the labeled GTP associated with the membrane indicates that the analyte is an inverse agonist, and a decrease in the labeled GTP associated with the membrane in the presence of S1P or known agonist indicates that the analyte is an antagonist.

In a further embodiment of the method, the cS1P₁ comprises the amino acid sequence of SEQ ID NO:2. In a further still aspect of the method, the membrane is provided by a recombinant cell comprising a nucleic acid encoding the cS1P₁. In a further still embodiment of the method, the medium comprises the analyte and a labeled sphingosine-1-phosphate (SIP). In further still embodiment, the labeled GTP is labeled GTPγS. In particular aspects of the above, the analyte is labeled.

Definitions

The term "cS1P₁ receptor" means that the S1P₁ receptor is of *Canis* (dog) origin, either isolated from dog tissue, produced from a nucleic acid obtained from the dog by recombinant means, produced from a nucleic acid synthesized *in vitro* but which encodes the cS1P₁ receptor, or synthesized *in vitro*. The term further includes biologically active fragments or portions of the cS1P₁ receptor, including fusion or chimeric proteins.

The term "S1P₁ receptor" means that the S1P₁ receptor is not of dog origin. The S1P₁ receptor can be from another organism, for example, a mammal such as rat and mouse, or a human. The S1P₁ receptor can either be isolated from tissue of the organism, produced from a nucleic acid obtained from the organism by recombinant means, produced from a nucleic acid synthesized *in vitro* but which encodes the S1P₁ receptor, or synthesized *in vitro*. The term further includes biologically active fragments or portions of the S1P₁ receptor, including fusion or chimeric proteins.

The term "promoter" refers to a recognition site on a DNA strand to which RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity of a nucleic acid sequence located downstream from the promoter. The promoter can be modified by including activating sequences termed "enhancers" or inhibiting sequences termed "silencers" within the promoter. The term further includes both promoters which are inducible and promoters which are constitutive.

The term "gene expression cassette" refers to a nucleotide or gene sequence that is to be expressed from a vector, for example, the nucleotide or gene sequence encoding the cS1P₁ receptor, a reporter gene, or a chimeric or promiscuous G protein. In general, a cassette comprises a gene sequence

inserted into a vector which in some embodiments provides regulatory sequences for expressing the nucleotide or gene sequence. In other embodiments, the nucleotide or gene sequence provides the regulatory sequences for its expression. In further embodiments, the vector provides some regulatory sequences and the nucleotide or gene sequence provides other regulatory sequences. For example, the vector can provide a promoter for transcribing the nucleotide or gene sequence and the nucleotide or gene sequence provides a transcription termination sequence. The regulatory sequences which can be provided by the vector or included in the nucleotide sequence include, but are not limited to, promoters, enhancers, transcription termination sequences, splice acceptor and donor sequences, introns, ribosome binding sequences, and poly(A) addition sequences.

The term "vector" refers to a means by which DNA fragments can be introduced into a host organism or host tissue. There are various types of vectors including plasmid, virus (including adenovirus, herpesvirus, and the like), bacteriophage, and cosmid.

A "conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (e.g., arginine for lysine; glutamic acid for aspartic acid).

The term "agonist" refers to an agent that mimics or upregulates (for example, potentiates or supplements) cS1P₁ receptor bioactivity (and, therefore, human S1P₁ activity). An agonist can also be an analyte that upregulates expression of the cS1P₁ receptor. Agonists include proteins, nucleic acids, carbohydrates, small molecules, or any other molecule which can activate the cS1P₁.

The term "antagonist" refers to an analyte that inhibits, decreases, or suppresses a bioactivity of cS1P₁ receptor (and, therefore, human S1P₁ receptor). An antagonist can be an analyte that decreases signaling from the cS1P₁ receptor, for example, an analyte that is capable of binding to the cS1P₁ or human S1P₁ receptor. A preferred antagonist inhibits or suppresses the interaction between cS1P₁ receptor (and, therefore, human S1P₁ receptor) and its ligand. Alternatively, an antagonist can be a compound that downregulates expression of the cS1P₁ receptor gene. Antagonists include proteins, nucleic acids, carbohydrates, antibodies, small molecules, or any other molecule which can decrease the activity of the S1P₁ receptor.

Both agonists and antagonists modulate activity of the cS1P₁ receptor.

As used herein, the term "modulate", refers to any change in the activity of the cS1P₁ receptor effected by the analyte. For example, modulation may cause an increase or a decrease in protein activity, binding characteristics, or any other biological, functional, or immunological properties of the S1P₁ receptor. A modulator of S1P₁ activity can be an agonist or an antagonist.

A "disorder" is any condition that would benefit from treatment with analytes identified by the methods described herein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

The term "mammalian" refers to any mammal, including a human being.

5 The term "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented.

10 The term "analyte" refers to compound, composition, drug, molecule, peptide, protein, carbohydrate, nucleic acid, peptidomimetics, and the like, which interact with directly or indirectly with the cS1P₁ receptor.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence encoding the cS1P₁ receptor (SEQ ID NO:1).

Figure 2 shows the amino acid sequence comprising the cS1P₁ receptor (SEQ ID NO:2).

15 Figure 3 shows the results of an S1P ligand binding assay using the cS1P₁ receptor.

Figure 4 shows the results of a GTPγS binding assay using the cS1P₁ receptor.

DETAILED DESCRIPTION OF THE INVENTION

20 The present invention provides nucleic acid molecules that encode the canis (dog) sphingosine-1-phosphate isoform 1 receptor (cS1P₁ or cEdg1 receptor) and provides methods for using the nucleic acid molecules and the cS1P₁ receptor produced therefrom in assays for identifying analytes (molecules, compounds, drugs, or compositions) that modulate the activity of the cS1P₁ receptor by interacting with or binding the cS1P₁ receptor or modulating the molecular or functional interaction between the cS1P₁ receptor and its ligand sphingosine-1-phosphate (S1P). Modulators of cS1P₁ activity
25 can be agonists, inverse agonists or antagonists. Many S1P₁ receptor agonists have immunosuppressive activities by producing lymphocyte sequestration in secondary lymphoid tissues (WO2003062252 to Bugianesi *et al.*). Thus, the assays disclosed herein are useful for identifying analytes with immunosuppressive activities. Analytes with immunosuppressive activity have been shown to be useful in treating a wide variety of autoimmune and chronic inflammatory diseases, including systemic lupus
30 erythematosus, chronic rheumatoid arthritis, type I diabetes mellitus, inflammatory bowel disease, biliary cirrhosis, uveitis, multiple sclerosis and other disorders such as Crohn's disease, ulcerative colitis, bullous pemphigoid, sarcoidosis, psoriasis, autoimmune myositis, Wegener's granulomatosis, ichthyosis, Graves ophthalmopathy, atopic dermatitis, and asthma (WO2003062252 to Bugianesi *et al.*). Analytes with immunosuppressive activity have also proved useful as part of chemotherapeutic regimens for the

treatment of cancers, lymphomas, and leukemias, and regimens for inhibiting tissue rejection in organ transplants (WO2003062252 to Bugianesi *et al.*).

Non-limiting examples of methods for identifying such analytes include (i) cell-based binding methods for identifying analytes which bind the cS1P₁ receptor, inhibit or suppress binding
5 between cS1P₁ receptor and its ligand, or interfere with the functional activation of G α proteins via the cS1P₁ receptor in eukaryote cells and (ii) cell-free binding methods for identifying analytes which bind the cS1P₁ receptor, inhibit or suppress binding between the cS1P₁ receptor and its ligand, or interfere with the functional activation of G α proteins via the cS1P₁ receptor. Thus, the present invention provides a means for identifying agonists and antagonists of the cS1P₁ receptor. The methods described herein are
10 useful tools for identifying analytes which modulate molecular and/or functional interactions between the cS1P₁ receptor and its ligand or G α proteins and, therefore, are modulators of the S1P₁-dependent signaling pathway.

The present invention is particularly useful for identifying analytes of pharmaceutical importance which can be used to design or develop therapies or treatments for diseases or disorders which
15 involve modulation of S1P₁ receptor activity. Therefore, in one aspect of the present invention, an isolated nucleic acid molecule is provided which comprises a sequence of nucleotides encoding an RNA molecule that can be translated *in vivo* or *in vitro* to produce the cS1P₁ receptor with the amino acid sequence as set forth in SEQ ID NO:2 (Figure 2). In further embodiments, the nucleic acid is substantially free from other nucleic acids of the dog or substantially free from other nucleic acids. In a
20 further embodiment, the isolated nucleic acid molecule comprises the nucleotide sequence set forth in SEQ ID NO:1 (Figure 1).

The isolated nucleic acid molecules include both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) molecules encoding the cS1P₁ receptor. The isolated nucleic acid molecules further include genomic DNA and complementary DNA (cDNA) encoding the cS1P₁ receptor, either of
25 which can be single- or double-stranded, as well as synthetic DNA, such as a synthesized, single stranded polynucleotide. When single-stranded, the DNA molecule can comprise either the coding (sense) strand or the non-coding (antisense) strand. For most cloning purposes, DNA is a preferred nucleic acid.

In further aspects of the present invention, modified cS1P₁ receptors are provided which have an amino acid sequence which is substantially similar to the amino acid sequence set forth in SEQ
30 ID NO:2 and nucleic acids which encode the cS1P₁ receptor for use in the analyte screening assays disclosed herein. Further provided are nucleic acids encoding the cS1P₁ receptor which have a nucleotide sequence substantially similar to the nucleotide sequence set forth in SEQ ID NO:1. As used herein, the term "substantially similar" with respect to SEQ ID NO:2 means that the cS1P₁ receptor contains mutations such as amino acid substitution or deletion mutations that do not abrogate the ability
35 of the cS1P₁ receptor to bind its ligand. The mutations include naturally occurring allelic variants and

variants produced by recombinant DNA methods. As used herein, the term “substantially similar” with respect to SEQ ID NO:1 means that the cS1P₁ receptor encoded by the nucleic acid contains mutations such as nucleotide substitution or deletion mutations which do not abrogate the ability of the cS1P₁ receptor to bind its ligand. The mutations include naturally occurring allelic variants and variants produced by recombinant DNA methods. In general, any of the foregoing mutations which do not abrogate the ability of the cS1P₁ receptor to bind its ligand S1P are conservative mutations.

The present invention further includes biologically active mutants of SEQ ID NO:1. In general, any such biologically active mutant will encode either a polypeptide which has properties or activity substantially similar to the properties or activity of the cS1P₁ receptor, including but not limited to the cS1P₁ receptor as set forth in SEQ ID NO:2. Any such polynucleotide includes, but is not limited to, nucleotide substitutions, deletions, additions, amino-terminal truncations, and carboxy-terminal truncations which do not substantially abrogate the properties or activities of the cS1P₁ receptor produced therefrom. Thus, the mutations of the present invention encode mRNA molecules that express a cS1P₁ receptor in a eukaryotic cell which has sufficient activity (ability to bind one or more of its receptors) to be useful in drug discovery. Further, the present invention provides biologically active fragments of SEQ ID NO:2 and mutants thereof and the DNA encoding such fragments. The biologically active fragments can include any combination of the ligand binding domain, transmembrane domain, and G protein binding domain. For example, the biologically active fragment can consist of the ligand binding domain and the transmembrane domain.

The present invention further includes synthetic DNAs (sDNA) which encode the cS1P₁ receptor wherein the nucleotide sequence of the sDNA differs from the nucleotide sequence of SEQ ID NO:1 but still encodes cS1P₁ receptor as set forth in SEQ ID NO:2 or mutant with substantially similar properties or activity. For example, to express or enhance expression of the cS1P₁ receptor in a particular cell type, it may be necessary to change the sequence comprising one or more of the codons encoding the cS1P₁ receptor to sequences to enable expression of the cS1P₁ receptor in the particular cell type. Such changes include modifications for codon usage peculiar to a particular host or removing cryptic cleavage or regulatory sites which would interfere with expression of the cS1P₁ receptor in a particular cell type. Therefore, the present invention discloses codon redundancies which may result in numerous DNA molecules expressing an identical protein. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein that do not alter or do not substantially alter the ultimate physical or functional properties of the expressed protein (in general, these mutations are referred to as conservative mutations). For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in the functionality of the polypeptide.

Included in the present invention are DNA sequences that hybridize to SEQ ID NO:1 under stringent conditions. By way of example, and not limitation, a procedure using conditions of high stringency is as follows. Prehybridization of filters containing DNA is carried out for about 2 hours to overnight at about 65°C in buffer composed of 6x SSC, 5x Denhardt's solution, and 100 µg/mL denatured salmon sperm DNA. Filters are hybridized for about 12 to 48 hrs at 65°C in prehybridization mixture containing 100 µg/mL denatured salmon sperm DNA and labeled DNA (for example, 5-20 x 10⁶ cpm of ³²P-labeled DNA). The filters are washed at 37°C for about 1 hour in a solution containing 2x SSC, 0.1% SDS. This is followed by a wash in 0.1x SSC, 0.1% SDS at 50°C for 45 minutes before autoradiography. Other procedures using conditions of high stringency would include either a hybridization step carried out in 5x SSC, 5x Denhardt's solution, 50% formamide at about 42°C for about 12 to 48 hours or a washing step carried out in 0.2x SSPE, 0.2% SDS at about 65°C for about 30 to 60 minutes. Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd Edition; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, (1989) or Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Edition. Cold Spring Harbor Laboratory Press, Plainview, NY (2001). In addition to the foregoing, other conditions of high stringency which may be used are also well known in the art.

In another aspect of the present invention, a substantially purified form of a cS1P₁ receptor which comprises a sequence of amino acids as disclosed in Figure 2 (SEQ ID NO:2) is provided. Further provided are biologically active fragments and/or mutants of the cS1P₁ receptor, which comprise at least a portion of the amino acid sequence set forth in SEQ ID NO: 2. These mutations or fragments include, but not limited to, amino acid substitutions, deletions, additions, amino terminal truncations, and carboxy-terminal truncations such that these mutations provide for proteins or protein fragments of diagnostic, therapeutic, or prophylactic use and are useful for screening assays for identifying analytes that interfere with the interaction of the cS1P₁ receptor and its ligand, such analytes being useful for treatment of diseases or disorders which involve modulation of cS1P₁ receptor activity. In a particular embodiment, the present invention provides an isolated nucleic acid molecule comprising a sequence that encodes a mutated cS1P₁ receptor comprising the sequence set forth in SEQ ID NO:2 with about 1 to 10 amino acid additions, deletions, or substitutions, wherein the mutated cS1P₁ receptor polypeptide is capable of binding its S1P ligand.

The cS1P₁ receptors of the present invention can be the "mature" protein or a fragment or portion thereof (e.g., ligand binding domain, transmembrane domain, or G protein binding domain), any of which can be a part of a larger protein such as a fusion protein. It is often advantageous to include covalently linked to the amino acid sequence of the cS1P₁ receptor, an additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which aid in purification of the

cS1P₁ receptors such as multiple histidine residues (polyHis) or antibody-binding epitopes, or one or more additional amino acid sequences which confer stability to the cS1P₁ receptor during recombinant production. Thus, cS1P₁ receptor fusion proteins are provided which comprise all or part of the cS1P₁ receptor linked at its amino or carboxyl terminus to proteins or polypeptides such as green fluorescent protein (GFP), c-myc epitope, alkaline phosphatase, protein A or G, glutathione S-transferase (GST), polyHis, peptide cleavage site, or antibody Fc region. Any such fusion construct can be expressed in a cell line of interest and used to screen for modulators of the cS1P₁ receptor disclosed herein. In a particular embodiment, the present invention provides an isolated nucleic acid molecule comprising a sequence that encodes a fusion cS1P₁ receptor comprising the sequence set forth in SEQ ID NO:2 or a fusion protein with amino acid additions, deletions, or substitutions, wherein the mutated cS1P₁ receptor is capable of binding its S1P ligand.

The present invention further provides vectors which comprise at least one of the nucleic acid molecules disclosed throughout this specification, preferably wherein the nucleic acid molecule is operably linked to a heterologous promoter. These vectors can comprise DNA or RNA. For most cloning purposes, DNA plasmid or viral expression vectors are preferred. Typical expression vectors include plasmids, modified viruses, bacteriophage, cosmids, yeast artificial chromosomes, and other forms of episomal or integrated DNA, any of which expresses the cS1P₁ receptor, polypeptide fragment thereof, or fusion protein comprising all or part of the cS1P₁ receptor encoded therein. It is well within the purview of the skilled artisan to determine an appropriate vector for a particular gene transfer or other use. As used herein, the term "recombinant cS1P₁ receptor" is intended to include any variation of cS1P₁ receptor disclosed herein which is expressed from a vector transfected into a eukaryote cell or transformed into a prokaryote cell. Transfected eukaryote cells and transformed prokaryote cells are referred to as recombinant host cells.

An expression vector containing DNA encoding a cS1P₁ receptor or any one of the aforementioned variations thereof wherein the DNA is preferably operably linked to a heterologous promoter can be used for expression of the recombinant cS1P₁ receptor in a recombinant host cell. Such recombinant host cells can be cultured under suitable conditions to produce recombinant cS1P₁ receptor or a biologically equivalent form, for example, as shown in the Examples. Expression vectors include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids, or specifically designed viruses.

Commercially available mammalian expression vectors which are suitable for recombinant cS1P₁ receptor expression include, but are not limited to, pcDNA3.neo (Invitrogen, Carlsbad, CA), pcDNA3.1 (Invitrogen, Carlsbad, CA), pcDNA3.1/Myc-His (Invitrogen), pCI-neo (Promega, Madison, WI), pLITMUS28, pLITMUS29, pLITMUS38 and pLITMUS39 (New England Biolabs, Beverly, MA), pcDNAI, pcDNAIamp (Invitrogen), pcDNA3 (Invitrogen), pMC1neo

(Stratagene, La Jolla, CA), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo (342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and lZD35 (ATCC 37565).

5 Also, a variety of bacterial expression vectors can be used to express recombinant cS1P₁ receptor in bacterial cells. Commercially available bacterial expression vectors which may be suitable for recombinant cS1P₁ receptor expression include, but are not limited to, pCR2.1 (Invitrogen), pET11a (Novagen, Madison, WI), lambda gt11 (Invitrogen), and pKK223-3 (Pharmacia).

10 In addition, a variety of fungal cell expression vectors may be used to express recombinant cS1P₁ receptor in fungal cells. Commercially available fungal cell expression vectors which are suitable for recombinant cS1P₁ receptor expression include, but are not limited to, pYES2 (Invitrogen) and *Pichia* expression vector (Invitrogen).

15 Also, a variety of insect cell expression vectors can be used to express recombinant cS1P₁ receptor in insect cells. Commercially available insect cell expression vectors which can be suitable for recombinant expression of cS1P₁ receptor include, but are not limited to, pBlueBacIII and pBlueBacHis2 (Invitrogen), and pAcG2T (Pharmlngen).

20 Viral vectors which can be used for expression of recombinant cS1P₁ receptor include, but are not limited to, adenovirus vectors, adeno-associated virus vectors, herpesvirus vectors, Sindbis virus vectors, Simliki forest virus vectors, pox virus vectors (such as vaccinia virus, fowl pox, canary pox, and the like), retrovirus vectors, and baculovirus vectors. Many of viral vectors are commercially available.

25 The nucleic acids of the present invention in the above vectors for expressing the S1P₁ or fragment thereof are preferably assembled into an expression cassette that comprises sequences which provide for efficient expression of the cS1P₁ receptor or variant thereof encoded thereon in a eukaryote cell, preferably a mammalian cell such as a CHO cell or variant thereof. The cassette preferably contains the full-length cDNA encoding the cS1P₁ receptor or a DNA encoding a fragment of the cS1P₁ receptor with homologous or heterologous transcriptional and translational control sequences operably linked to the DNA. Such control sequences include at least a transcription promoter (constitutive or inducible) and transcription termination sequences and can further include other regulatory elements such as
30 transcription enhancers, ribosome binding sequences, splice junction sequences, and the like. In most embodiments, the promoter is a heterologous promoter; however, in particular embodiments, the promoter can be the natural cS1P₁ receptor promoter. In either embodiment, the expression cassette allows for ectopic expression of the cS1P₁ receptor in various host cells of non-canine origin. In a particularly useful embodiment, the promoter is the constitutive cytomegalovirus immediate early
35 promoter with or without the intron A sequence (CMV_{ie} promoter) although those skilled in the art will

recognize that any of a number of other known promoters such as the strong immunoglobulin promoter, Rous sarcoma virus long terminal repeat promoter, SV40 small or large T antigen promoter, or the like. A transcriptional terminator can be the bovine growth hormone terminator although other known transcriptional terminators such as SV40 termination sequences can also be used.

5 The present invention further provides recombinant host cells transformed or transfected with a vector comprising any one of the aforementioned nucleic acid molecules, particularly host cells transformed or transfected with a vector comprising any one of the aforementioned nucleic acid molecules wherein the nucleic acid molecule is operably linked to a promoter. Recombinant host cells include bacteria such as *E. coli*, fungal cells such as yeast, plant cells, mammalian cells including, but not limited to, cell lines of bovine, porcine, monkey, human, or rodent origin; and insect cells including, but not limited to, *Drosophila* and silkworm-derived cell lines. For instance, one insect expression system utilizes *Spodoptera frugiperda* (Sf21) insect cells (Invitrogen) in tandem with a baculovirus expression vector (pAcG2T, Pharmingen, San Diego, CA). Mammalian cells which may be suitable and which are commercially available, include but are not limited to, L cells L-M(TK-) (ATCC CCL-1.3), L cells L-M (ATCC CCL-1.2), Saos-2 cells (ATCC HTB-85), 293 cells (ATCC CRL-1573), Raji cells (ATCC CCL-86), CV-1 cells (ATCC CCL-70), COS-1 cells (ATCC CRL-1650), COS-7 cells (ATCC CRL-1651), CHO-K1 cells (ATCC CCL-61), 3T3 cells (ATCC CCL-92), NIH/3T3 cells (ATCC CRL-1658), HeLa cells (ATCC CCL-2), C1271 cells (ATCC CRL-1616), BS-C-1 cells (ATCC CCL-26), MRC-5 cells (ATCC CCL-171), HEK293T cells (ATCC CRL-1573), ST2 cells (Riken Cell bank, Tokyo, Japan

10 RCB0224), C3H10T1/2 cells (JCRB0602, JCRB9080, JCRB0003, or IFO50415), and CPAE cells (ATCC CCL-209). Such recombinant host cells can be cultured under suitable conditions to produce cS1P₁ receptor or a biologically equivalent form. Recombinant eukaryote cells include both transiently infected cells and stably transfected cells in which the expression cassette or vector is integrated into the genome of the cell.

25 As noted above, an expression vector containing DNA encoding cS1P₁ receptor or any one of the aforementioned variations thereof can be used to express the cS1P₁ receptor encoded therein in a recombinant host cell. Therefore, the present invention provides a process for expressing a cS1P₁ receptor or any one of the aforementioned variations thereof in a recombinant host cell comprising introducing the vector comprising a nucleic acid that encodes the cS1P₁ receptor into a suitable host cell and culturing the host cell under conditions which allow expression of the cS1P₁ receptor and preferably, integration of the cS1P₁ receptor into the cell's membrane. In a further embodiment, the cS1P₁ receptor has an amino acid sequence substantially as set forth in SEQ ID NO:2 and binds at least its ligand S1P, and the nucleic acid encoding the cS1P₁ receptor is operably linked to a heterologous promoter which can be constitutive or inducible. Thus, the present invention further provides a cell comprising a nucleic acid

35 encoding the cS1P₁ receptor which has an amino acid sequence substantially as set forth in SEQ ID

NO:2, which preferably binds at least its ligand S1P, and wherein the nucleic acid encoding the cS1P₁ receptor is operably linked to a heterologous promoter.

Following expression of cS1P₁ receptor or any one of the aforementioned variations of the cS1P₁ receptor in a host cell, cS1P₁ receptor or variant thereof can be recovered to provide cS1P₁ receptor in a form capable of binding to its ligand. Several cS1P₁ receptor purification procedures are available and suitable for use. The cS1P₁ receptor can be purified from cell lysates and extracts by various combinations of, or individual application of, salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography, or hydrophobic interaction chromatography. In addition, cS1P₁ receptor can be separated from other cellular polypeptides by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for cS1P₁ receptor or a particular epitope thereof. Alternatively, in the case of fusion polypeptides comprising all or a portion of the cS1P₁ receptor fused to a second polypeptide, purification can be achieved by affinity chromatography comprising a reagent specific for the second polypeptide such as an antibody or metal.

Cloning, expression vectors, transfections and transformations, and protein isolation of expressed proteins are well known in the art and have been described, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual* 2nd Edition; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, (1989) or Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, 3rd Edition. Cold Spring Harbor Laboratory Press, Plainview, NY (2001). For example, any of a variety of procedures may be used to clone DNA encoding cS1P₁ receptor from RNA isolated from the dog. These methods include, but are not limited to, the method shown in Examples 1-3 and the following methods.

(1) RACE PCR cloning methods such as disclosed in Frohman et al., *Proc. Natl. Acad. Sci. USA* 85: 8998-9002 (1988)). 5' and/or 3' RACE can be performed to generate a full-length cDNA sequence. This strategy involves using gene-specific oligonucleotide primers for PCR amplification of cS1P₁ receptor cDNA. These gene-specific primers are designed through identification of an expressed sequence tag (EST) nucleotide sequence which has been identified by searching any number of publicly available nucleic acid and protein databases.

(2) Direct functional expression of the cS1P₁ receptor cDNA following the construction of a cS1P₁ receptor containing cDNA library in an appropriate expression vector system.

(3) Screening a cS1P₁ receptor -containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a labeled degenerate oligonucleotide probe designed from the amino acid sequence of the cS1P₁ receptor.

(4) Screening a cS1P₁ receptor-containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a partial cDNA encoding the cS1P₁ receptor. This partial cDNA is obtained by the specific PCR amplification of cS1P₁ receptor DNA fragments through the design of

degenerate oligonucleotide primers from the amino acid sequence known for other membrane proteins which are related to the cS1P₁ receptor.

(5) Screening a cS1P₁ receptor-containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a partial cDNA or oligonucleotide with homology to a mammalian cS1P₁ receptor protein. This strategy may also involve using gene-specific oligonucleotide primers for PCR amplification of cS1P₁ receptor cDNA identified as an EST as described above.

(6) Designing 5' and 3' gene specific oligonucleotides using SEQ ID NO: 1 as a template so that either the full-length cDNA can be generated by known RACE techniques or a portion of the coding region can be generated by these same known RACE techniques to generate and isolate a portion of the coding region to use as a probe to screen one of numerous types of cDNA and/or genomic libraries in order to isolate a full-length version of the nucleotide sequence encoding cS1P₁ receptor.

It would be readily apparent to those skilled in the art that other types of libraries, as well as libraries constructed from other cell types or species types, may be useful for isolating a cS1P₁ receptor-encoding DNA or a cS1P₁ receptor homologue. Other types of libraries include, but are not limited to, cDNA libraries derived from other cells. The selection of cells or cell lines for use in preparing a cDNA library to isolate a cDNA encoding cS1P₁ receptor can be done by first measuring cell-associated cS1P₁ receptor activity using any known assay available for such a purpose.

Preparation of cDNA libraries can be performed by standard techniques well known in the art. Well known cDNA library construction techniques can be found for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd Edition; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, (1989) or Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Edition. Cold Spring Harbor Laboratory Press, Plainview, NY (2001). Complementary DNA libraries may also be obtained from numerous commercial sources, including but not limited to Clontech Laboratories, Inc. (Palo Alto, CA) and Stratagene (La Jolla, CA).

The DNA molecules, RNA molecules, and recombinant polypeptides of the present invention can be used to screen and measure levels of cS1P₁ receptor expression in homologous or heterologous cells. The recombinant polypeptides, DNA molecules, and RNA molecules lend themselves to the formulation of kits suitable for the detection and typing of cS1P₁ receptors. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant cS1P₁ receptor or anti-cS1P₁ receptor antibodies suitable for detecting cS1P₁ receptors. The carrier may also contain a means for detection such as labeled antigen or enzyme substrates or the like. The kit enables identification of polymorphic forms of cS1P₁ receptor which can then be used in the previously described methods to determine the effect the polymorphism has on binding between the polymorphic cS1P₁ receptor and its ligand.

In accordance with yet another embodiment of the present invention, there are provided antibodies having specific affinity for the cS1P₁ receptor or epitope thereof. The term "antibodies" is intended to be a generic term which includes polyclonal antibodies, monoclonal antibodies, Fab fragments, single V_H chain antibodies such as those derived from a library of camel or llama antibodies or camelized antibodies (Nuttall et al., *Curr. Pharm. Biotechnol.* 1: 253-263 (2000); Muyldermans, J. *Biotechnol.* 74: 277-302 (2001)), and recombinant antibodies. The term "recombinant antibodies" is intended to be a generic term which includes single polypeptide chains comprising the polypeptide sequence of a whole heavy chain antibody or only the amino terminal variable domain of the single heavy chain antibody (V_H chain polypeptides) and single polypeptide chains comprising the variable light chain domain (V_L) linked to the variable heavy chain domain (V_H) to provide a single recombinant polypeptide comprising the Fv region of the antibody molecule (scFv polypeptides)(*See*, Schmiedl et al., *J. Immunol. Meth.* 242: 101-114 (2000); Schultz et al., *Cancer Res.* 60: 6663-6669 (2000); Dübel et al., *J. Immunol. Meth.* 178: 201-209 (1995); and in U.S. Patent No. 6,207,804 B1 to Huston et al.). Construction of recombinant single V_H chain or scFv polypeptides which are specific against an analyte can be obtained using currently available molecular techniques such as phage display (de Haard et al., *J. Biol. Chem.* 274: 18218-18230 (1999); Saviranta *et al.*, *Bioconjugate* 9: 725-735 (1999); de Greeff *et al.*, *Infect. Immun.* 68: 3949-3955 (2000)) or polypeptide synthesis. In further embodiments, the recombinant antibodies include modifications such as polypeptides having particular amino acid residues or ligands or labels such as horseradish peroxidase, alkaline phosphatase, fluors, and the like. Further still embodiments include fusion polypeptides which comprise the above polypeptides fused to a second polypeptide such as a polypeptide comprising protein A or G.

The antibodies specific for cS1P₁ receptor can be produced by methods known in the art. For example, polyclonal and monoclonal antibodies can be produced by methods well known in the art, as described, for example, in Harlow and Lane, *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY (1988). The cS1P₁ receptor or fragments thereof can be used as immunogens for generating such antibodies. Alternatively, synthetic peptides can be prepared (using commercially available synthesizers) and used as immunogens. Amino acid sequences can be analyzed by methods well known in the art to determine whether they encode hydrophobic or hydrophilic domains of the corresponding polypeptide. Altered antibodies such as chimeric, humanized, camelized, CDR-grafted, or bifunctional antibodies can also be produced by methods well known in the art. Such antibodies can also be produced by hybridoma, chemical synthesis or recombinant methods described, for example, in Sambrook et al., *supra.*, and Harlow and Lane, *supra.* Both anti-peptide and anti-fusion protein antibodies can be used. (*See*, for example, Bahouth et al., *Trends Pharmacol. Sci.* 12: 338 (1991); Ausubel et al., *Current Protocols in Molecular Biology* (John Wiley and Sons, N.Y. (1989)).

Antibodies so produced can be used for the immunoaffinity or affinity chromatography purification of the cS1P₁ receptor or cS1P₁ receptor/ligand complexes. The above referenced anti-cS1P₁ receptor antibodies can also be used to modulate the activity of the cS1P₁ receptor in living animals, in humans, or in biological tissues isolated therefrom. Accordingly, contemplated herein are compositions comprising a carrier and an amount of an antibody having specificity for cS1P₁ receptor effective to block naturally occurring cS1P₁ receptor from binding its ligand.

Therefore, the nucleic acids encoding cS1P₁ receptor or variant thereof, vectors containing the same, host cells transformed with the nucleic acids or vectors which express the cS1P₁ receptor or variants thereof, the cS1P₁ receptor and variants thereof, as well as antibodies specific for the cS1P₁ receptor, can be used in *in vivo* or *in vitro* methods for screening a plurality of analytes to identify analytes that are modulators of the cS1P₁ receptor /ligand interaction. These methods provide information regarding the function and activity of the cS1P₁ receptor and variants thereof which can lead to the identification and design of molecules, compounds, or compositions capable of specific interactions with canine and ultimately, the human S1P₁ receptor. In preferred embodiments, the methods identify analytes which interfere with the binding of the cS1P₁ receptor to its ligand or activity of the cS1P₁ receptor. Such analytes are useful either alone or in combination with other compounds for treating a wide variety of autoimmune and chronic inflammatory diseases, including systemic lupus erythematosus, chronic rheumatoid arthritis, type I diabetes mellitus, inflammatory bowel disease, biliary cirrhosis, uveitis, multiple sclerosis and other disorders such as Crohn's disease, ulcerative colitis, bullous pemphigoid, sarcoidosis, psoriasis, autoimmune myositis, Wegener's granulomatosis, ichthyosis, Graves ophthalmopathy, atopic dermatitis, and asthma; and, as part of chemotherapeutic regimens for the treatment of cancers, lymphomas, and leukemias, and in organ transplantation. Accordingly, the present invention provides methods (screening assays) for identifying analytes that modulate the binding of cS1P₁ receptor to its ligand or activity of the cS1P₁ receptor and which can be used for treating the aforementioned diseases or disorders. The method involves identifying analytes that bind to the cS1P₁ receptor and/or have a stimulatory or inhibitory effect on the biological activity of the cS1P₁ receptor or its expression and then determining which of these analytes has an effect on symptoms or diseases regarding the aforementioned disorders and diseases in an *in vivo* assay.

The screening assays include (i) cell-based methods for identifying analytes which bind the cS1P₁ receptor, inhibit or suppress binding between cS1P₁ receptor and its ligand, or modulate activity of the cS1P₁ receptor, and (ii) cell-free methods for identifying analytes which bind the cS1P₁ receptor, inhibit or suppress binding between the cS1P₁ receptor and its ligand, or modulate activity of the cS1P₁ receptor. Analytes that bind or modulate activity of the cS1P₁ receptor include both agonists and antagonists. Thomsen *et al.*, Curr. Drug. Discovery, January: 13-18 (2004), provide a review of

screening assays for identifying modulators of G-protein-coupled receptors, any one of which can be used to identify modulators of the cS1P₁ receptor.

Cell-based methods for identifying analytes that bind or modulate the activity of the cS1P₁ receptor can be accomplished by any method suitable for measuring the activity of mammalian S1P₁ receptors, which include for example, many methods suitable for measuring the activity of a G-protein-coupled receptor or any other seven transmembrane receptor. Methods for measuring activity of G-protein coupled receptors (functional assays) include, but are not limited to, measuring alterations in the concentration of intracellular Ca²⁺, inositol triphosphate (IP₃), diacylglycerol (DAG), or adenosine cyclic 3', 5'-monophosphate (cAMP) in response to an analyte; activation of phospholipase C or protein kinase C (PKC), or alterations in the concentration or activation of other signaling molecules.

Analytes that bind the cS1P₁ receptor can be identified in a competitive binding cell-based assay using cells which express the cS1P₁ on the cell surface and labeled-S1P as a competitor. Example 3 illustrates a competitive cell-based binding assay. In a typical competitive binding assay, eukaryote cells which have been transiently or stably transfected with an expression vector that expresses the cS1P₁ receptor are incubated in a cell culture medium suitable for the cells for a time sufficient for the cS1P₁ receptor to become integrated into the membranes of the cells. The cells can be adherent cells or non adherent cells. For example, the cells can be adherent cells such as CHO cells which are incubated in cell culture dishes in a medium suitable for growing the CHO cells such that the CHO cells grow in the culture dishes as a monolayer. Alternatively, the cells can be non-adherent cells such as HeLa S cells which are incubated in culture bottles under agitation, e.g., spinner culture bottles. After sufficient time has elapsed to allow a significant number of cS1P₁ receptors to be expressed and become integrated into the membranes of the cells, the cells are harvested. In the case of adherent cells, the transfected cells are harvested with an enzyme-free dissociation solution to dislodge the cells from the surface of the tissue culture dishes without causing damage to the cS1P₁ integrated into the membranes of the cells. The cells are pelleted by low speed centrifugation and suspended in a buffer. Aliquots of the cells are transferred to buffer containing labeled S1P and analyte to be tested. After incubating for a time sufficient for S1P and/or analyte to bind the cS1P₁, unbound labeled S1P and analyte are removed and the amount of labeled S1P is then detected by a method suitable for detecting the label. Non-specific binding can be defined as the amount of label bound to the cS1P₁ receptor on the cells in the presence of an excess of unlabeled S1P (e.g., about 200 nM unlabeled S1P).

In variations of the assay, a plurality of cell aliquots are mixed with aliquots of the mixture containing different concentrations of the analyte to be tested. Analytes which cause a decrease in the amount of label retained relative to controls comprising the labeled S1P and no analyte are analytes that bind to the cS1P₁ receptor. Serial dilutions of the analyte in the presence of a fixed amount of labeled S1P enable the affinity of the analyte for the cS1P₁ receptor to be determined. In an alternative

embodiment of the above assay, the S1P is unlabeled and the analyte is labeled. In this case, analytes which bind the cS1P₁ receptor are determined by detecting the amount of labeled analyte bound in the absence and presence of various concentrations of S1P. In a further alternative of the competitive binding assay, the assay is performed as a cell-free assay wherein membranes comprising the cS1P₁ receptor are
5 prepared as described below and incubated with S1P and analyte as above.

In the assays disclosed herein, determination of the amount of binding in the presence of varying concentrations of analyte and S1P and analysis of the data by a computer program such as the PRISM software (GraphPad Software, Inc. San Diego, CA) can be used to measure the affinity of the analyte for the cS1P₁ receptor. Specificity of analytes for the cS1P₁ receptor can be determined by
10 measuring the level of labeled S1P binding in the presence of the analyte to related S1P receptors (S1P₁, S1P₂, S1P₃, S1P₄, S1P₅, canis or non-canis) in similar binding assays using membranes prepared from cells transfected with each respective receptor.

Analytes that can bind to the cS1P₁ receptor and which can act as an agonist or antagonist can be determined in a functional or signaling assay. Examples of cell-based functional assays
15 include, but are not limited to, measuring alterations in the concentration of intracellular Ca²⁺ (calcium flux), inositol triphosphate (IP₃), diacylglycerol (DAG), or adenosine cyclic 3', 5'-monophosphate (cAMP) in response to an analyte; or activation of phospholipase C (PLC) or protein kinase C (PKC) in response to an analyte.

Measuring calcium flux in response to an analyte can be used to identify analytes that are
20 G-protein-coupled receptor agonists or antagonists. Binding of a ligand to a G-protein-coupled receptor coupled to G_{αq} activates PLC_β. The PLC_β hydrolyzes PIP₂ to DAG and IP₃. The IP₃ then acts to effect an increase in intracellular concentrations of Ca²⁺ via release of the Ca²⁺ from intracellular Ca²⁺ stores. DAG can activate specific protein kinase C (PKC) isoforms such PKC_α and PKC_β. The increase in intracellular Ca²⁺ can be conveniently assessed using fluorescence-based Ca²⁺ release measurements.
25 The cS1P₁ receptor is primarily a G_{αi/o}-coupled receptor which in the presence of a ligand can also stimulate Ca²⁺ release (Okamoto et al., J. Biol. Chem. 273: 27104-27110 (1998)). Therefore, in a further aspect of the present invention, a gene expression cassette encoding the cS1P₁ receptor is transfected into eukaryote cells such as CHO K1 cells. An aliquot of the cotransfected cells is then incubated in a medium containing an analyte, an aliquot with a known agonist as a positive control, and an aliquot with
30 neither the analyte nor the agonist as a negative control. Each of the aliquots further comprises a means for detecting release of Ca²⁺ from intracellular Ca²⁺ stores. Such means include further cotransfecting into the cell a gene expression cassette encoding aequorin, which in the presence of Ca²⁺, emits photons that are detectable with a luminometer, or including a calcium-sensitive dye such as fluor-3 or fluor-4 which fluoresces in the presence of Ca²⁺ and which fluorescence is detectable with a fluorometer. An
35 analyte that is an agonist causes a detectable increase in the release of Ca²⁺ from the intracellular stores.

To detect an analyte which is an antagonist, aliquots of the cells are incubated in a serial dilution of the analyte in a medium and the Ca^{2+} flux measured for each of the aliquots. Then, to each of the aliquots, an agonist is added and the Ca^{2+} flux measured. An antagonist results in a decrease in the release of Ca^{2+} from the intracellular stores in the presence of the known agonist. WO0168922 to Grant *et al.*

discloses a high throughput calcium flux assay for G-protein-coupled receptors.

While the S1P₁ receptor can stimulate Ca^{2+} release, it has been found that the stimulation can be enhanced by coexpressing the S1P₁ receptor with a chimeric G protein such as $\text{G}\alpha_{\text{qo5}}$ or $\text{G}\alpha_{\text{q55}}$ or co-expressing with certain promiscuous G proteins such as $\text{G}\alpha_{15}$ or $\text{G}\alpha_{16}$. The G proteins are capable of interacting with $\text{G}\alpha_{\text{i/o}}$ and $\text{G}\alpha_{\text{q}}$ and allowing an increase in the intracellular Ca^{2+} response to an agonist. Assays that link $\text{G}\alpha_{\text{i/o}}$ -coupled receptors to calcium flux have been described by Coward *et al.*, Anal. Biochem. 270: 242-249 (1999), Kazmi *et al.*, Biochem. 39: 3734-3744 (2000), Gopalakrishnan *et al.*, Anal. Biochem. 321: 192-201 (2003); and Knight *et al.*, Anal. Biochem. 320: 88-103 (2003).

Therefore, in a further aspect of the present invention, a first gene expression cassette encoding the cS1P₁ receptor is cotransfected with a second gene expression cassette encoding a chimeric or promiscuous G protein into eukaryote cells. An aliquot of the cotransfected cells is then incubated in a medium containing an analyte, an aliquot with a known agonist as a positive control, and an aliquot with neither the analyte nor the agonist as a negative control. Detecting release of Ca^{2+} from intracellular Ca^{2+} stores is as described above. An analyte which is an agonist causes a detectable increase in the release of Ca^{2+} from the intracellular stores. To detect an analyte that is an antagonist, aliquots of the cells are incubated in a serial dilution of the analyte in a medium and the Ca^{2+} flux measured for each of the aliquots. Then, to each of the aliquots, an agonist is added and the Ca^{2+} flux measured.

Agonists or antagonists of the cS1P₁ receptor can be identified by measuring the change in IP₃ or DAG concentrations or activity of PKC in response to an analyte. The IP₃ or DAG can be measured using antibodies specific for the IP₃ or the DAG. Commercially available assays for measuring IP₃ include HITHUNTER (DiscoverX Corp., Fremont, CA) and ALPHASCREEN IP₃ (Perkin Elmer Life and Analytical, Boston, MA). WO2003021220 to Brandish and Hill describes an assay for measuring inositol phosphate concentrations. Commercially available assays for measuring DAG include the BIOTRAK DAG Assay (Amersham Bioscience, Piscataway, NJ). Commercially available assays for measuring PKC activity include the RPN77 Protein Kinase C BIOTRAK Assay System (Amersham Bioscience).

Therefore, in a further aspect of the present invention, a gene expression cassette encoding the cS1P₁ receptor is transfected into eukaryote cells such as CHO K1 cells. An aliquot of the cotransfected cells is then incubated in a medium containing an analyte, an aliquot with a known agonist as a positive control, and an aliquot with neither the analyte nor the agonist as a negative control. Each of

the aliquots further comprises a means for detecting IP₃ or DAG. An agonist results in an increase in IP₃ or DAG levels relative to the negative control. To detect an analyte that is an antagonist, aliquots of the cells are incubated in a serial dilution of the analyte in a medium and IP₃ or DAG measured for each of the aliquots. Then, to each of the aliquots, an agonist is added and the IP₃ or DAG measured. An antagonist results in a decrease in IP₃ or DAG levels in the presence of the known agonist.

To enhance the stimulation of PLC β activity on PIP₂, the first gene expression cassette encoding the cS1P₁ receptor is cotransfected with a second gene expression cassette encoding a chimeric or promiscuous G protein into eukaryote cells. An aliquot of the cotransfected cells are then incubated in a medium containing an analyte, an aliquot with a known agonist as a positive control, and an aliquot with neither the analyte nor the agonist as a negative control. Each of the aliquots further comprise a means for detecting IP₃ or DAG as described above. An analyte which is an agonist causes a detectable increase in IP₃ or DAG. To detect an analyte that is an antagonist, aliquots of the cells are incubated in a serial dilution of the analyte in a medium and the IP₃ or DAG measured for each of the aliquots. Then, to each of the aliquots, an agonist is added and the IP₃ or DAG measured.

Agonists or antagonists of the cS1P₁ receptor can also be identified by measuring activation of the PLC β . Examples of assays for detecting PLC β activity include the following. Várnai and Balla, J. Cell. Biol. 143: 501-510 (1998) describe a method for measuring PLC β activation that uses a fusion protein consisting of the pleckstrin homology (PH) domain of PLC δ 1 fused to a green fluorescent protein (GFP). In the absence of PLC β activity, the fusion protein is associated with PIP₂ along the cytosol side of the cell membrane. In the presence of PLC β activity, the PIP₂ is hydrolyzed to DAG and IP₃, which releases the fusion protein to the cytosol. Van der Wal *et al.*, J. Biol. Chem. 276: 15337-15344 (2001), describe a method for measuring PLC β activation that measures fluorescence resonance energy transfer (FRET) between PH domains in which one domain is tagged with a fluorescence donor and the other is tagged with a fluorescence acceptor. In the absence of PLC activity, the PH domains bind PIP₂ and because the acceptor and donor fluors are in close proximity, the fluorescence energy from the donor fluor is transferred to the acceptor fluor fluoresces at a first wavelength. In the presence of PLC β activity, the PH domains are released to the cytosol. Because the PH domains are no longer in close proximity, the donor fluor fluoresces at a second wavelength.

Therefore, in a further aspect of the present invention, a gene expression cassette encoding the cS1P₁ receptor is transfected into eukaryote cells such as CHO K1 cells. An aliquot of the cotransfected cells is then incubated in a medium containing an analyte, an aliquot with a known agonist as a positive control, and an aliquot with neither the analyte nor the agonist as a negative control. Each of the aliquots further comprises a means for detecting PLC β activity such as disclosed above. An analyte that is an agonist results in a detectable increase in PLC β activity with respect to the negative control. To detect an analyte that is an antagonist, aliquots of the cells are incubated in a serial dilution of the analyte

in a medium and PLC β activity measured for each of the aliquots. Then, to each of the aliquots, an agonist is added and the PLC β activity measured. An antagonist results in a decrease in PLC β activity relative to the positive control in the presence of the known agonist.

To enhance the stimulation of the PLC β activity, the first gene expression cassette
5 encoding the cS1P₁ receptor is cotransfected with a second gene expression cassette encoding a chimeric or promiscuous G protein into eukaryote cells. An aliquot of the cotransfected cells is then incubated in a medium containing an analyte, an aliquot with a known agonist as a positive control, and an aliquot with neither the analyte nor the agonist as a negative control. Each of the aliquots further comprise a means for detecting PLC β activity as disclosed above. To detect an analyte that is an antagonist, aliquots of the
10 cells are incubated in a serial dilution of the analyte in a medium and the PLC β activity measured for each of the aliquots. Then, to each of the aliquots, an agonist is added and the PLC β activity measured.

Activation of S1P₁ receptors by S1P results in inhibition of adenylate cyclase activity with a concomitant decrease in cAMP levels. The decrease in cAMP levels results in a decrease in expression of genes regulated by a cAMP-responsive promoter. Agonists and antagonists of the cS1P₁
15 receptor can be identified in an assay that measures inhibition of adenylate cyclase via the decrease in cAMP. Chen *et al.*, Anal.Biochem. 226: 349-354 (1995), describes a colorimetric assay that uses a recombinant cell transfected with an expression vector encoding a G-protein coupled receptor with a second expression vector containing a promoter with a cAMP responsive element operably linked to the β -galactosidase reporter gene. An alternative assay using enzyme fragment complementation to assay
20 cAMP activity is described in Golla and Seethala, J. Biomol. Screen, 7: 515-525 (2002). Commercially available kits include HITHUNTER cAMP from DiscoverRx Corp. and cAMP DIRECT BIOTRAK kit (Amersham Biosciences). Other methods for measuring changes in cAMP levels are well known in the art.

Therefore, in a further aspect of the present invention, a first gene expression cassette
25 encoding the cS1P₁ receptor and a second gene expression cassette encoding a reporter gene encoding an assayable product operably linked to a cAMP responsive promoter, i.e., a promoter comprising one or more cAMP response elements, are transfected into eukaryote cells such as CHO K1 cells. An aliquot of the cotransfected cells is then incubated in a medium containing an analyte, an aliquot with a known agonist as a positive control, and an aliquot with neither the analyte nor the agonist as a negative control.
30 Each of the aliquots further comprises a means for detecting the reporter gene product. An agonist results in a reduction in expression of the reporter gene relative to the negative control. To detect an analyte that is an antagonist, aliquots of the cells are incubated in a serial dilution of the analyte in a medium and reporter gene expression measured for each of the aliquots. Then, to each of the aliquots, an agonist is added and reporter gene expression measured. An antagonist results in expression of the reporter gene
35 relative to the positive control in the presence of the known agonist.

In a further still aspect, a first gene expression cassette encoding the cS1P₁ receptor, a second gene expression cassette encoding a reporter gene encoding an assayable product (e.g., a reporter gene encoding luciferase, β -lactamase, secreted alkaline phosphatase (SEAP), or the like) operably linked to a promoter comprising a cAMP response element, and a third gene expression cassette encoding a chimeric or promiscuous G protein are cotransfected into eukaryote cells. An aliquot of the cotransfected cells are then incubated in a medium containing an analyte, an aliquot with a known agonist as a positive control, and an aliquot with neither the analyte nor the agonist as a negative control. Each of the aliquots further comprises a means for detecting reporter gene expression as described above. To detect an analyte which is an antagonist, aliquots of the cells are incubated in a serial dilution of the analyte in a medium and reporter gene expression measured for each of the aliquots. Then, to each of the aliquots, an agonist is added and reporter gene expression measured.

In an alternative embodiment for measuring the effect on cAMP synthesis, a first gene expression cassette encoding the cS1P₁ receptor is transfected into eukaryote cells such as CHO K1 cells. An aliquot of the cotransfected cells is then incubated in a medium containing an analyte, an aliquot with a known agonist as a positive control, and an aliquot with neither the analyte nor the agonist as a negative control. The levels of cAMP are then measured using any one of a number of commercially available assays for measuring cAMP levels, e.g., the HITHUNTER cAMP kit or the cAMP DIRECT BIOTRAK kit (Amersham Biosciences).

Cell-free assays include contacting cS1P₁ receptor (or variant thereof, for example, full-length, a biologically active fragment thereof, or a fusion protein comprising all or a portion of the cS1P₁ receptor) with an analyte and determining the ability of the analyte to bind to the S1P₁ receptor or modulate activity of the cS1P₁ receptor. Binding of the analyte to the cS1P₁ receptor can be determined either directly or indirectly. In one aspect, the assay includes contacting the cS1P₁ receptor with a known analyte that binds the cS1P₁ receptor to form an assay mixture, contacting the assay mixture with an analyte, and determining the ability of the analyte to interact with the cS1P₁ receptor, wherein determining the ability of the analyte to interact with the cS1P₁ receptor comprises determining the ability of the analyte to preferentially bind to the cS1P₁ receptor as compared to an analyte which is known to bind the cS1P₁ receptor. Detection of binding can be direct, for example, wherein the analyte is labeled, or indirectly, for example, in competition assays wherein the analyte competes for binding to the cS1P₁ receptor with S1P or other analyte known to bind the S1P.

The cell-free assays of the present invention can use either a membrane-bound form of cS1P₁ receptor or a soluble fragment thereof. In the case of cell-free assays comprising the membrane-bound form of the cS1P₁ receptor, it may be desirable to use a solubilizing agent such that the membrane-bound form of the cS1P₁ receptor is maintained in solution. Examples of such solubilizing agents include but are not limited to non-ionic detergents such as n-octylglucoside, n-dodecyl glucoside, n-

dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methyl glucarnide, Triton X-100, Triton X-114, Thesit, Isotridecypoly(ethylene glycol 5 ether)n, 3-[(3-cholamidopropyl) dimethylamminio]-1-propane sulfonate (CHAPS), 3 [(3-cholamidopropyl) dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), N-dodecyl=N,N-dimethyl-3-ammonio-1-propanesulfonate.

5 Analytes that bind the cS1P₁ receptor can be identified in a competitive binding cell-free assay using membranes from cells which express the cS1P₁ on the cell surface and labeled-S1P as a competitor. In a typical competitive binding assay, eukaryote cells, which have been transiently or stably transfected with an expression vector that expresses the cS1P₁ receptor, are incubated in a cell culture medium suitable for the cells for a time sufficient for the cS1P₁ receptor to become integrated into the
10 membranes of the cells. The cells can be adherent cells or non adherent cells. For example, the cells can be adherent cells such as CHO cells which are incubated in cell culture dishes in a medium suitable for growing the CHO cells such that the CHO cells grow in the culture dishes as a monolayer. Alternatively, the cells can be non-adherent cells such as HeLa S cells which are incubated in culture bottles under agitation, e.g., spinner culture bottles. After sufficient time has elapsed to allow a significant number of
15 cS1P₁ receptors to be expressed and become integrated into the membranes of the cells, the cells are harvested. In the case of adherent cells, the transfected cells are harvested with an enzyme-free dissociation solution to dislodge the cells from the surface of the tissue culture dishes without causing damage to the cS1P₁ integrated into the membranes of the cells. The cells are pelleted by low speed centrifugation and suspended in a buffer. Membranes are prepared from the cells and aliquots of the
20 membranes are transferred to buffer containing labeled S1P and analyte to be tested. After incubating for a time sufficient for S1P and/or analyte to bind the cS1P₁, unbound labeled S1P and analyte are removed and the amount of labeled S1P is then detected by a method suitable for detecting the label. Non-specific binding can be defined as the amount of label bound to the cS1P₁ receptor on the cells in the presence of about 200 nM unlabeled S1P.

25 In variations of the assay, a plurality of membrane aliquots are mixed with aliquots of the mixture containing different concentrations of the analyte to be tested. Analytes that cause a decrease in the amount of label retained relative to controls comprising the labeled S1P and no analyte are analytes that bind to the cS1P₁ receptor. Serial dilutions of the analyte in the presence of a fixed amount of labeled S1P enable the affinity of the analyte for the cS1P₁ receptor to be determined. In an alternative
30 embodiment of the above assay, the S1P is unlabeled and the analyte is labeled. In this case, analytes which bind the cS1P₁ receptor are determined by detecting the amount of labeled analyte bound in the absence and presence of various concentrations of S1P.

 A GTP binding assay is an example of a cell-free method which can be used to not only measure binding of an analyte to the cS1P₁ receptor but also to determine whether the analyte can
35 modulate activity of the cS1P₁ receptor. Therefore, in a further aspect of a cell-free assay for determining

whether an analyte is an agonist or antagonist, a labeled-GTP γ S cell-free binding assay method can be used. In this assay, membranes are prepared from transfected cells and aliquots incubated in a mixture with GDP, various concentrations of the analyte, and labeled GTP γ S. After incubating for a time sufficient for the labeled GTP γ S to bind the G protein, the reaction is terminated and the bound labeled
5 GTP γ S is measured by a means suitable for detecting the label. The GTP γ S can be labeled by any standard technique known in the art, such as radiolabeling, fluorescence labeling, Europium labeling, or the like. In variations of the assay, a plurality of membrane aliquots are mixed with aliquots of the mixture containing different concentrations of the analyte to be tested. Controls include S1P in the absence of the analyte.

10 When the method is performed in the absence of S1P, analytes that stimulate labeled GTP γ S binding greater than the endogenous level (or non-specific binding level) are agonists while compounds that inhibit the endogenous level of labeled GTP γ S are inverse agonists. This is detected as label associated with the membrane. On the other hand, antagonists are detected in a labeled GTP γ S binding assay in the presence of a submaximal level of S1P or other known agonist where they reduce the
15 labeled GTP γ S binding that is stimulated by S1P. Determination of the amount of binding in the presence of varying concentrations of analyte and analysis of the data by a computer program such as PRISM software (GraphPad) can measure the affinity of analytes for the cS1P₁ receptor. Specificity of analytes for the cS1P₁ receptor can be determined by measuring the level of labeled GTP γ S binding in the presence of the analyte to other G protein coupled receptors (e.g., S1P₂, S1P₃, S1P₄, S1P₅, or the like) in
20 similar binding assays using membranes prepared from cells transfected with each respective receptor.

In a further aspect of the method, the analyte is labeled with a label that is different from the label of the GTP γ S. For example, the analyte can be labeled with a first fluorescent label which fluoresces at a first wavelength and the GTP γ S is labeled with a second fluorescent label which fluoresces at a second wavelength or a radioisotope such as ³⁵S or europium. In this embodiment, a labeled analyte,
25 which is an agonist, will bind to the cS1P₁ receptor on the membrane and will stimulate binding of the labeled GTP γ S to the G protein of the membrane. Both labels will be substantially associated with the membrane and detectable. That is association of both labels with the membrane will be greater than the endogenous level or the non-specific binding level. In contrast, a labeled analyte, which is an antagonist, will bind to the cS1P₁ receptor on the membrane but will not stimulate binding of the labeled GTP γ S to
30 the G protein of the membrane. The label of the analyte will be substantially associated with the membrane and detectable at a level greater than the endogenous level or the non-specific binding level. However, the labeled GTP γ S will not be detectable at a level greater than the endogenous level or the non-specific binding level.

In further aspects of the GTP γ S-based method, detection of agonist or antagonist activity
35 of an analyte is determined by determining whether in the presence of the analyte the G α subunit is

activated or rendered inactive. Detection of activated or inactivated G α subunit can be achieved by including in the assay or subsequent to the assay an antibody or peptide which is specific for and binds either the activated or inactivated form of the G α subunit. Preferably, the antibody or peptide is labeled.

In various embodiments of the above cell-free assay methods, it may be desirable to immobilize the cS1P₁ receptor or a target protein of the cS1P₁ receptor to facilitate separation of complexed from uncomplexed forms of one or both, as well as to accommodate automation of the assay. Binding of an analyte to cS1P₁ receptor, or interaction of the cS1P₁ receptor with a target protein in the presence and absence of an analyte, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, microarrays, and microcentrifuge tubes.

In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase (GST) fusion proteins or glutathione-S-transferase fusion proteins can be adsorbed onto glutathione SEPHAROSE beads (Sigma Chemical; St. Louis, Mo.) or glutathione derivatized microtitre plates, which are then combined with the analyte or the analyte and either the non-adsorbed target protein or the cS1P₁ receptor, and the mixture incubated under conditions conducive to complex formation (for example, at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components and complex formation is measured. Alternatively, the complexes can be dissociated from the matrix, and the level of binding or activity of the cS1P₁ receptor can be determined using standard techniques. Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, either the cS1P₁ receptor or its target protein can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated cS1P₁ receptor or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (for example, biotinylation kit, Pierce Chemicals, Rockford, Ill.), and immobilized in the wells of streptavidin coated plates (Pierce Chemical). Alternatively, antibodies reactive with the cS1P₁ receptor or target proteins but which do not interfere with binding of the cS1P₁ receptor to its target protein can be derivatized to the wells of the plate and unbound target protein or cS1P₁ receptor trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described herein for the GST-immobilized complexes, include immunodetection of complexes using antibodies specific for the cS1P₁ receptor or target protein, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the cS1P₁ receptor or target protein.

The present invention further provides screening assays for monitoring the expression of the cS1P₁ receptor. For example, regulators of expression of the cS1P₁ receptor can be identified in a method in which a cell is contacted with an analyte and the expression of cS1P₁ receptor (protein or mRNA) in the cell is determined. The level of expression of the cS1P₁ receptor in the presence of the

analyte is compared to the level of expression of the cS1P₁ receptor in the absence of the analyte wherein a change in the level of expression indicates that the analyte can regulate expression of the cS1P₁ receptor. For example, an increase in cS1P₁ receptor levels in the presence of an analyte indicates that the analyte is a stimulator or inducer of cS1P₁ receptor expression. Conversely, an analyte that causes a decrease in cS1P₁ receptor levels is an inhibitor of cS1P₁ receptor expression. The level of cS1P₁ receptor in the cells can be determined by methods well known in the art such as RT-PCR (preferably real-time RT-PCR), Northern blotting, or Western blotting. Preferably, the nucleic acid encoding the cS1P₁ receptor is operably linked to its native promoter or an S1P₁ promoter from a non-canis organism.

The method of the present invention can be used for high throughput screening (HTS) of analytes to identify analytes that bind cS1P₁ and/or are modulators of cS1P₁ receptor activity. Often chemical entities with useful properties are generated by identifying a chemical compound (called a "lead compound") with some desirable property or activity, creating variants of the lead compound, and evaluating the property and activity of those variant compounds. The current trend is to shorten the time scale for all aspects of drug discovery. Because of the ability to test large numbers quickly and efficiently, high throughput screening (HTS) methods are replacing conventional lead compound identification methods.

In one aspect, high throughput screening methods involve providing a library containing a large number of potential cS1P₁ modulators (candidate compounds). Such "combinatorial chemical libraries" are then screened in one or more of the assays described herein, to identify those library members particular chemical species or subclasses that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential cS1P₁ modulators.

Devices for the preparation of combinatorial libraries are commercially available (*See*, for example, 357 MPS, 390 MPS, Advanced Chem Tech, Louisville, KY; Symphony, Rainin, Woburn, MA; 433A Applied Biosystems, Foster City, CA; 9050 Plus, Millipore, Bedford, MA). A number of well known robotic systems have also been developed for solution phase chemistries. These systems include automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, MA; Orca, Hewlett-Packard, Palo Alto, CA) which mimic the manual synthetic operations performed by a chemist. Any of the above devices are suitable for use with the present invention. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein will be apparent to persons skilled in the relevant art. In addition, numerous combinatorial libraries are themselves commercially available (*See*, for example, ComGenex, Princeton, NJ; Asinex, Moscow, Russia; Tripos, Inc., St. Louis, MO.; ChemStar, Ltd, Moscow, Russia; 3D Pharmaceuticals, Exton, PA; Martek Biosciences, Columbia, MD).

Any of the assays described herein are amenable to high throughput screening. As described above, the cS1P₁ receptor modulators are preferably screened by the methods disclosed herein. High throughput systems for such screening are well known to those of skill in the art. Thus, for example, U.S. Pat. No. 5,559,410 discloses high throughput screening methods for protein binding, while
5 U.S. Pat. Nos. 5,576,220 and 5,541,061 disclose high throughput methods of screening for ligand/antibody binding.

In addition, high throughput screening systems are commercially available (*See*, for example, Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA). These systems typically automate entire
10 procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization. The manufacturers of such systems provide detailed protocols. Thus, for example, Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand
15 binding, and the like.

The following examples are intended to promote a further understanding of the present invention.

EXAMPLE 1

20 A full length nucleotide sequence encoding the cS1P₁ receptor was obtained by RT-PCR and RACE (rapid amplification of cDNA end) PCR methods using dog genomic DNA (Novagen) and cDNA libraries as templates. The cDNA libraries were prepared from dog heart and brain RNA using a SMART RACE cDNA Amplification kit (BD Bioscience). RACE primers were designed from a partial cS1P₁ receptor sequence (GenBank database accession number AY011741) and are listed in Table 1 as
25 CM012, CM012-r, CM001-r, and CM004. In the primer sequences shown, M is A or C, R is A or G, Y is C or T, S is C or G, and N is any nucleotide.

For RACE amplification, reagents from the SMART RACE cDNA Amplification Kit were combined with 200nM each primer (Qiagen) and 2.5uL of heart or brain cDNA. An Applied Biosystem GeneAmp PCR 9700 instrument was used with the following cycling conditions: 5 cycles at
30 94°C for 5 seconds and 72°C for 3 minutes; 5 cycles at 94°C for 5 seconds, 70°C for 10 minutes, and 72°C for 3 minutes; 25 cycles at 94°C for 5 seconds and 68°C for 10 seconds; and, 1 cycle at 72°C for 3 minutes. RACE amplification products were then used as templates to perform nested RACE reactions using primers CM012, CM012-r, CM001-r, CM004, and CM001 and the same cycling parameters as listed above. RACE products were cloned into the TA cloning vector pCR4-TOPO (Invitrogen) and

transformed into TOP10 E. coli cells (Invitrogen). Single colonies were inoculated into LB media with ampicillin and grown overnight at 37°C. DNA was purified from colonies using QIAPREP Turbo Miniprep Kit (QIAGEN). Plasmid DNA was sent for primer extension sequencing (SEQWRIGHT) using gene specific and universal primers. Sequence analysis, based on amino acid homology with the human S1P₁ gene, indicated a complete sequence at the 3' end including the stop codon and some 3' untranslated sequence, and an incomplete RACE product at the 5' end that did not include the predicted start codon.

To obtain the full length 5' end of the cS1P₁, degenerate primers D5p-1, D5p-2, D5p-3, and D5p-4r were designed upstream of the start codon based on the nucleotide alignment of the full length human, mouse, and rat S1P₁ sequences with the partial dog cS1P₁ sequence. PCR was performed using dog genomic DNA (Novagen) as template with the degenerate and RACE primers and Platinum Pfx enzyme (Invitrogen). PCR conditions were as follows: 1 cycle at 94°C for 30 seconds; 5 cycles at 94°C for 10 seconds and 72°C for 2 minutes; 5 cycles at 94°C for 30 seconds and 66°C for 2 minutes; 25 cycles at 94°C for 10 seconds, 63°C for 3 minutes, and 72°C for 1 minutes; and, 1 cycle at 72°C for 7 minutes. PCR products were cloned into the TA cloning vector pCR4-TOPO (Invitrogen) and transformed into TOP10 E. coli cells (Invitrogen). Single colonies were inoculated into LB media with ampicillin and grown overnight at 37°C. DNA was purified from colonies using QIAPREP Turbo Miniprep Kit (QIAGEN). Plasmid DNA was sent for primer extension sequencing (SEQWRIGHT).

The full length cS1P₁ gene had 1146 bp of nucleotide sequence shown as SEQ ID NO:1.

The 382 amino acid protein shown as SEQ ID NO:2 had greater than 90% identity to the human and rat S1P₁ receptors.

Table 1

Name	Sequence 5' to 3'	SEQ ID NO:	T _m	% GC	Location (in part seq)
CM001	GCAACAGCTTCCGCTCCTTCCTGC	3	70.5	62.5	316-339
CM001-r	GCAGGAAGGAGCGGAAGCTGTTGC	4	70.5	62.5	316-339
CM012	CATCATGGGCTGGAAGTGCATCGGCG	5	71.1	61.5	386-411
CM012-r	CGCCGATGCAGTTCCAGCCCATGATG	6	71.1	61.5	386-411
CM004-r	TCTTGCGGAAGGTCAGGCGGCGG	7	72.4	69.6	548-570
CM014-r	CAGGTCAGACAGGGCCAGGTTGC	8	70.6	65.2	106-129
CM015-r	GTACATAGGTCGGTGGAAGTTC	9	63.4	50.0	74-95
CM016-r	GCCAGGTTGCCGATGAAATAGTAC	10	65.4	50.0	92-114
D5p-1	GCCCTCTCCAGCCAAGGAAAARCTMC	11	69.6	57.7	upstream ATG
D5p-2	CCTCGCCCTCTAGCGTTYGYCTGGAG	12	72.7	65.4	upstream ATG
D5p-3	CTCRNCCTCGCCCTCTAGCGTT	13	69.0	63.6	upstream ATG
D5p-4r	SAACTATGATATCATMGTCGGGC	14	62.6	45.6	ATG
D5p-5r	CRGTGGTGTTTCATYCTCATCTGCTGC	15	68.0	53.8	-3-23
DE1-HF	cAAGCTTatgggggtccaccagcgtcc	16	71	61.5	-
D5r	CTCCCCCATGGGTCGGCGGCTTTCAGTTCTCGA G	17	76.7		

EXAMPLE 2

To obtain a clone encoding the entire cS1P₁ gene, dog genomic DNA was PCR amplified using primers DE1-HF and D5r (Table 1) designed from the sequencing information. PCR reactions with Pfu Turbo (Stratagene) polymerase were conducted using the following cycling conditions: 1 cycle at 94°C for 30 seconds; 5 cycles at 94°C for 30 seconds and 72°C for 2 minutes; 5 cycles at 94°C for 30 seconds and 70°C for 2 minutes; 25 cycles at 94°C for 30 seconds, 68°C for 1 minutes, and 72°C for 2 minutes; and, 1 cycle at 72°C for 7 minutes. The PCR product was cloned into the pCR2.1-TOPO cloning vector and transformed into TOP10 E. coli cells (Invitrogen). The clone chosen for expression

studies had one base pair change in the nucleotide sequence compared to the sequence shown in SEQ ID NO:1; however, the sequence encoded the same amino acid sequence as shown in SEQ ID NO:2. The plasmid DNA was cut with *EcoRI* and fragment containing cS1P₁ was ligated into pcDNA3.1 (+) zeocin. The sequence was confirmed and DNA was prepared using an endotoxin-free maxi-prep kit (QIAGEN).

Chinese Hamster Ovary (CHO) cells (about 1.6×10^6) were transfected with 6 μ g of DNA and 8 μ L of lipofectamine using a modified protocol from Life Technologies. After a 5 hour recovery, zeocin selection was added to the cells, which were split to pools the next day. Cells were subsequently split several times to enrich the population of zeocin resistant cells. Pools were screened for gene expression using a whole cell S1P binding assay. Membranes prepared from pools were screened for expression using the S1P binding assay and the GTP γ S binding assay.

EXAMPLE 3

The transfected CHO cells from Example 2 expressing the cS1P₁ receptor were evaluated for the ability of the cS1P₁ receptor expressed in the cells to properly integrate into the cell membrane and bind S1P.

Transfected CHO cells from Example 2 were harvested with enzyme-free dissociation solution (Specialty Media, Lavallete, NJ). The cells were washed once in cold PBS and suspended in S1P binding buffer (50mM HEPES-Na, pH 7.5, 5mM MgCl₂, 1mM CaCl₂, 0.5% FAF-BSA). ³³P-S1P was synthesized enzymatically from γ ³³P-ATP and sphingosine using a crude extract with sphingosine kinase activity in a reaction mix containing 50 mM KH₂PO₄, 1 mM mercaptoethanol, 1 mM Na₃VO₄, 25 mM KF, 2 mM semicarbazide, 1 mM Na₂EDTA, 5 mM MgCl₂, 50 mM sphingosine, 0.1% TritonX-114, and 1 mCi γ ³³P-ATP (NEN; specific activity 2000 Ci/mmol). Reaction products were extracted with butanol and ³³P-S1P was purified by HPLC.

³³P-S1P (0.1 uCi/ml) was sonicated with 0.2 nM S1P dissolved in binding buffer; 100 μ L of the mixture was added to 100 μ L cells (1×10^5). Binding was performed for 60 minutes at room temperature. Cells were then collected onto GF/B filter plates with a Packard Filtermate Universal Harvester. After drying the filter plates for 30 minutes, 50 μ L of MICROSCINT 20 was added to each well and binding was measured with a Packard Top Count. Non-specific binding was defined as the amount of radioactivity remaining in the presence of 200 nM cold S1P.

The results shown in Figure 3 show that transfected cells ectopically expressing the cS1P₁ receptor were able to bind the labeled S1P. As shown by the results in Figure 3, the EC₅₀ was about 0.37 nM. This indicates that the cS1P₁ receptor expressed in the CHO cells was properly integrated into the cell membrane and was able to bind the S1P.

Alternatively, ligand binding can be measured with membranes prepared from cells expressing cS1P₁. Membranes are prepared from transfected cells by homogenization in ice cold 20 mM HEPES pH 7.4, 10 mM EDTA using a Kinematica polytron (setting 5, for 10 seconds). Homogenates are centrifuged at 48,000 x g for 15 minutes at 4°C and the pellet suspended in 20 mM HEPES pH 7.4, 0.1 mM EDTA. Following a second centrifugation, the final pellet is suspended in 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM MgCl₂. The ³³P-S1P mixture is mixed with membranes diluted in binding buffer to a protein concentration of 5 to 25 µg/ml, and binding is performed as described for cells.

EXAMPLE 4

A cell-free assay was used to show that the cS1P₁ receptor ectopically expressed in CHO cells was able to functionally couple GTP to Gα.

Functional coupling of the cS1P₁ receptor to G proteins was measured in a ³⁵S-GTPγS binding assay. Membranes were prepared from transfected cells by homogenization in ice cold 20 mM HEPES pH 7.4, 10 mM EDTA using a Kinematica polytron (setting 5, for 10 seconds). Homogenates were centrifuged at 48,000 x g for 15 minutes at 4°C and the pellet suspended in 20 mM HEPES pH 7.4, 0.1 mM EDTA. Following a second centrifugation, the final pellet was suspended in 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM MgCl₂. Membranes were incubated in 200 µL with 5 µM GDP, various concentrations of S1P (sonicated in 1 % BSA), and 100 pM ³⁵S-GTPγS (NEN; specific activity 1250 Ci/mmol) in 96 well microtiter dishes. Binding was performed for one hour at room temperature, and terminated by harvesting the membranes onto GF/B filter plates with a Packard Filtermate Universal Harvester. After drying the filter plates for 30 min, 40 µL of MICROSCINT 20 was added to each well and binding was measured on a Packard Top Count scintillation counter.

The results shown in Figure 4 show that the cS1P₁ receptor in the presence of its ligand was capable of coupling GTP to Gα. As shown by the results in Figure 4, the EC₅₀ was about 0.42 nM.

This result indicates that the ectopically expressed the cS1P₁ receptor was functional.

EXAMPLE 5

A cell-based competition assay for detecting analytes that are competitor of S1P binding to cS1P₁ receptor and determining their affinity for the cS1P₁ receptor can be preformed as follows.

Transfected CHO cells from Example 2 are harvested with enzyme-free dissociation solution (Specialty Media, Lavallete, NJ). The cells were washed once in cold PBS and suspended in S1P binding buffer (50mM HEPES-Na, pH 7.5, 5mM MgCl₂, 1mM CaCl₂, 0.5% FAF-BSA). ³³P-SIP is synthesized enzymatically from γ³³P-ATP and sphingosine using a crude extract with sphingosine kinase

activity in a reaction mix containing 50 mM KH_2PO_4 , 1 mM mercaptoethanol, 1 mM Na_3VO_4 , 25 mM KF, 2 mM semicarbazide, 1 mM Na_2EDTA , 5 mM MgCl_2 , 50 mM sphingosine, 0.1% TritonX-114, and 1 mCi $\gamma^{33}\text{P}$ -ATP (NEN; specific activity 2000 Ci/mmol). Reaction products are extracted with butanol and ^{33}P -SIP is purified by HPLC.

5 ^{33}P -SIP (0.1 uCi/ml) is sonicated with 0.2 nM sphingosine-1-phosphate and analyte dissolved in binding buffer and 100 μL of the mixture is added to 100 μL cells (1×10^5) or membranes prepared from cells. Binding is performed for 60 minutes at room temperature. Cells are then collected onto GF/B filter plates with a Packard Filtermate Universal Harvester. After drying the filter plates for 30 minutes, 50 μL of MICROSCINT 20 is added to each well and binding is measured with a Packard Top
10 Count. Non-specific binding is defined as the amount of radioactivity remaining in the presence of 200 nM cold SIP. Controls consist of the above assay performed in the absence of the analyte and the above assay performed in the absence of the ^{33}P -SIP.

Determination of the amount of binding in the presence of varying concentrations of analyte and analysis of the data by PRISM software (GraphPad Software, Inc. San Diego, CA) is used to
15 measure the affinity of analytes for the cSIP₁ receptor. Specificity of analytes for the cSIP₁ receptor is determined by measuring the level of ^{33}P -SIP binding in the presence of the analyte to related SIP receptors (SIP₁, SIP₃, SIP₄, SIP₅, canis or non-canis) in similar binding assays using membranes prepared from cells transfected with each respective receptor.

20 EXAMPLE 6

A cell-free competition binding assay for detecting analytes that are competitors of SIP binding to the cSIP₁ receptor and determining the analytes' affinity for the cSIP₁ receptor is described.

Transfected CHO cells from Example 2 are harvested with enzyme-free dissociation solution (Specialty Media, Lavallete, NJ). ^{33}P -SIP is synthesized enzymatically from $\gamma^{33}\text{P}$ -ATP and
25 sphingosine using a crude extract with sphingosine kinase activity in a reaction mix containing 50 mM KH_2PO_4 , 1 mM mercaptoethanol, 1 mM Na_3VO_4 , 25 mM KF, 2 mM semicarbazide, 1 mM Na_2EDTA , 5 mM MgCl_2 , 50 mM sphingosine, 0.1% TritonX-114, and 1 mCi $\gamma^{33}\text{P}$ -ATP (NEN; specific activity 2000 Ci/mmol). Reaction products are extracted with butanol and ^{33}P -SIP is purified by HPLC.

Membranes are prepared from transfected by homogenization in ice cold 20 mM HEPES
30 pH 7.4, 10 mM EDTA using a Kinematica polytron (setting 5, for 10 seconds). Homogenates are centrifuged at 48,000 x g for 15 minutes at 4°C and the pellet suspended in 20 mM HEPES pH 7.4, 0.1 mM EDTA. Following a second centrifugation, the final pellet is suspended in 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM MgCl_2 . Membranes are incubated in 200 μL of ^{33}P -SIP (0.1 uCi/ml) sonicated with 0.2 nM sphingosine-1-phosphate and analyte dissolved in binding buffer. Binding is performed for

60 minutes at room temperature and terminated by harvesting the membranes onto GF/B filter plates with a Packard Filtermate Universal Harvester. After drying the filter plates for 30 min, 40 μ L of MICROSCINT 20 is added to each well and binding is measured on a Packard Top Count scintillation counter. Controls consist of the above assay performed in the absence of the analyte and the above assay performed in the absence of the 33 -P-S1P.

Determination of the amount of binding in the presence of varying concentrations of analyte and analysis of the data by PRISM software (GraphPad Software, Inc. San Diego, CA) is used to measure the affinity of analytes for the cS1P₁ receptor. Specificity of analytes for the cS1P₁ receptor is determined by measuring the level of 33 P-S1P binding in the presence of the analyte to related S1P receptors (S1P₂, S1P₃, S1P₄, S1P₅, canis or non-canis) in similar binding assays using membranes prepared from cells transfected with each respective receptor.

EXAMPLE 7

A cell-free assay for determining whether an analyte is an agonist or an antagonist and its affinity for the cS1P₁ receptor can use the 35 S-GTP γ S binding assay of Example 4 as follows.

Membranes are prepared from transfected cells (made as in Example 2) by homogenization in ice cold 20 mM HEPES pH 7.4, 10 mM EDTA using a Kinematica polytron (setting 5, for 10 seconds). Homogenates are centrifuged at 48,000 x g for 15 minutes at 4°C and the pellet suspended in 20 mM HEPES pH 7.4, 0.1 mM EDTA. Following a second centrifugation, the final pellet is suspended in 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM MgCl₂. Membranes are incubated in 200 μ L with 5 μ M GDP, various concentrations of analyte in DMSO, methanol, or other solvent (preferably, sonicated in 1 % BSA), and 100 pM 35 S-GTP γ S (NEN; specific activity 1250 Ci/mmol) in 96 well microtiter dishes. Binding is performed for one hour at room temperature and terminated by harvesting the membranes onto GF/B filter plates with a Packard Filtermate Universal Harvester. After drying the filter plates for 30 min, 40 μ L of MICROSCINT 20 is added to each well and binding is measured on a Packard Top Count scintillation counter. Controls consist of performing the above assay in the absence of S1P to determine the endogenous level of GTP γ S binding.

Determination of the amount of binding in the presence of varying concentrations of compound and analysis of the data by PRISM software (GraphPad) is used to measure the affinity of compounds for the cS1P₁ receptor. Specificity of compounds for the cS1P₁ receptor is determined by measuring the level of 35 S-GTP γ S binding in the presence of the analyte to other G protein coupled receptors in similar binding assays using membranes prepared from cells transfected with each respective receptor. When the method is performed in the absence of S1P, analytes that stimulate labeled GTP γ S binding above the endogenous level are agonists while compounds that inhibit the endogenous level of

labeled GTP γ S are considered inverse agonists. On the other hand, antagonists are detected in a labeled GTP γ S binding assay in the presence of a submaximal level of S1P or other known agonist where they reduce the labeled GTP γ S binding that is stimulated by the agonist.

EXAMPLE 8

This example describes a method for making polyclonal antibodies specific for the cS1P₁ receptor or particular peptide fragments or epitope thereof.

The cS1P₁ receptor is produced in *E. coli* transformed with the vector of Example 2. Antibodies are generated in New Zealand white rabbits over a 10-week period. The cS1P₁ receptor or peptide fragment or epitope thereof is emulsified by mixing with an equal volume of Freund's complete adjuvant and injected into three subcutaneous dorsal sites for a total of about 0.1 mg S1P₁ receptor per immunization. A booster containing about 0.1 mg cS1P₁ receptor emulsified in an equal volume of Freund's incomplete adjuvant is administered subcutaneously two weeks later. Animals are bled from the articular artery. The blood is allowed to clot and the serum collected by centrifugation. The serum is stored at -20°C.

For purification, the cS1P₁ receptor is immobilized on an activated support. Antisera is passed through the sera column and then washed. Specific antibodies are eluted via a pH gradient, collected, and stored in a borate buffer (0.125M total borate) at -0.25 mg/mL. The anti-cS1P₁ receptor antibody titers are determined using ELISA methodology with free cS1P₁ receptor bound in solid phase (1 pg/well). Detection is obtained using biotinylated anti-rabbit IgG, HRP-SA conjugate, and ABTS.

EXAMPLE 9

This example describes a method for making monoclonal antibodies specific for the cS1P₁ receptor.

BALB/c mice are immunized with an initial injection of about 1 μ g of purified cS1P₁ receptor per mouse mixed 1:1 with Freund's complete adjuvant. After two weeks, a booster injection of about 1 μ g of the antigen is injected into each mouse intravenously without adjuvant. Three days after the booster injection serum from each of the mice is checked for antibodies specific for the S1P₁ receptor.

The spleens are removed from mice positive for antibodies specific for the cS1P₁ receptor and washed three times with serum-free DMEM and placed in a sterile Petri dish containing about 20 mL of DMEM containing 20% fetal bovine serum, 1 mM pyruvate, 100 units penicillin, and 100 units streptomycin. The cells are released by perfusion with a 23 gauge needle. Afterwards, the cells are pelleted by low-speed centrifugation and the cell pellet is resuspended in 5 mL 0.17 M ammonium

chloride and placed on ice for several minutes. Then 5 mL of 20% bovine fetal serum is added and the cells pelleted by low-speed centrifugation. The cells are then resuspended in 10 mL DMEM and mixed with mid-log phase myeloma cells in serum-free DMEM to give a ratio of 3:1. The cell mixture is pelleted by low-speed centrifugation, the supernatant fraction removed, and the pellet allowed to stand for 5 minutes. Next, over a period of 1 minute, 1 mL of 50% polyethylene glycol (PEG) in 0.01 M HEPES, pH 8.1, at 37°C is added. After 1 minute incubation at 37°C, 1 mL of DMEM is added for a period of another 1 minute, then a third addition of DMEM is added for a further period of 1 minute. Finally, 10 mL of DMEM is added over a period of 2 minutes. Afterwards, the cells are pelleted by low-speed centrifugation and the pellet resuspended in DMEM containing 20% fetal bovine serum, 0.016 mM thymidine, 0.1 hypoxanthine, 0.5 μ M aminopterin, and 10% hybridoma cloning factor (HAT medium). The cells are then plated into 96-well plates.

After 3, 5, and 7 days, half the medium in the plates is removed and replaced with fresh HAT medium. After 11 days, the hybridoma cell supernatant is screened by an ELISA assay. In this assay, 96-well plates are coated with the cS1P₁ receptor. One hundred μ L of supernatant from each well is added to a corresponding well on a screening plate and incubated for 1 hour at room temperature. After incubation, each well is washed three times with water and 100 μ L of a horseradish peroxidase conjugate of goat anti-mouse IgG (H+L), A, M (1:1,500 dilution) is added to each well and incubated for 1 hour at room temperature. Afterwards, the wells are washed three times with water and the substrate OPD/hydrogen peroxide is added and the reaction is allowed to proceed for about 15 minutes at room temperature. Then 100 μ L of 1 M HCl is added to stop the reaction and the absorbance of the wells is measured at 490 nm. Cultures that have an absorbance greater than the control wells are removed to two cm² culture dishes, with the addition of normal mouse spleen cells in HAT medium. After a further three days, the cultures are re-screened as above and those that are positive are cloned by limiting dilution. The cells in each two cm² culture dish are counted and the cell concentration adjusted to 1×10^5 cells per mL. The cells are diluted in complete medium and normal mouse spleen cells are added. The cells are plated in 96-well plates for each dilution. After 10 days, the cells are screened for growth. The growth positive wells are screened for antibody production; those testing positive are expanded to 2 cm² cultures and provided with normal mouse spleen cells. This cloning procedure is repeated until stable antibody producing hybridomas are obtained. The stable hybridomas are progressively expanded to larger culture dishes to provide stocks of the cells.

Production of ascites fluid is performed by injecting intraperitoneally 0.5 mL of pristane into female mice to prime the mice for ascites production. After 10 to 60 days, 4.5×10^6 cells are injected intraperitoneally into each mouse and ascites fluid is harvested between 7 and 14 days later.

While the present invention is described herein with reference to illustrated embodiments, it should be understood that the invention is not limited hereto. Those having ordinary

skill in the art and access to the teachings herein will recognize additional modifications and embodiments within the scope thereof. Therefore, the present invention is limited only by the claims attached herein.

WHAT IS CLAIMED:

1. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a canis sphingosine-1-phosphate isoform 1 (cS1P₁) receptor or fragment thereof.

2. The isolated nucleic acid of Claim 1 wherein the cS1P₁ receptor or fragment thereof comprises an amino acid sequence of SEQ ID NO:2.

3. The isolated nucleic acid of Claim 1 wherein the nucleic acid is a DNA.

4. The isolated nucleic acid of Claim 1 wherein the nucleic acid is an RNA.

5. The isolated nucleic acid of Claim 1 wherein the nucleic acid is a cDNA.

6. The isolated nucleic acid of Claim 1 wherein the nucleotide sequence comprises a nucleotide sequence of SEQ ID NO:1.

7. An isolated protein comprising the amino acid sequence or part thereof of SEQ ID NO:2.

8. An antibody which binds a protein comprising the amino acid sequence or part thereof of SEQ ID NO:2.

9. A vector comprising a nucleic acid encoding a canis sphingosine-1-phosphate receptor isoform 1 (cS1P₁) receptor or fragment thereof.

10. The vector of Claim 9 wherein the S1P₁ receptor or fragment thereof comprises an amino acid sequence of SEQ ID NO:2.

11. A gene expression cassette comprising a nucleic acid encoding a canis sphingosine-1-phosphate receptor isoform 1 (cS1P₁) receptor or fragment thereof, having an amino acid sequence of SEQ ID NO:2 operably linked to a heterologous promoter.

12. A cell comprising a nucleic acid encoding a canis sphingosine-1-phosphate receptor isoform 1 (cS1P₁) receptor or fragment thereof.

13. The cell of Claim 12 wherein the cS1P₁ receptor or fragment thereof comprises an amino acid sequence of SEQ ID NO:2.

5 14. The cell of Claim 12 wherein the nucleic acid is operably linked to a heterologous promoter.

15. The cell of Claim 12 wherein the nucleic acid is integrated into the genome of the cell.

10 16. A method for producing a canis sphingosine-1-phosphate isoform 1 (cS1P₁) receptor comprising:

(a) providing a nucleic acid encoding the cS1P₁ receptor operably linked to a heterologous promoter;

15 (b) introducing the nucleic acid into a cell to produce a recombinant cell; and

(c) culturing the recombinant cell under conditions which allows expression of the cS1P₁ receptor encoded by the nucleic acid to produce the cS1P₁ receptor.

20 17. The method of Claim 16 wherein nucleic acid is integrated into the genome of the recombinant cell.

18. The method of Claim 16 or 17 wherein the cS1P₁ receptor comprises the amino acid sequence of SEQ ID NO:2.

25 19. A method for identifying an analyte which binds to a canis sphingosine-1-phosphate receptor isoform 1 (cS1P₁) receptor, which comprises:

(a) providing a recombinant cell which produces the cS1P₁ receptor;

(b) incubating the recombinant cell in a medium with the analyte; and

30 (c) determining the amount of analyte bound to the recombinant cell.

20. The method of Claim 19 wherein the cS1P₁ comprises the amino acid sequence of SEQ ID NO:2.

21. The method of Claim 19 wherein the cS1P1 is encoded by a nucleic acid which is integrated into the genome of the recombinant cell.

22. The method of Claim 19 wherein the medium comprises the analyte and a labeled sphingosine-1-phosphate (SIP).

23. The method of Claim 19 or 22 wherein the analyte is labeled.

24. A method for determining whether an analyte is a canis sphingosine-1-phosphate receptor isoform 1 (cS1P1) receptor agonist, which comprises:

(a) providing a membrane which has the cS1P1 receptor integrated therein;
(b) incubating the membrane in the presence of the analyte and labeled GTP for a time sufficient for the labeled GTP to be associated with the membrane when the analyte is an agonist; and

(c) separating the membrane from unbound labeled GTP and determining the amount of labeled GTP associated with the membrane wherein presence of the labeled GTP associated with the membrane indicates that the analyte is an agonist.

25. The method of Claim 24 wherein the cS1P1 comprises the amino acid sequence of SEQ ID NO:2.

26. The method of Claim 24 wherein the membrane is provided by a recombinant cell comprising a nucleic acid encoding the cS1P1.

27. The method of Claim 24 wherein the medium comprises the analyte and a labeled sphingosine-1-phosphate (SIP).

28. The method of Claim 24 or 27 wherein the analyte is labeled.

29. A method for identifying an analyte that modulates activity of a canis sphingosine-1-phosphate receptor isoform 1 (cS1P1) receptor, which comprises:

(a) providing a recombinant cell which produces the cS1P1 receptor;
(b) incubating the recombinant cell in a medium with the analyte; and
(c) determining the activity of the cS1P1 receptor wherein a change in the activity of the cS1P1 receptor indicates the analyte modulates activity of the cS1P1 receptor.

30. The method of Claim 29 wherein the activity of the cS1P₁ is determined by measuring a change in the intracellular concentration of Ca²⁺ in the presence of the analyte.

5 31. The method of Claim 29 wherein the activity of the cS1P₁ is determined by measuring a change in the intracellular concentration of a metabolite selected from the group consisting of inositol triphosphate (IP₃) and diacylglycerol (DAG) in the presence of the analyte.

10 32. The method of Claim 29 wherein the activity of the cS1P₁ is determined by measuring a change in the activity of phospholipase C beta (PLC β) or protein kinase C (PKC) in the presence of the analyte.

33. The method of any one of Claims 29 to 32 wherein the recombinant cell further produces a chimeric protein G or a promiscuous G protein.

15 34. The method of Claim 29 wherein the activity of the cS1P₁ is determined by measuring a change in the synthesis of cyclic AMP (cAMP) in the presence of the analyte.

20 35. The method of Claim 29 wherein the cS1P₁ comprises the amino acid sequence of SEQ ID NO:2.

36. A method for screening for analytes with an immunosuppressive activity useful for treating autoimmune and inflammatory diseases, as a component of a chemotherapeutic regimen for treating cancers, or a regimen for inhibiting tissue rejection of transplants in a mammal, which comprises:

25 (a) determining activity of canis sphingosine-1-phosphate receptor isoform 1 (cS1P₁) receptor in the presence of a particular concentration of the analyte or in the absence of the analyte, and

(b) determining the activity of the cS1P₁ receptor at a different concentration of the analyte.

30 37. The method of Claim 36 wherein the activity is determined in a cell-based assay.

38. The method of Claim 36 wherein the activity is determined in a cell-free assay.

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FIG. 1

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FIG. 2

2/2

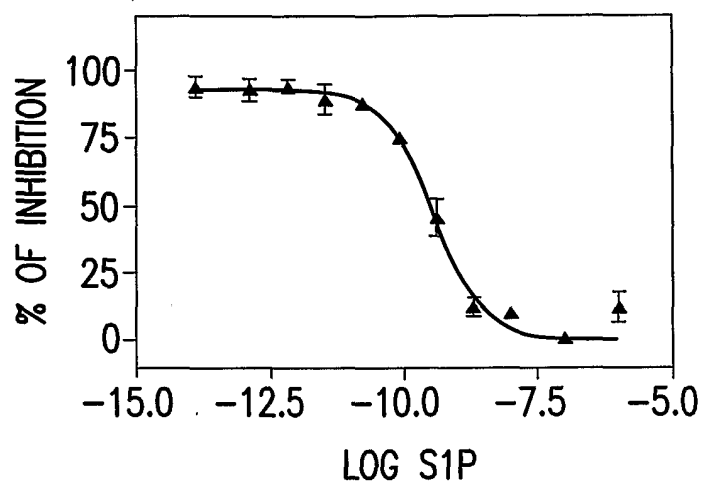
dogEDG1
S1P BINDING

FIG.3

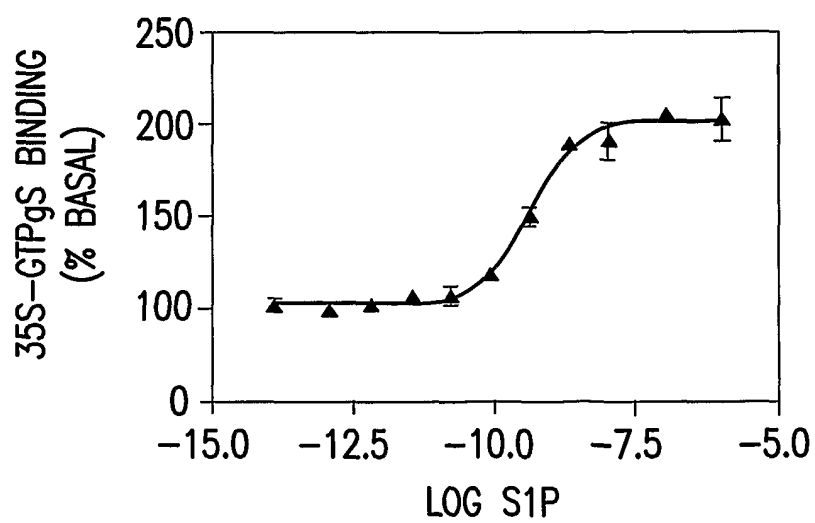
dog EDG1
GTP γ S BINDING

FIG.4

SEQUENCE LISTING

<110> Merck & Co., Inc.
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Meyers, Cheryl
Shei, Gan Ju

<120> CANIS SPHINGOSINE 1-PHOSPHATE RECEPTOR
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<130> PCT 21433

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35 40 45
Ser Val Val Phe Ile Leu Ile Cys Cys Phe Ile Ile Leu Glu Asn Ile
50 55 60
Phe Val Leu Leu Thr Ile Trp Lys Thr Lys Lys Phe His Arg Pro Met
65 70 75 80
Tyr Tyr Phe Ile Gly Asn Leu Ala Leu Ser Asp Leu Leu Ala Gly Val
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Ala Tyr Thr Ala Asn Leu Leu Leu Ser Gly Ala Thr Thr Tyr Lys Leu
100 105 110
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Thr Met Leu Lys Met Lys Leu His Asn Gly Ser Asn Ser Phe Arg Ser
145      150      155      160
Phe Leu Leu Ile Ser Ala Cys Trp Val Ile Ser Leu Val Leu Gly Gly
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Leu Pro Ile Met Gly Trp Asn Cys Ile Gly Ala Leu Ala Ser Cys Ser
      180      185      190
Thr Val Leu Pro Leu Tyr His Lys His Tyr Ile Leu Phe Cys Thr Thr
      195      200      205
Val Phe Thr Leu Leu Leu Leu Ala Ile Val Ile Leu Tyr Cys Arg Ile
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Tyr Ser Leu Val Arg Thr Arg Ser Arg Arg Leu Thr Phe Arg Lys Asn
225      230      235      240
Ile Ser Lys Ala Ser Arg Ser Ser Glu Lys Ser Leu Ala Leu Leu Lys
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Thr Val Ile Ile Val Leu Ser Val Phe Ile Ala Cys Trp Ala Pro Leu
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Phe Ile Leu Leu Leu Leu Asp Val Gly Cys Lys Val Lys Thr Cys Asp
      275      280      285
Ile Leu Phe Arg Ala Glu Tyr Phe Leu Val Leu Ala Val Leu Asn Ser
      290      295      300
Gly Thr Asn Pro Ile Ile Tyr Thr Leu Thr Asn Lys Glu Met Arg Arg
305      310      315      320
Ala Phe Ile Arg Ile Leu Ser Cys Cys Lys Cys Pro Gly Gly Asp Pro
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Ala Gly Lys Phe Lys Arg Pro Ile Ile Ala Gly Val Glu Phe Ser Arg
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