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(54) Title: METHOD OF MODULATING CELLULAR ACTIVITY INVOLVING SPHINGOSINE KINASE AND AGENTS FOR SAME, AND SPHINGOSINE KINASE VARIANTS

(57) Abstract: The present invention relates generally to a method of modulating cellular activity and to agents for use therein. More particularly, the present invention provides a method of modulating cellular activity by modulating intracellular translocation of sphingosine kinase to the cell membrane. In a related aspect, the present invention provides a method of modulating sphingosine kinase mediated signalling via modulation of its intracellular translocation and agents for use therein. The present invention still further extends to sphingosine kinase variants and to functional derivatives, homologues and analogues thereof, exhibiting ablated or reduced capacity to undergo translocation. The method and molecules of the present invention are useful, inter alia, in the treatment and/or prophylaxis of conditions characterised by aberrant, unwanted or otherwise inappropriate cellular functional activity and/or aberrant, unwanted or otherwise inappropriate sphingosine kinase mediated signalling. The present invention is further directed to methods for identifying and/or designing agents capable of modulating sphingosine kinase intracellular translocation.

Methods of Modulating Cellular Activity Involving Sphingosine Kinase and Agents for Same, and Sphingosine Kinase Variants.

FIELD OF THE INVENTION

The present invention relates generally to a method of modulating cellular activity and to agents for use therein. More particularly, the present invention provides a method of modulating cellular activity by modulating intracellular translocation of sphingosine kinase to the cell membrane. In a related aspect, the present invention provides a method of modulating sphingosine kinase mediated signalling via modulation of its intracellular translocation and agents for use therein. The present invention still further extends to sphingosine kinase variants and to functional derivatives, homologues and analogues thereof, exhibiting ablated or reduced capacity to undergo translocation. The method and molecules of the present invention are useful, *inter alia*, in the treatment and/or prophylaxis of conditions characterised by aberrant, unwanted or otherwise inappropriate cellular functional activity and/or aberrant, unwanted or otherwise inappropriate sphingosine kinase mediated signalling. The present invention is further directed to methods for identifying and/or designing agents capable of modulating sphingosine kinase intracellular translocation.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications referred to by author in this specification are collected alphabetically at the end of the description.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

Sphingosine kinases catalyze the formation of sphingosine 1-phosphate (S1P), a bioactive lipid that regulates a diverse range of cellular processes, including cell growth, survival,

differentiation, motility, and cytoskeletal organization (Pyne *et al.*, 2000, *Biochem. J.* 349:385-402; Spiegel *et al.*, 2002, *J. Biol. Chem.* 277:25851-25854). Some of these cellular processes are mediated by five S1P-specific G protein-coupled receptors (Kluk *et al.*, 2002, *Biochim. Biophys. Acta* 1582:72-80; Spiegel *et al.*, 2002, *Trends Cell. Biol.* 12:236-242), while other effects appear controlled by intracellular S1P.

S1P is mitogenic in various cell types and triggers a diverse range of important regulatory pathways including; mobilisation of intracellular calcium by an inositol triphosphate independent pathway (Mattie, M *et al.* (1994) *J Biol Chem* 269, 3181-3188), activation of phospholipase D (Desai *et al.*, 1992, *J Biol Chem* 267, 23122-23128), inhibition of c-Jun N-terminal kinase (JNK) (Cuvilliver *et al.*, 1998, *J Biol Chem* 273, 2910-2916), inhibition of caspases (Cuvilliver *et al.* 1998, *supra*), adhesion molecule expression (Xia *et al.*, 1998, *Proc Natl Acad Sci USA* 95, 14196-14201), and stimulation of DNA binding activity of NF-κB (Xia *et al.*, 2002, *J. Biol. Chem.* 277:7996-8003) and transcription factor activator protein-1 (AP-1) (Su *et al.*, 1994, *J Biol Chem* 269, 16512-16517).

Cellular levels of S1P are largely mediated by the activity of sphingosine kinase, and to a lesser extent by its degradation by S1P lyase (Van Veldhoven *et al.*, 2000, *Biochim Biophys Acta* 1487, 128-134) and S1P phosphatase (Mandala *et al*, 2000, *Proc Natl Acad Sci USA* 97, 7859-7964) activities. Basal levels of S1P in the cell are generally low (Spiegel *et al.*, 1998, *Ann N Y Acad Sci* 845, 11-18), but can increase rapidly and transiently when cells are exposed to various mitogenic agents. This response is a direct consequence of an increase in sphingosine kinase activity in the cytosol and can be prevented by the addition of sphingosine kinase inhibitors. This places sphingosine kinase, and its activation, in a central and obligatory role in mediating the observed effects attributed to S1P in the cell. However, at present almost nothing is known of the mechanism(s) leading to sphingosine kinase activation.

Sphingosine kinase can be very rapidly activated by a wide variety of cell agonists. While the response differs between cell types, these stimuli include TNFα (Xia et al. 1998, *supra*); Pitson *et al., 2000, Biochem. J.* 350:429–441) (Fig 1), platelet-derived growth

factor (Olivera *et al.*, 1993, *Nature* 365, 557-560), epidermal growth factor (Meyer zu Heringdorf *et al.*, 1998, *EMBO J* 17, 2830-2838), nerve growth factor (Rius *et al.*, 1997, *FEBS Lett* 417, 173-176), vitamin D3 (Kleuser *et al.*, 1998, *Cancer Res* 58, 1817-1823), phorbol esters (Pitson *et al.* 2000, *supra*; Buehrer *et al.* 1996), acetylcholine (muscarinic agonists) (Meyer zu Heringdorf *et al.* 1998, *supra*), and crosslinking of the immunoglobulin receptors FcεR1 (Choi *et al.*, 1996, *Nature* 380, 634-639) and FcγR1 (Melendez *et al.*, 1998, *J Biol Chem* 273, 9393-9402). In all cases this sphingosine kinase activation increases the V_{max} of the reaction while leaving the substrate affinities (K_m) unaltered.

Two human sphingosine kinase isoforms exist (1 and 2), which differ in their tissue distribution, developmental expression, catalytic properties, and somewhat in their substrate specificity (Pitson *et al., 2000, supra*; Liu *et al.*, 2000, *J. Biol. Chem.* 275:19513-19520). A number of studies have shown the effects of sphingosine kinase 1 in enhancing cell proliferation and suppressing apoptosis (Olivera *et al.*, 1999, *J. Cell Biol.* 147:545–558; Xia *et al.*, 2000, *Curr. Biol.* 10:1527–1530; Edsall *et al.*, 2001, *J. Neurochem.* 76:1573-1584). Furthermore, overexpression of human sphingosine kinase 1 (hSK1) in NIH3T3 fibroblasts results in acquisition of the transformed phenotype and the ability to form tumors in nude mice, demonstrating the oncogenic potential of this enzyme (Xia *et al.*, 2000, *supra*). More recent work has shown the involvement of hSK1 in estrogen-dependent regulation of breast tumor cell growth and survival (Nava *et al.*, 2002, *Expt. Cell Res.* 281:115-127; Sukocheva *et al.*, 2003, *Mol. Endocrinol.* 17:2002-2012), while other studies have shown elevated hSK1 mRNA in a variety of human solid tumors and inhibition of tumor growth in vivo by sphingosine kinase inhibitors (French *et al.*, 2003, *Cancer Res.* 63:5962-5969).

Thus, the involvement of hSK1 in cell growth, survival and tumorigenesis is now well established. Less clear, however, are the mechanisms by which hSK1 brings about these effects. Recent studies have indicated it is independent of G protein-coupled receptors (Olivera *et al.*, 2003, *J. Biol. Chem.* 278:46452–46460), suggesting these effects are mediated solely by intracellular S1P levels and the associated, but as yet unidentified,

intracellular targets. While these direct targets are unknown, hSK1 has been implicated in a number of pro-proliferative and pro-survival pathways, such as activation of ERK1/2 (Pitson *et al.*, 2000, *J. Biol. Chem.* 275:33945–33950; Shu *et al.*, 2002, *Mol. Cell. Biol.* 22:7758-7768), phosphatidylinositol-3-kinase (Osawa *et al.*, 2001, *J. Immunol.* 167:173-180) and NF-(B (Xia *et al.*, 2002, *supra*), and inhibition of caspase activation (Edsall *et al.*, 2001, *supra*).

Accordingly, as detailed above, although the central role of sphingosine kinase in the context of its regulation of a wide variety of cellular activities is well established, the precise mechanisms by which this occurs have been only partially determined. Accordingly, there is an ongoing need to elucidate those mechanisms in order to provide better means for developing methods of regulating cellular activities via regulation of the sphingosine kinase signalling pathway.

In work leading up to the present invention, the inventors have surprisingly determined that although activation of sphingosine kinase is induced by its phosphorylation, the subsequent increase in catalytic activity of the phosphorylated sphingosine kinase molecule is not the only regulatory event which enables sphingosine kinase mediated cellular functioning to occur. Rather, it has been determined that the phosphorylation induced intracellular translocation of sphingosine kinase is crucial in this regard. However, most unexpectedly, particularly in light of the fact that phosphorylation of sphingosine increases the level of its intrinsic catalytic activity, it has been determined that modulation of sphingosine kinase mediated cellular activities can be effected merely by modulating the intracellular translocation of the sphingosine kinase molecule, *irrespective of* the phosphorylation state of the sphingosine kinase molecule. Still further, the site of the sphingosine kinase molecule which binds the translocation mediator calmodulin has also now been identified and characterised. These findings have now enabled the development of simple and streamlined methods of modulating sphingosine kinase mediated cellular functioning based on regulating its intracellular translocation, irrespective of its phosphorylation status. Accordingly, this has provided for the development of highly effective methods for therapeutically or prophylactically treating

conditions characterised by unwanted or inappropriate cellular functioning, in particular inappropriate cellular proliferation such as neoplastic proliferation.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The subject specification contains nucleotide and amino acid sequence information prepared using the programme PatentIn Version 3.1, presented herein after the bibliography. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (eg. <210>1, <210>2, etc). The length, type of sequence (DNA, protein, etc) and source organism for each nucleotide or amino acid sequence is indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are identified by the indicator SEQ ID NO: followed by the sequence identifier (eg. SEQ ID NO:1, SEQ ID NO:2, etc.). The sequence identifier referred to in the specification correlates to the information provided in numeric indicator field <400> in the sequence listing, which is followed by the sequence identifier (eg. <400>1, <400>2, etc). That is SEQ ID NO:1 as detailed in the specification correlates to the sequence indicated as <400>1 in the sequence listing

Specific mutations in amino acid sequence are represented herein as "Xaa_1nXaa_2" where Xaa_1 is the original amino acid residue before mutation, n is the residue number and Xaa_2 is the mutant amino acid. The abbreviation "Xaa" may be the three letter or single letter amino acid code. A mutation in single letter code is represented, for example, by X_1nX_2 where X_1 and X_2 are the same as Xaa_1 and Xaa_2 respectively. In terms of both the mutation and the human sphingosine kinase protein sequence in general, the amino acid residues for human sphingosine kinase 1 are numbered with the residue phenylalamine (F) in the motif RFTLGTFLRLAALRTY of SEQ ID NO:2 being numbered 197.

One aspect of the present invention provides a method of modulating sphingosine kinase

mediated signalling, said method comprising contacting sphingosine kinase with an effective amount of an agent for a time and under conditions sufficient to modulate the intracellular localisation of said sphingosine kinase wherein upregulating sphingosine kinase cell membrane localisation upregulates said signalling and downregulating said sphingosine kinase cell membrane localisation downregulates said signalling.

Another aspect of the present invention provides a method of modulating human sphingosine kinase 1 mediated signalling, said method comprising contacting said human sphingosine kinase 1 with an effective amount of an agent for a time and under conditions sufficient to modulate intracellular localisation of said human sphingosine kinase 1 wherein upregulating said sphingosine kinase cell membrane localisation upregulates said signalling and downregulating said sphingosine kinase cell membrane localisation downregulates said signalling.

Yet another aspect of the present invention provides a method of modulating human sphingosine kinase 2 mediated signalling, said method comprising contacting said human sphingosine kinase 2 with an effective amount of an agent for a time and under conditions sufficient to modulate intracellular localisation of said human sphingosine kinase 2 wherein upregulating said sphingosine kinase cell membrane localisation upregulates said signalling and downregulating said sphingosine kinase cell membrane localisation downregulates said signalling.

In still another aspect there is provided a method of modulating human sphingosine kinase mediated signalling, said method comprising contacting said human sphingosine kinase with an effective amount of an agent for a time and under conditions sufficient to modulate intracellular localisation of said human sphingosine kinase wherein upregulating said sphingosine kinase cell membrane localisation upregulates said signalling and downregulating said sphingosine kinase cell membrane localisation downregulates said signalling and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

Still yet another aspect of the present invention provides a method of modulating human sphingosine kinase mediated signalling, said method comprising contacting said human sphingosine kinase with an effective amount of an agent for a time and under conditions sufficient to modulate the interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2, wherein inducing or agonising said interaction upregulates sphingosine kinase cell membrane localisation and antagonising said interaction downregulates sphingosine kinase cell membrane localisation and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

Yet still another aspect of the present invention is directed to a method of modulating sphingosine kinase mediated cellular activity, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate the intracellular localisation of sphingosine kinase wherein upregulating sphingosine kinase cell membrane localisation upregulates said cellular activity and downregulating sphingosine kinase cell membrane localisation downregulates said cellular activity.

A further aspect of the present invention is directed to a method of modulating sphingosine kinase mediated cellular activity, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate the interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2, wherein inducing or agonising said interaction upregulates sphingosine kinase cell membrane localisation and antagonising said interaction downregulates sphingosine kinase cell membrane localisation and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

Yet another further aspect of the present invention is directed to a method for the treatment and/or prophylaxis of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase mediated cellular activity, said method comprising administering to said mammal an effective amount of an

agent for a time and under conditions sufficient to modulate intracellular localisation of sphingosine kinase wherein upregulating sphingosine kinase cell membrane localisation upregulates said cellular activity and downregulating sphingosine kinase cell membrane localisation downregulates said cellular activity.

Still another further aspect of the present invention is directed to a method for the treatment and/or prophylaxis of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase functional activity, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate intracellular localisation of sphingosine kinase wherein upregulating sphingosine kinase cell membrane localisation upregulates said sphingosine kinase activity and downregulating sphingosine kinase cell membrane localisation downregulates said sphingosine kinase activity.

Yet still another further aspect of the present invention is directed to a method for the treatment and/or prophylaxis of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise in appropriate sphingosine kinase mediated cellular activity, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2, wherein inducing or agonising said interaction upregulates sphingosine kinase membrane localisation and antagonising said interaction downregulates sphingosine kinase cell membrane localisation and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

Still another aspect of the present invention is directed to a method for the treatment and/or prophylaxis of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase functional activity, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient modulate the to interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2, wherein inducing

or agonising said interaction upregulates sphingosine kinase cell membrane localisation and antagonising said interaction downregulates sphingosine kinase cell membrane localisation and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

Another aspect of the present invention contemplates a method for the treatment and/or prophylaxis of a condition characterised by aberrant, unwanted or otherwise inappropriate cell growth in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the intracellular localisation of sphingosine kinase wherein downregulating sphingosine kinase cell membrane localisation downregulates the subject cell growth.

In yet another aspect the present invention contemplates a method for the treatment and/or prophylaxis of a condition characterised by aberrant, unwanted or otherwise inappropriate cell growth in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to antagonise the interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2, wherein antagonising said interaction downregulates sphingosine kinase cell membrane localisation and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

Still yet another aspect of the present invention contemplates the use of an agent, as hereinbefore defined, in the manufacture of medicament for the treatment of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase mediated cellular activity, wherein said agent modulates the intracellular localisation of sphingosine kinase and wherein upregulating sphingosine kinase cell membrane localisation upregulates said cellular activity and downregulating sphingosine kinase cell membrane localisation down regulates said cellular activity.

Still another aspect of the present invention contemplates the use of an agent, as hereinbefore defined, in the manufacture of medicament for the treatment of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase mediated signalling, wherein said agent modulates the intracellular localisation of sphingosine kinase and wherein upregulating sphingosine kinase cell membrane localisation upregulates said sphingosine kinase mediated signalling and downregulating sphingosine kinase cell membrane localisation downregulates said sphingosine kinase mediated signalling.

A further aspect of the present invention provides a method for detecting an agent capable of modulating the intracellular localisation of sphingosine kinase or its functional equivalent or derivative thereof said method comprising contacting a cell or extract thereof containing said sphingosine kinase or its functional equivalent or derivative with a putative agent and detecting an altered expression phenotype associated with cell membrane localisation.

Another further aspect of the present invention is directed to sphingosine kinase variants comprising a mutation in a region of said sphingosine kinase which region comprises a translocation mediator binding site, wherein said variant exhibits ablated or reduced translocation capacity relative to wild type sphingosine kinase or a functional derivative, homologue or analogue of said sphingosine kinase variant.

In still another further aspect there is provided a human sphingosine kinase variant comprising an amino acid sequence with a single or multiple amino acid substitution and/or deletion of amino acids 191-206 wherein said variant exhibits ablated or reduced translocation capacity relative to wild-type sphingosine kinase or a functional derivative, homologue or analogue of said sphingosine kinase variant.

In yet another aspect, the present invention extends to genetically modified animals, which animals have been modified to express a sphingosine kinase variant as hereinbefore defined.

Single and three letter abbreviations used throughout the specification are defined in Table 1.

TABLE 1

Single and three letter amino acid abbreviations

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	The	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any residue	Xaa	X

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphical representation of the phosphorylation and plasma membrane localization of hSK1 enhances cell proliferation. Growth of NIH3T3 cells stably transfected with plasmids encoding for wild type hSK1 (o), $hSK1^{S225A}$ (▲), Lck-hSK1 (●) and Lck-$hSK1^{S225A}$ (■) or the empty vector (□) over five days in; **A**, serum free medium (containing 0.1% BSA); **B**, medium containing 1% FCS, or; **C**, 5% FCS. **D**, Sphingosine kinase activity in these cells and protein expression of the various hSK1 constructs as determined by Western blot via their FLAG epitope. **E**, Cell proliferation of these cells as measured by BrdU incorporation into nacent DNA. **F**, Serum-deprivation induced apoptosis of these cells as measured by nuclear condensation and fragmentation Data are representative of three independent experiments.

Figure 2 is an image of the localization of hSK1 to the plasma membrane by the Lck N-terminal motif. **A**, Lysates from NIH3T3 cells stably transfected with wild type hSK1, $hSK1^{S225A}$, Lck-hSK1, and Lck-$hSK1^{S225A}$ were fractionated into cytosol and membranes and probed via Western blot with anti- FLAG. Data are representative of three independent experiments. **B**, Fluorescence microscopy of the same stably transfected NIH3T3 cells. Images are representative of >50% of cells observed in three independent experiments.

Figure 3 is an image of the phosphorylation and plasma membrane localization of hSK1 leads to transformation. **A**, NIH3T3 cells stably transfected with empty vector, or plasmids encoding for wild type hSK1 or $hSK1^{S225A}$, either alone or co-transfected with plasmid encoding for an activated mutant H-Ras (V12- Ras) were cultured on soft agar. Colonies formed after 3 weeks were visualised by MTT staining as described previously (Xia *et al.*, 2000, *supra*). **B**, Quantitation of colony formation in soft agar. Data are mean (± SD) from three independent experiments.

Figure 4 is a graphical representation of the phosphorylation and plasma membrane localization of hSK1 lead to increased intracellular and extracellular sphingosine 1-phosphate levels. Intracellular and extracellular S1P levels were determined in NIH3T3

cells stably transfected with empty vector, or plasmids encoding for wild type hSK1, $hSK1^{S225A}$, Lck-hSK1 and $Lck\text{-}hSK1^{S225A}$. Data are mean (± SD) from three independent experiments.

Figure 5 is a schematic representation of the analysis of the putative CaM binding regions of hSK1 (SEQ ID NO:2). Boxed residues are those predicted to be possible CaM binding regions. Residues underlined constitute the regions of hSK1 incorporated in GST-fusion proteins. Triangles indicate the location of tryptic cleavage sites in hSK1 that are protected by the presence of CaM during limited proteolysis.

Figure 6 is a schematic representation of the site-directed mutagenesis of predicted CaM binding regions of hSK1. *A*, The selective binding of the hSK1 mutants to CaM-Sepharose (CaM) was examined using extracts from HEK293T cells expressing the various hSK1 mutants (Load). Bound hSK1 proteins were visualised by Western blotting via their FLAG epitope. Binding to Sepharose CL-4B (CL4B) was used as a control to account for any non-specific binding to the Sepharose 4B beads. *B*, Relative catalytic activity of the hSK1 mutants.

Figure 7 is an image depicting the limited proteolysis of hSK1 reveals protection of tryptic cleavage sites by CaM. *A*, Coomassie stained gel of tryptic peptides generated from limited proteolysis of recombinant hSK1 alone, purified CaM alone, or both proteins together. B, Western blot with anti-His antibodies of limited trypsinolysis of *C*-terminally His-tagged hSK1.

Figure 8 is an image depicting association of hSK1-derived peptides with CaM. The selective binding of the hSK1-derived peptides to CaM-Sepharose (CaM) was examined using GST-peptide fusion proteins generated in *E. coli* (Load). Bound fusion proteins were visualised by Western blotting with anti-GST antibodies. Binding to Sepharose CL-4B (CL4B) was used as a control to account for any non-specific binding to the Sepharose 4B beads.

Figure 9 is a schematic representation depicting the functional outcome of site-directed mutagenesis of PCB3 of hSK1. *A*, The selective binding of the hSK1 mutants to CaM-Sepharose (CaM) was examined using extracts from HEK293T cells expressing the various hSK1 mutants (Load). Bound hSK1 proteins were visualised by Western blotting via their FLAG epitope. Binding to Sepharose CL-4B (CL4B) was used as a control to account for any non-specific binding to the Sepharose 4B beads. *B*, Relative catalytic activity of the hSK1 mutants.

Figure 10 is a schematic representation depicting that hSK2 associates with CaM via a binding site conserved with hSK1. *A*, Association of hSK1 and hSK2 with CaM-Sepharose (CaM) was examined in the presence of 5 mM $CaCl_2$ or 5 mM EGTA using extracts from HEK293T cells expressing either hSK1 or hSK2 (Load). Bound hSK1 or hSK2 were visualised by Western blotting via their FLAG epitopes. Binding to Sepharose CL-4B (CL4B) was used as a control to account for any non-specific binding to the Sepharose 4B beads. *B*, Ablation of CaM-Sepharose binding by the V327A/L328Q mutations of hSK2 was examined using extracts from HEK293T cells expressing the form of hSK2. *C*, Relative catalytic activity of the hSK2 mutant.

Figure 11 is a schematic representation depicting that mutation of the hSK1 CaM-binding site ablates agonist-induced translocation of hSK1 to the plasma membrane. *A*, Fluorescence microscopy of HEK293T cells transfected with either wild type hSK1-GFP or $hSK1^{F197A/L198Q}$-GFP with or without PMA (10 ng/ml) for 30 min. Images are representative of >50% of cells observed in two independent experiments. Phosphorylation (*B*) and activation (*C*) of hSK1 in transiently transfected HEK293T cells was followed by Western blot using the phospho-hSK1 specific polyclonal antibodies (anti-p-hSK1) and sphingosine kinase enzyme assays following treatment of cells overexpressing wild type hSK1 or $hSK1^{F197A/L198Q}$ with TNFα (1 ng/ml) or PMA (10 ng/ml) for 30 min. Total hSK1 levels were determined via the FLAG epitope.

Figure 12 is an image depicting that calmyrin associates with hSK1 in a calcium-dependent manner. Association of hSK1 with GST-calmyrin bound to glutathione-

Sepharose was examined in the presence of 5 mM $CaCl_2$ or 5 mM EGTA using extracts from HEK293T cells expressing hSK1 (Load).

Figure 13 is an image depicting that mutation of the hSK1 CaM-binding site ablates calmyrin binding. The involvement of the CaM-binding site of hSK1 in its association with calmyrin was assessed using GST-calmyrin bound to glutathione-Sepharose and extracts from HEK293T cells expressing either wildtype hSK1 or $hSK1^{F197A/L198Q}$ (Load). Bound hSK1 was visualised by Western blotting via the FLAG epitope. GST alone was used as a control to account for any non-specific binding.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated, in part, on the surprising determination that sphingosine kinase mediated cellular activity is regulated by the translocation of sphingosine kinase from the cytosol to the cell membrane. Still further, it has been determined that although phosphorylation of sphingosine kinase is a highly significant event in that it both increases the catalytic activity of sphingosine kinase and effects its intracellular translocation, the translocation of sphingosine kinase, irrespective of the state of its phosphorylation, will achieve modulation of cellular activities which are mediated by sphingosine kinase signalling events. Finally, there has been the identification and characterisation of a sphingosine kinase translocation factor binding site itself. These determinations now permit the rational design of therapeutic and/or prophylactic methods for treating conditions characterised by aberrant or unwanted cellular activity and/or sphingosine kinase functional activity, in particular neoplastic conditions. Further, there is facilitated the identification and/or design of agents which specifically modulate sphingosine kinase translocation.

Accordingly, one aspect of the present invention provides a method of modulating sphingosine kinase mediated signalling, said method comprising contacting sphingosine kinase with an effective amount of an agent for a time and under conditions sufficient to modulate the intracellular localisation of said sphingosine kinase wherein upregulating sphingosine kinase cell membrane localisation upregulates said signalling and downregulating said sphingosine kinase cell membrane localisation downregulates said signalling.

Reference to "sphingosine kinase mediated signalling" should be understood as a reference to a signalling pathway in which the sphingosine kinase molecule forms a functional component. In this regard, it is thought that sphingosine kinase is central to the generation of sphingosine-1-phosphate during activation of this pathway. It should be understood that modulation of sphingosine kinase mediated signalling encompasses both up and downregulation of the signalling events, for example the induction or cessation of a given

signalling event or a change to the level or degree of any given signalling event.

In accordance with the present invention antagonising translocation of sphingosine kinase to the cell membrane prevents the completion of a sphingosine kinase mediated signalling event while agonising or otherwise inducing translocation of sphingosine kinase to the cell membrane promotes sphingosine kinase mediated signalling. It should also be understood that the degree or level of a sphingosine kinase mediated signalling event can be modulated by increasing or decreasing the concentration of sphingosine kinase molecules which are localised to the cell membrane. Accordingly, the modulation of signalling need not necessarily equate to the onset or inhibition of signalling but may be designed to regulate the level of sphingosine kinase mediated signalling which occurs.

Reference to "sphingosine kinase" should be understood to include reference to all forms of sphingosine kinase protein and derivatives, mutants, homologues or analogues thereof. In this regard, "sphingosine kinase" should be understood as being a molecule which is, *inter alia*, involved in the generation of sphingosine-1-phosphate during activation of the sphingosine kinase signalling pathway. This includes, for example, all protein forms of sphingosine kinase and its functional derivatives, mutants, homologues or analogues thereof, including, for example, any isoforms which arise from alternative splicing of sphingosine kinase mRNA or allelic or polymorphic variants of sphingosine kinase.

Without limiting the present invention to any one theory or mode of action, two human sphingosine kinase isoforms exist (1 and 2), which differ in their tissue distribution, developmental expression, catalytic properties, and somewhat in their substrate specificity (Pitson *et al., 2000, supra*; Liu *et al.*, 2000, *supra*). A number of studies have shown the effects of sphingosine kinase 1 in enhancing cell proliferation and suppressing apoptosis (Olivera *et al.*, 1999, *supra*; Xia *et al.*, 2000, *supra*; Edsall *et al.*, 2001, *supra*). Furthermore, overexpression of human sphingosine kinase 1 (hSK1) in NIH3T3 fibroblasts has been shown to result in acquisition of the transformed phenotype and the ability to form tumors in nude mice, demonstrating the oncogenic potential of this enzyme (Xia *et al.*, 2000, *supra*). More recent work has shown the involvement of hSK1 in estrogen-

dependent regulation of breast tumor cell growth and survival (Nava *et al.*, 2002, *supra*; Sukocheva *et al.*, 2003, *supra*), while other studies have shown elevated hSK1 mRNA in a variety of human solid tumors and inhibition of tumor growth in vivo by sphingosine kinase inhibitors (French *et al.*, 2003, *supra*).

Reference to a "functional" derivative, mutant, homologue or analogue thereof should be understood as a reference to a molecule which exhibits any one or more of the functional activities of sphingosine kinase.

Preferably, said sphingosine kinase is sphingosine kinase 1 or 2 and more preferably human sphingosine kinase 1 or 2.

Accordingly, in one preferred embodiment, the present invention provides a method of modulating human sphingosine kinase 1 mediated signalling, said method comprising contacting said human sphingosine kinase 1 with an effective amount of an agent for a time and under conditions sufficient to modulate intracellular localisation of said human sphingosine kinase 1 wherein upregulating said sphingosine kinase cell membrane localisation upregulates said signalling and downregulating said sphingosine kinase cell membrane localisation downregulates said signalling.

In another preferred embodiment, the present invention provides a method of modulating human sphingosine kinase 2 mediated signalling, said method comprising contacting said human sphingosine kinase 2 with an effective amount of an agent for a time and under conditions sufficient to modulate intracellular localisation of said human sphingosine kinase 2 wherein upregulating said sphingosine kinase cell membrane localisation upregulates said signalling and downregulating said sphingosine kinase cell membrane localisation downregulates said signalling.

Reference to "translocation" and "localisation" of the subject sphingosine kinase (those terms being utilised interchangeably) should be understood as a reference to the intracellular physical location of this molecule, irrespective of any physical or functional

characteristic of the subject sphingosine kinase molecules, such as its level of catalytic activity or its degree of phosphorylation. As detailed hereinbefore, the present invention is predicated on the determination that localisation of sphingosine kinase to the cell membrane is crucial in order to complete the sphingosine kinase signalling event and thereby effect cellular functional activities such as proliferation. Without limiting the present invention to any one theory or mode of action, it is known that sphingosine kinase translocates from the cytosol to the plasma membrane upon exposure of cells to certain agonists (Pitson *et al.*, 2003, *supra*; Rosenfeldt *et al.*, 2001, *FASEB J.* 15:2649–2659; Johnson *et al.*, 2002, *J. Biol. Chem.* 277:35257-35262l; Melendez *et al.*, 2002, *J. Biol. Chem.* 277:17255– 17262; Young *et al.*, 2003, *Cell Calcium* **33**:119–128). Further, it is known that this translocation is dependent on phosphorylation of sphingosine kinase at serine 225. Still further, it has been determined that one of the critical regions for the binding of a factor which facilitates the translocation of sphingosine kinase corresponds to residues 191-206 of human sphingosine kinase 1 and the corresponding conserved region of human sphingosine kinase 2. In particular, Phe197 and Leu198 are critically involved in this interaction. However, it has been surprisingly determined both that this translocation event is crucial to effect the biological outcomes of a sphingosine kinase mediated signalling event and, further, that this can be achieved even by translocating an unphosphorylated sphingosine kinase molecule. Accordingly, the present invention provides a means of regulating sphingosine kinase mediated signalling in a manner which circumvents the need to consider or modulate the phosphorylation state of sphingosine kinase.

Accordingly, in a preferred embodiment, there is provided a method of modulating human sphingosine kinase mediated signalling, said method comprising contacting said human sphingosine kinase with an effective amount of an agent for a time and under conditions sufficient to modulate intracellular localisation of said human sphingosine kinase wherein upregulating said sphingosine kinase cell membrane localisation upregulates said signalling and downregulating said sphingosine kinase cell membrane localisation downregulates said signalling and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

More particularly, the present invention provides a method of modulating human sphingosine kinase mediated signalling, said method comprising contacting said human sphingosine kinase with an effective amount of an agent for a time and under conditions sufficient to modulate the interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2, wherein inducing or agonising said interaction upregulates sphingosine kinase cell membrane localisation and antagonising said interaction downregulates sphingosine kinase cell membrane localisation and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

Preferably, said amino acid is one or both of Phe197 or Leu198.

According to these preferred embodiments, said sphingosine kinase is sphingosine kinase 1 or sphingosine kinase 2.

Reference to "modulating" either sphingosine kinase signalling events or sphingosine kinase localisation should be understood as a reference to upregulating or downregulating the subject signalling or localisation event. Reference to upregulating or downregulating in this regard should be understood to include increasing or decreasing the level, degree or rate at which the signalling or localisation event occurs, in addition to including reference to inducing or ablating the subject signalling or localisation event. Accordingly, the agent which is utilised in accordance with the method of the present invention may be an agent which induces the subject event, agonises an event which has already undergone onset, antagonises a pre-existing event, entirely prevents the onset of such an event.

Accordingly, reference to "upregulating sphingosine kinase cell membrane localisation" should be understood as a reference to:

(i) inducing the intracellular cell membrane localisation of sphingosine kinase, for example inducing the interaction of sphingosine kinase with an agent which effects localisation to the cell membrane;

(ii) upregulating, enhancing or otherwise agonising an existing cell membrane-localisation event, for example increasing the affinity of or otherwise stabilising the interaction of sphingosine kinase with an agent which effects its localisation to the cell membrane.

Conversely, "downregulating sphingosine kinase cell membrane localisation" should be understood as a reference to:

(i) preventing the interaction of sphingosine kinase with an agent, such as an endogeneous translocation factor, which would otherwise lead to translocation of sphingosine kinase from the cytosol to the cell membrane.

(ii) antagonising an existing interaction between sphingosine kinase and a translocation agent, for example, such that the translocation of sphingosine kinase is rendered ineffective or less effective.

It should be understood that modulation (either in the sense of upregulation or downregulation) of the cell membrane localisation of sphingosine kinase may be partial or complete. Partial modulation occurs where only some of the sphingosine kinase cell membrane localisation events which would normally occur in a given cell are affected by the method of the present invention while complete modulation occurs where all sphingosine kinase localisation events are modulated.

Modulation of the intracellular localisation of sphingosine kinase may be achieved by any one of a number of techniques including, but not limited to:

(i) introducing into a cell a proteinaceous or non-proteinaceous agent which antagonises the localisation of sphingosine kinase to the cell membrane, such as by interacting with sphingosine kinase in order to block its interaction either with the cell membrane directly or with an intermediate molecule which would normally act

to facilitate membrane localisation.

(ii) introducing into a cell a proteinaceous or non-proteinaceous agent which agonises the localisation of sphingosine kinase to the cell membrane, such as by interacting with sphingosine kinase in order to facilitate its interaction with the cell membrane either directly or via the agent or to facilitate its interaction with an intermediate molecule (such as a translocation factor) which would normally act to facilitate membrane localisation.

(iii) introducing into a cell a sphingosine kinase molecule variant which has been designed to exhibit cell membrane localisation properties.

(iv) introducing into a cell a nucleic acid molecule which encodes a proteinaceous agent as described in any one of (i)-(iii).

Preferably, said agent modulates the interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 and most preferably Phe197 or Leu198.

Reference to "agent" should be understood as a reference to any proteinaceous or non-proteinaceous molecule which modulates (i.e. upregulates or downregulates) the intracellular localisation of sphingosine kinase to the cell membrane, for example the molecules detailed in points (i) –(iii) above. The subject agent may be linked, bound or otherwise associated with any proteinaceous or non-proteinaceous molecule. For example, it may be associated with a molecule which permits targeting to a specific tissue.

Said proteinaceous molecule may be derived from natural, recombinant or synthetic sources including fusion proteins or following, for example, natural product screening. Said non-proteinaceous molecule may be derived from natural sources, such as for example natural product screening or may be chemically synthesised. For example, the present invention contemplates chemical analogues of a translocation factor capable of acting as agonists or antagonists of sphingosine kinase localisation. Chemical agonists

may not necessarily be derived from the translocation factor but may share certain conformational similarities. Alternatively, chemical agonists may be specifically designed to mimic or upregulate certain physiochemical properties of the translocation factor. For example, agonists include agents which induce elevated calcium levels, for example, calcium ionophores such as ionomycin. Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing sphingosine kinase localisation. Antagonists include antibodies (such as monoclonal and polyclonal antibodies) specific for sphingosine kinase, or parts of said sphingosine kinase, or a translocation factor. Antagonists also include sphingosine kinase peptides which are designed to express the translocation factor binding residues at positions 197 and 198 of SEQ ID No:2, thereby functioning as a competitive inhibitor of intracellular translocation factor binding to wild-type sphingosine kinase. Other examples of antagonists include agents which decrease the intracellular level of free calcium, for example calcium chelators such as BAPTA or MAPTAM, or antagonists of the translocation factors themselves, such as W7 which is an antagonist of calmodulin. Modulation of expression may also be achieved utilising antigens, RNA, ribosomes, DNAzymes, RNA aptamers, or molecules suitable for use in co-suppression. The proteinaceous and non-proteinaceous molecules referred to in points (i)-(iv), above, are herein collectively referred to as "modulatory agents".

It should be understood that reference to "translocation factor" is intended as a reference to any molecule which binds to sphingosine kinase and facilitates its intracellular localisation to the cell membrane. For example, one might use calmodulin, calmyrin or other calmodulin-related protein.

Screening for the modulatory agents hereinbefore defined can be achieved by any one of several suitable methods including, but in no way limited to, contacting a cell expressing sphingosine kinase or functional equivalent or derivative thereof with an agent and screening for the modulation of sphingosine kinase localisation to the cell membrane. This can be achieved by analysing sphingosine kinase localisation directly or by analysing a downstream event such as cellular proliferation. Detecting such modulation can be achieved utilising techniques such as Western blotting, electrophoretic mobility shift

assays and/or the readout of reporters of sphingosine kinase activity such as luciferases, CAT, proliferation assays and the like.

It should be understood that the sphingosine kinase gene or functional equivalent or derivative thereof may be naturally occurring in the cell which is the subject of testing or it may have been transfected into the host cell for the purpose of testing. Further, to the extent that a sphingosine kinase nucleic acid molecule is transfected into a cell, that molecule may comprise the entire sphingosine kinase gene or it may merely comprise a portion of the gene such as the portion which regulates localisation of the sphingosine kinase expression product.

In another example, the subject of detection could be a downstream sphingosine kinase regulatory target (for example, sphingosine-1-phosphate), rather than sphingosine kinase itself. Yet another example includes sphingosine kinase localisation related binding sites ligated to a minimal reporter. In another example, modulation of sphingosine kinase localisation can be detected by screening for the modulation of the proliferation of the host cell. This is an example of an indirect system, where modulation of sphingosine kinase localisation, *per se*, is not the subject of detection. Rather, modulation of the molecules which cell membrane-localised sphingosine kinase regulates the expression of, are monitored. These methods provide a mechanism for performing high throughput screening of putative modulatory agents such as the proteinaceous or non-proteinaceous agents comprising synthetic, combinatorial, chemical and natural libraries.

The agents which are utilised in accordance with the method of the present invention may take any suitable form. For example, proteinaceous agents may be glycosylated or unglycosylated, phosphorylated or dephosphorylated to various degrees and/or may contain a range of other molecules fused, linked, bound or otherwise associated with the proteins such as amino acids, lipids, carbohydrates or other peptides, polypeptides or proteins. Similarly, the subject non-proteinaceous molecules may also take any suitable form. Both the proteinaceous and non-proteinaceous agents herein described may be linked, bound otherwise associated with any other proteinaceous or non-proteinaceous

molecules. For example, in one embodiment of the present invention said agent is associated with a molecule which permits its targeting to a localised region, such as a specific tissue.

The term "expression" refers to the transcription and translation of a nucleic acid molecule. Reference to "expression product" is a reference to the product produced from the transcription and translation of a nucleic acid molecule. Reference to "modulation" should be understood as a reference to upregulation or downregulation.

"Derivatives" of the molecules herein described (for example sphingosine kinase or other proteinaceous or non-proteinaceous agents) include fragments, parts, portions or variants from either natural or non-natural sources. Non-natural sources include, for example, recombinant or synthetic sources. By "recombinant sources" is meant that the cellular source from which the subject molecule is harvested has been genetically altered. This may occur, for example, in order to increase or otherwise enhance the rate and volume of production by that particular cellular source. Parts or fragments include, for example, active regions of the molecule. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in a sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences include fusions with other peptides, polypeptides or proteins, as detailed above.

Derivatives also include fragments having particular epitopes or parts of the entire protein fused to peptides, polypeptides or other proteinaceous or non-proteinaceous molecules. For example, sphingosine kinase or derivative thereof may be fused to a molecule such as the 10 amino acid lck protein tyrosine kinase dual acylation motif in order to facilitate cell

membrane localisation. Analogs of the molecules contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecules or their analogs.

Derivatives of nucleic acid sequences which may be utilised in accordance with the method of the present invention may similarly be derived from single or multiple nucleotide substitutions, deletions and/or additions including fusion with other nucleic acid molecules. The derivatives of the nucleic acid molecules utilised in the present invention include oligonucleotides, PCR primers, antisense molecules, molecules suitable for use in cosuppression and fusion of nucleic acid molecules. Derivatives of nucleic acid sequences also include degenerate variants.

A "variant" of sphingosine kinase should be understood to mean a molecule which exhibits at least some of the functional activity of the form of sphingosine kinase of which it is a variant. A variation may take any form and may be naturally or non-naturally occurring. A mutant molecule is one which exhibits modified functional activity.

By "homologue" is meant that the molecule is derived from a species other than that which is being treated in accordance with the method of the present invention.

Chemical and functional equivalents should be understood as molecules exhibiting any one or more of the functional activities of the subject molecule, which functional equivalents may be derived from any source such as being chemically synthesised or identified via screening processes such as natural product screening. For example chemical or functional equivalents can be designed and/or identified utilising well known methods such as combinatorial chemistry or high throughput screening of recombinant libraries or following natural product screening. These methods may also be utilised to screen for any of the modulatory agents which are useful in the method of the present invention.

For example, libraries containing small organic molecules may be screened, wherein organic molecules having a large number of specific parent group substitutions are used. A general synthetic scheme may follow published methods (eg., Bunin *et al.* (1994) *Proc. Natl. Acad. Sci. USA*, *91*:4708-4712; DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. USA*, **90**:6909-6913). Briefly, at each successive synthetic step, one of a plurality of different selected substituents is added to each of a selected subset of tubes in an array, with the selection of tube subsets being such as to generate all possible permutation of the different substituents employed in producing the library. One suitable permutation strategy is outlined in US. Patent No. 5,763,263.

There is currently widespread interest in using combinational libraries of random organic molecules to search for biologically active compounds (see for example U.S. Patent No. 5,763,263). Ligands discovered by screening libraries of this type may be useful in mimicking or blocking natural ligands or interfering with the naturally occurring ligands of a biological target. In the present context, for example, they may be used as a starting point for developing sphingosine kinase translocation agonists or antagonists. Sphingosine kinase or a relevant part thereof may, according to the present invention, be used in combination libraries formed by various solid-phase or solution-phase synthetic methods (see for example U.S. Patent No. 5,763,263 and references cited therein). By use of techniques, such as that disclosed in U.S. Patent No. 5,753,187, millions of new chemical and/or biological compounds may be routinely screened in less than a few weeks. Of the large number of compounds identified, only those exhibiting appropriate biological activity are further analysed.

With respect to high throughput library screening methods, oligomeric or small-molecule library compounds capable of interacting specifically with a selected biological agent, such as a biomolecule, a macromolecule complex, or cell, are screened utilising a combinational library device which is easily chosen by the person of skill in the art from the range of well-known methods, such as those described above. In such a method, each member of the library is screened for its ability to interact specifically with the selected agent. In practising the method, a biological agent is drawn into compound-containing tubes and

allowed to interact with the individual library compound in each tube. The interaction is designed to produce a detectable signal that can be used to monitor the presence of the desired interaction. Preferably, the biological agent is present in an aqueous solution and further conditions are adapted depending on the desired interaction. Detection may be performed for example by any well-known functional or non-functional based method for the detection of substances.

"Analogues" of sphingosine kinase or agonistic or antagonistic agents contemplated herein include, but are not limited to, modifications to side chains, incorporating unnatural amino acids and/or derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the analogues. The specific form which such modifications can take will depend on whether the subject molecule is proteinaceous or non-proteinaceous. The nature and/or suitability of a particular modification can be routinely determined by the person of skill in the art.

For example, examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH4; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivatisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carboethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during protein synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acids contemplated herein is shown in Table 1.

TABLE 1

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl- -aminobutyrate	Mgabu

	D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
	D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
	D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
	D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
5	D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
	D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
	D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
	D-α-methylleucine	Dmleu	α-napthylalanine	Anap
10	D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
	D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
	D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
15	D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
	D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
	D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
	D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
20	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
25	D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
30	D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe

	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
5	D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
	γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
	L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
	L-ethylglycine	Etg	penicillamine	Pen
10	L-homophenylalanine	Hphe	L-α-methylalanine	Mala
	L-α-methylarginine	Marg	L-α-methylasparagine	Masn
	L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
	L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
	L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
15	L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
	L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
	L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
	L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
	L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
20	L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
	L-α-methylserine	Mser	L-α-methylthreonine	Mthr
	L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
	L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
	N-(N-(2,2-diphenylethyl)	Nnbhm	N-(N-(3,3-diphenylpropyl)	Nnbhe
25	carbamylmethyl)glycine		carbamylmethyl)glycine	
	1-carboxy-1-(2,2-diphenyl-	Nmbc		
	ethylamino)cyclopropane			

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional

reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety.

Without limiting the present invention to any one theory or mode of action, site direction mutagenesis, proteolysis and peptide interaction analysis have been utilised to determine that the residues spanning the region 191-206 of human sphingosine kinase (SEQ ID NO:2) are one of the sphingosine kinase sites involved in inducing the translocation of sphingosine kinase from the cytoplasm to the plasma membrane. In particular, it has been determined that residues Phe197 and Leu198 of human sphingosine kinase 1 are critically involved in this interaction. It has still further been determined that a corresponding and conserved region of the human sphingosine kinase 2 molecule exhibits corresponding functional activity. Using a mutated form of human sphingosine kinase 1 (Phe197Ala and Leu198Gln) it has been shown that although hSK1 phosphorylation and catalytic activation remains unchanged in these mutant molecules, agonist-induced translocation of hSK1 from the cytoplasm to the plasma membrane is ablated.

Since sphingosine kinase is a molecule which is central to the functioning of an intracellular signalling pathway, the method of the present invention provides a means of modulating cellular activity which is regulated or controlled by sphingosine kinase signalling. For example, the sphingosine kinase signalling pathway is known to regulate cellular activities such as those which lead to inflammation, cellular transformation, apoptosis, cell proliferation, upregulation of the production of inflammatory mediators such as cytokines, chemokines, eNOS and upregulation of adhesion molecule expression. Said upregulation may be induced by a number of stimuli including, for example, inflammatory cytokines such as tumour necrosis factor α and interleukin 1, endotoxin, oxidised or modified lipids, radiation or tissue injury. In this regard, reference to "modulating cellular activity" is a reference to upregulating or downregulating any one or more of the activities which a cell is capable of performing pursuant to sphingosine kinase signalling such as, but not limited, one or more of chemokine production, cytokine production or cellular proliferation. Although the preferred method is to downregulate sphingosine kinase activity, thereby downregulating unwanted cellular activity, most

preferably unwanted cellular proliferation, the present invention should nevertheless be understood to encompass upregulating of cellular activity which may be desirable in certain circumstances.

Accordingly, yet another aspect of the present invention is directed to a method of modulating sphingosine kinase mediated cellular activity, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate the intracellular localisation of sphingosine kinase wherein upregulating sphingosine kinase cell membrane localisation upregulates said cellular activity and downregulating sphingosine kinase cell membrane localisation downregulates said cellular activity.

Preferably, said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

More preferably, the present invention is directed to a method of modulating sphingosine kinase mediated cellular activity, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate the interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2, wherein inducing or agonising said interaction upregulates sphingosine kinase cell membrane localisation and antagonising said interaction downregulates sphingosine kinase cell membrane localisation and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

Most preferably, said amino acid is one or both of Phe197 or Leu198 and said sphingosine kinase is human sphingosine kinase 1 or sphingosine kinase 2.

In accordance with these embodiments, said cellular activity is preferably cell growth and, even more preferably, neoplastic cell growth.

Most preferably, said neoplastic cell growth is downregulated by antagonising or otherwise downregulating translocation of sphingosine kinase to the cell membrane.

Without limiting this preferred aspect of the present invention to any one theory or mode of action, it has been determined that the oncogenic activity of sphingosine kinase is in particular related to its aberrant overexpression. By "overexpression" is meant the upregulation of intracellular sphingosine kinase to a *functional* level which is greater than that expressed under the normal physiological conditions for a given cell type or to the upregulation of sphingosine kinase levels to any level of functionality but where that upregulation event is one which is artificially effected rather than being an increase which has occurred in the subject cell due to the effects of naturally occurring physiology. It should be understood, however, that the means by which upregulation is achieved may be artificial means which seek to mimic a physiological pathway – for example introducing a hormone or other stimulatory molecule. Accordingly, the term "expressing" in this context is not intended to be limited to the notion of sphingosine kinase gene transcription and translation. Rather, it is a reference to an outcome, being the establishment of a higher functional level of sphingosine kinase than is found under normal physiological conditions in a cell at a particular point in time (ie. it includes non-naturally occurring increases in sphingosine kinase level and increases in the level of activity of existing sphingosine kinase concentrations as opposed to just increases in intracellular concentrations, *per se*, of sphingosine kinase).

Still without limiting the present invention to any one theory or mode of action, it is known that the signalling cascade stimulated by the lipid kinase, sphingosine kinase, has a major role in oncogenesis. Specifically, constitutive activation of sphingosine kinase by overexpression in cells causes cell transformation and tumour formation, thereby indicating that a wild type human lipid kinase is by itself oncogenic. Furthermore, sphingosine kinase is also involved in Ras but not v-Src induced transformation. Finally inhibition of sphingosine kinase activity utilising a sphingosine kinase inhibitor not only reverses transformation in cells overexpressing sphingosine kinase but does so also in Ras transformed cells. In this regard, reference to "modulating" the growth of a cell should be

understood as a reference to upregulating or downregulating the growth of a cell. More specifically, reference to "downregulating" should be understood as a reference to preventing, reducing or otherwise inhibiting one or more aspects of the growth of a cell (including inducing the apoptosis of or otherwise killing a cell) while reference to "upregulating" should be understood to have the converse meaning, and includes induction of the formation of neoplastic cells/cellular transformation (i.e. the conversion of a normal cell to a neoplastic cell). Reference to the "growth" of a cell should be understood in its broadest sense to include reference to all aspects of cell division/proliferation.

It should be understood that reference to "cell" in the context of the present invention is a reference to any form or type of cell, irrespective of its origin. For example, the cell may be a naturally occurring cell or it may be manipulated, modified or otherwise treated either *in vitro* or *in vivo* such as a cell which has been freezed/thawed or genetically, biochemically or otherwise modified either *in vitro* or *in vivo* (including, for example, cells which are the result of the fusion of two distinct cell types). By "neoplastic cell" is meant a cell exhibiting uncontrolled proliferation. The neoplastic cell maybe a benign cell or a malignant cell. Preferably the cell is malignant. In one particular embodiment, the neoplastic cell is a malignant cell the proliferation of which would form a solid tumour such as a malignant cell derived from the mammary gland (breast), colon, stomach, lung, brain, bone, oesophagus or pancreas.

Preferably the neoplastic cell is a malignant cell derived from the colon, stomach, lung, brain, bone, oesophagus, pancreas, mammary gland (breast), ovary or uterus.

It should be understood that the cell which is treated according to the method of the present invention may be located *ex vivo* or *in vivo*. By "*ex vivo*" is meant that the cell has been removed from the body of a subject wherein the modulation of its growth will be achieved *in vitro*. For example, the cell may be a non-neoplastic cell which is to be immortalised by upregulating sphingosine kinase activity. In accordance with the preferred aspects of the present invention, the cell may be a neoplastic cell, such as a malignant cell, located *in vivo* (such as in the colon or breast) and the downregulation of its growth will be achieved by

applying the method of the present invention *in vivo* to downregulate the level of sphingosine kinase functional activity. It should also be understood that where reference is made to a specific cell type which is located *in vivo*, such as a malignant colorectal cell, this cell may be located in the colorectal area of the patient. If a colorectal primary malignancy has metastasised, the subject colorectal cell may be located in another region of the patient's body. For example, it may form part of a secondary tumour (metastasis) which is located, for example, in the liver, lymph node or bone.

Although the preferred method is to downregulate the proliferation of a neoplastic cell, for example as a therapeutic treatment for cancer, it may also be desirable to upregulate cell growth. For example, it may be desirable to immortalise a population of cells *in vitro*, to facilitate their long term *in vitro* use or, for example, to facilitate the *in vitro* growth of tissues such as skin. In another example, it may be useful to adapt cell lines to less fastidious growth conditions such as a capacity to grow in low serum conditions.

A further aspect of the present invention relates to the use of the invention in relation to the treatment and/or prophylaxis of disease conditions. Without limiting the present invention to any one theory or mode of action, the broad range of cellular functional activities which are regulated via the sphingosine kinase signalling pathway renders the regulation of sphingosine kinase functioning an integral component of every aspect of both healthy and disease state physiological processes. Accordingly, the method of the present invention provides a valuable tool for modulating aberrant or otherwise unwanted cellular functional activity which is regulated via the sphingosine kinase signalling pathway.

Accordingly, yet another aspect of the present invention is directed to a method for the treatment and/or prophylaxis of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase mediated cellular activity, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate intracellular localisation of sphingosine kinase wherein upregulating sphingosine kinase cell membrane localisation

upregulates said cellular activity and downregulating sphingosine kinase cell membrane localisation downregulates said cellular activity.

Still another aspect of the present invention is directed to a method for the treatment and/or prophylaxis of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase functional activity, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate intracellular localisation of sphingosine kinase wherein upregulating sphingosine kinase cell membrane localisation upregulates said sphingosine kinase activity and downregulating sphingosine kinase cell membrane localisation downregulates said sphingosine kinase activity.

Preferably, said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

Accordingly, in a preferred embodiment the present invention is directed to a method for the treatment and/or prophylaxis of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase mediated cellular activity, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2, wherein inducing or agonising said interaction upregulates sphingosine kinase membrane localisation and antagonising said interaction downregulates sphingosine kinase cell membrane localisation and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

Still another embodiment of the present invention is directed to a method for the treatment and/or prophylaxis of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase functional activity, said method comprising administering to said mammal an effective amount of an agent for a

time and under conditions sufficient modulate the to interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2, wherein inducing or agonising said interaction upregulates sphingosine kinase cell membrane localisation and antagonising said interaction downregulates sphingosine kinase cell membrane localisation and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

More preferably, said amino acid is one or both of Phe197 or Leu198 and said sphingosine kinase is human sphingosine kinase 1 or sphingosine kinase 2.

Reference to "aberrant, unwanted or otherwise inappropriate" cellular activity should be understood as a reference to overactive cellular activity, to physiological normal cellular activity which is inappropriate in that it is unwanted or to insufficient cellular activity. This definition applies in an analogous manner in relation to "aberrant, unwanted or otherwise, inappropriate" sphingosine kinase activity. For example, TNF production during tumour cell growth has been shown to support cellular proliferation and to provide anti-apoptotic characteristics to the neoplastic cells. Accordingly, to the extent that a cell is neoplastic, it is desirable that the promotion of cellular proliferation and anti-apoptotic characteristics be down-regulated. Similarly, diseases which are characterised by inflammation, such as rheumatoid arthritis, atherosclerosis, asthma, autoimmune disease and inflammatory bowel disease, are known to involve cellular activation by cytokines such as TNF, leading to the synthesis and secretion of inflammatory mediators, such as adhesion molecules. In such situations, it is also desirable to down-regulate such activity. In other situations, it may be desirable to agonise or otherwise induce sphingosine kinase cell membrane localisation in order to stimulate cellular proliferation.

As detailed hereinbefore and without limiting the present invention to any one theory or mode of action, constitutive activation of sphingosine kinase causes cell transformation and tumour development, thereby indicating that sphingosine kinase is by itself oncogenic. However, sphingosine kinase inhibition is also effective in downregulating neoplastic cell proliferation where the subject cell has been transformed by certain unrelated oncogenes

such as Ras induced transformation. Accordingly, the method of the present invention is particularly useful, but in no way limited to, use in the treatment of primary and secondary malignancies such as those associated with solid tumours of the colon, stomach, lung, mammary gland (breast), brain, bone, oesophagus and pancreas and, in particular, tumours which arise from the proliferation of Ras transformed cells or estrogen-dependent breast cell tumours. Although the preferred method is to downregulate uncontrolled cellular proliferation in a subject, by inhibiting cell membrane localisation of sphingosine kinase, upregulation of cell growth may also be desirable in certain circumstances such as to promote wound healing, angiogenesis or other healing process.

Accordingly, the present invention contemplates a method for the treatment and/or prophylaxis of a condition characterised by aberrant, unwanted or otherwise inappropriate cell growth in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the intracellular localisation of sphingosine kinase wherein downregulating sphingosine kinase cell membrane localisation downregulates the subject cell growth.

Preferably, the present invention contemplates a method for the treatment and/or prophylaxis of a condition characterised by aberrant, unwanted or otherwise inappropriate cell growth in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to antagonise the interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2, wherein antagonising said interaction downregulates sphingosine kinase cell membrane localisation and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

Preferably said uncontrolled cell proliferation is caused by the transformation of the cell by oncogene upregulation or by sphingosine kinase overexpression oncogenic activity.

Still more preferably said cell is a malignant cell which forms a solid tumour of the colon, stomach, lung, brain, bone, oesophagus, pancreas, mammary gland (breast), ovary or uterus.

The most preferred embodiment of this aspect of the present invention preferably facilitates the subject proliferation being reduced, retarded or otherwise inhibited. Reference to "reduced, retarded or otherwise inhibited" should be understood as a reference to inducing or facilitating the partial or complete inhibition of cell proliferation. Said inhibition may occur by either direct or indirect mechanisms and includes the induction of cellular apoptosis or other cellular killing mechanisms.

The subject of the treatment or prophylaxis is generally a mammal such as but not limited to human, primate, livestock animal (eg. sheep, cow, horse, donkey, pig), companion animal (eg. dog, cat), laboratory test animal (eg. mouse, rabbit, rat, guinea pig, hamster), captive wild animal (eg. fox, deer). Preferably the mammal is a human or primate. Most preferably the mammal is a human.

An "effective amount" means an amount necessary at least partly to attain the desired response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of a particular condition being treated. The amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the degree of protection desired, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Reference herein to "treatment" and "prophylaxis" is to be considered in its broadest context. The term "treatment" does not necessarily imply that a subject is treated until total recovery. Similarly, "prophylaxis" does not necessarily mean that the subject will not eventually contract a disease condition. Accordingly, treatment and prophylaxis include amelioration of the symptoms of a particular condition or preventing or otherwise reducing

the risk of developing a particular condition. The term "prophylaxis" may be considered as reducing the severity or onset of a particular condition. "Treatment" may also reduce the severity of an existing condition.

The present invention further contemplates a combination of therapies, such as the administration of the agent together with subjection of the mammal to other agents, drugs or treatments which may be useful in relation to the treatment of the subject condition such as cytotoxic agents or radiotherapy in the treatment of cancer.

Administration of the modulatory agent, in the form of a pharmaceutical composition, may be performed by any convenient means. The modulatory agent of the pharmaceutical composition is contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the modulatory agent chosen. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 mg to about 1 mg of modulatory agent may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation.

The modulatory agent may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intraperitoneal, intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). The modulatory agent may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as

magnesium stearate.

Routes of administration include, but are not limited to, respiratorally, intratracheally, nasopharyngeally, intravenously, intraperitoneally, subcutaneously, intracranially, intradermally, intramuscularly, intraoccularly, intrathecally, intracereberally, intranasally, infusion, orally, rectally, *via* IV drip patch and implant.

In accordance with these methods, the agent defined in accordance with the present invention may be coadministered with one or more other compounds or molecules. By "coadministered" is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or different routes. For example, the subject agent may be administered together with an agonistic agent in order to enhance its effects. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two types of molecules. These molecules may be administered in any order.

Another aspect of the present invention contemplates the use of an agent, as hereinbefore defined, in the manufacture of medicament for the treatment of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase mediated cellular activity, wherein said agent modulates the intracellular localisation of sphingosine kinase and wherein upregulating sphingosine kinase cell membrane localisation upregulates said cellular activity and downregulating sphingosine kinase cell membrane localisation down regulates said cellular activity.

Still another aspect of the present invention contemplates the use of an agent, as hereinbefore defined, in the manufacture of medicament for the treatment of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase mediated signalling, wherein said agent modulates the intracellular localisation of sphingosine kinase and wherein upregulating sphingosine kinase cell membrane localisation upregulates said sphingosine kinase mediated signalling

and downregulating sphingosine kinase cell membrane localisation downregulates said sphingosine kinase mediated signalling.

More particularly, said agent modulates the interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2, most particularly Phe197 or Leu198, wherein inducing or agonising said interaction upregulates sphingosine kinase cell membrane localisation and antagonising said interaction downregulates sphingosine kinase cell membrane localisation.

Preferably, said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

More preferably, said sphingosine kinase is human sphingosine kinase 1 or 2.

Even more preferably, said cellular activity is cellular proliferation.

Most preferably said cellular proliferation is neoplastic cell proliferation and said proliferation is downregulated.

In yet another further aspect, the present invention contemplates a pharmaceutical composition comprising the modulatory agent as hereinbefore defined together with one or more pharmaceutically acceptable carriers and/or diluents. These agents are referred to as the active ingredients.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene

glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a

suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule encoding a modulatory agent. The vector may, for example, be a viral vector.

Yet another aspect of the present invention relates to the agent as hereinbefore defined, when used in the method of the present invention.

The present invention should also be understood to encompass a method for screening for agents which modulate the intracellular localisation of sphingosine kinase, particularly agents which agonise or antagonise the interaction of a translocation factor which interacts with, or itself interact with, one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2, in particular Phe197 or Leu198.

Screening for the modulatory agents hereinbefore defined can be achieved by any one of several suitable methods including, but in no way limited to, contacting a cell comprising sphingosine kinase and a translocation mediator (such as calmodulin) with an agent and screening for the modulation of sphingosine kinase localisation or modulation of the activity or expression of a downstream sphingosine kinase cellular target such as NF-κB. This is particularly useful for screening for agonists or antagonists of a mediator of translocation. The present method is also useful for screening for molecules which, themselves, bind to sphingosine kinase and induce its translocation to the plasma membrane. Detecting such modulation can be achieved utilising techniques such as Western blotting, electrophoretic mobility shift assays and/or the readout of reporters of sphingosine kinase activity such as luciferases, CAT and the like.

It should be understood that the sphingosine kinase or mediator of translocation may be naturally occurring in the cell which is the subject of testing or the genes encoding them may have been transfected into a host cell for the purpose of testing. Further, the naturally occurring or transfected gene may be constitutively expressed - thereby providing a model useful for, *inter alia*, screening for agents which down-regulate sphingosine kinase translocation or the gene may require activation - thereby providing a model useful for, inter alia, screening for agents which modulate sphingosine kinase translocation under certain stimulatory conditions. Further, to the extent that a sphingosine kinase nucleic acid molecule is transfected into a cell, that molecule may comprise the entire sphingosine kinase gene or it may merely comprise a portion of the gene such as a portion comprising amino acid residues 191-206 of SEQ ID NO:2.

In another example, the subject of detection could be a downstream sphingosine kinase regulatory target, rather than sphingosine kinase itself, such as NF-κB. Yet another example includes sphingosine kinase binding sites ligated to a minimal reporter. For example, modulation of sphingosine kinase translocation can be detected by screening for the modulation of the downstream signalling components of an appropriately stimulated cell. Where the cell which is the subject of the screening system is a neoplastic cell, for

example, modulation of sphingosine kinase translocation could be detected by screening for the cessation of proliferation of that cell.

Suitable agents may also be identified and/or designed utilising well known methods such as combinatorial chemistry or high throughput screening of recombinant libraries or following natural product screening.

For example, libraries containing small organic molecules may be screened, wherein organic molecules having a large number of specific parent group substitutions are used. A general synthetic scheme may follow published methods (eg., Bunin BA, et al. (1994) Proc. Natl. Acad. Sci. USA, 91:4708-4712; DeWitt SH, et al. (1993) Proc. Natl. Acad. Sci. USA, 90:6909-6913). Briefly, at each successive synthetic step, one of a plurality of different selected substituents is added to each of a selected subset of tubes in an array, with the selection of tube subsets being such as to generate all possible permutation of the different substituents employed in producing the library. One suitable permutation strategy is outlined in US. Patent No. 5,763,263.

There is currently widespread interest in using combinational libraries of random organic molecules to search for biologically active compounds (see for example U.S. Patent No. 5,763,263). Ligands discovered by screening libraries of this type may be useful in mimicking or blocking natural ligands or interfering with the naturally occurring ligands of a biological target. In the present context, for example, they may be used as a starting point for developing sphingosine kinase translocation agonists or antagonists which exhibit properties such as more potent pharmacological effects. Sphingosine kinase or a functional part thereof and/or translocation factor may according to the present invention be used in combination libraries formed by various solid-phase or solution-phase synthetic methods (see for example U.S. Patent No. 5,763,263 and references cited therein). By use of techniques, such as that disclosed in U.S. Patent No. 5,753,187, millions of new chemical and/or biological compounds may be routinely screened in less than a few weeks. Of the large number of compounds identified, only those exhibiting appropriate biological activity are further analysed.

With respect to high throughput library screening methods, oligomeric or small-molecule library compounds capable of interacting specifically with a selected biological agent, such as a biomolecule, a macromolecule complex, or cell, are screened utilising a combinational library device which is easily chosen by the person of skill in the art from the range of well-known methods, such as those described above. In such a method, each member of the library is screened for its ability to interact specifically with the selected agent. In practising the method, a biological agent is drawn into compound-containing tubes and allowed to interact with the individual library compound in each tube. The interaction is designed to produce a detectable signal that can be used to monitor the presence of the desired interaction. Preferably, the biological agent is present in an aqueous solution and further conditions are adapted depending on the desired interaction. Detection may be performed for example by any well-known functional or non-functional based method for the detection of substances.

Accordingly, another aspect of the present invention provides a method for detecting an agent capable of modulating the intracellular localisation of sphingosine kinase or its functional equivalent or derivative thereof said method comprising contacting a cell or extract thereof containing said sphingosine kinase or its functional equivalent or derivative with a putative agent and detecting an altered expression phenotype associated with cell membrane localisation.

Reference to "sphingosine kinase" should be understood as a reference to either sphingosine kinase expression product or to a portion or fragment of sphingosine kinase such as the cell membrane localisation region or the region which interacts with a translocation factor being the region defined by amino acids 191-206 of SEQ ID NO:2. In this regard, the sphingosine kinase expression product is expressed in a cell. The cell may be a host cell which has been transfected with the sphingosine kinase nucleic acid molecule or it may be a cell which naturally contains the sphingosine kinase gene. Reference to "extract thereof" should be understood as a reference to a cell free transcription system.

Reference to detecting an "altered expression phenotype associated with said cell membrane localisation" should be understood as the detection of cellular changes associated with modulation of the intracellular localisation of sphingosine kinase. These may be detectable, for example, as intracellular changes or changes observable extracellularly, such as changes in proliferation levels.

Still another aspect of the present invention is directed to agents identified in accordance with the screening method defined herein and to said agents for use in the methods of the present invention. Said agents should be understood to extend to monoclonal antibodies which bind to all or part of the region defined by amino acids 191-206 of SEQ ID NO:2, and in particular to Phe197 and/or Leu198 of human sphingosine kinase or corresponding region.

Still a further aspect of the present invention is directed to sphingosine kinase variants comprising a mutation in a region of said sphingosine kinase which region comprises a translocation mediator binding site, wherein said variant exhibits ablated or reduced translocation capacity relative to wild type sphingosine kinase or a functional derivative, homologue or analogue of said sphingosine kinase variant.

The present invention also extends to variants which exhibit enhanced or up-regulated activity due to the nature of the mutation of an existing translocation mediator binding site or the incorporation of additional such sites.

Reference to "mutation" should be understood as a reference to any change, alteration or other modification, whether occurring naturally or non-naturally, which modulates the capacity of said sphingosine kinase to undergo translocation. Said modulation may be up-regulation or down-regulation. Although the present invention is preferably directed to variants which exhibit ablated activation capacity, it should be understood that the present invention extends to the generation of variants which exhibit improved translocation capacity. Reference to a "functional" derivative, homologue, or analogue in this context

should be understood as a reference to the subject molecule exhibiting the defined modulated translocation capacity.

The change, alteration or other modification may take any form including, but not limited to, a structural modification (such an alteration in the secondary, tertiary or quaternary structure of the sphingosine kinase molecule), a molecular modification (such as an addition, substitution or deletion of one or more amino acids from the sphingosine kinase protein) or a chemical modification. The subject modification should also be understood to extend to the fusion, linking or binding of a proteinaceous or non-proteinaceous molecule to the sphingosine kinase protein or to the nucleic acid molecule encoding a sphingosine kinase protein. It should also be understood that although it is necessary that the subject mutation is expressed by the sphingosine kinase expression product, the creation of the mutation may be achieved by any suitable means including either mutating a wild-type sphingosine kinase protein, synthesising a sphingosine kinase variant or modifying a nucleic acid molecule encoding a wild-type sphingosine kinase protein such that the expression product of said mutated nucleic acid molecule is a sphingosine kinase protein variant. Preferably, said mutation is a single or multiple amino acid sequence substitution, addition and/or deletion.

In accordance with this preferred embodiment there is provided a human sphingosine kinase variant comprising an amino acid sequence with a single or multiple amino acid substitution and/or deletion of amino acids 191-206 wherein said variant exhibits ablated or reduced translocation capacity relative to wild-type sphingosine kinase or a functional derivative, homologue or analogue of said sphingosine kinase variant.

Preferably, amino acid is amino acid Phe197 and/or Leu198 and even more preferably said substitution is a Phe197 Ala and/or Leu198 Gln substitution.

In terms of the present invention, reference to "wild-type" sphingosine kinase is a reference to the forms of sphingosine kinase expressed by most individuals in a given population. There may be greater than one wild-type form of sphingosine kinase (for example due to

allelic or isoform variation) and the level or extent of translocation ability exhibited by said wild-type sphingosine kinase molecules may fall within a range of levels. However, it should be understood that "wild-type" does not include reference to a naturally occurring form of sphingosine kinase which cannot be translocated. Such a variant form of sphingosine kinase may, in fact, constitute a naturally occurring mutant form of sphingosine kinase within the context of the present invention.

Reference to "agent" as hereinbefore defined should be understood to include reference to the sphingosine kinase variants herein defined.

In yet another aspect, the present invention extends to genetically modified animals, which animals have been modified to express a sphingosine kinase variant as hereinbefore defined.

The present invention is described by reference to the following non-limiting examples.

EXAMPLE 1

SPHINGOSINE KINASE 1 PHOSPHORYLATION AND TRANSLOCATION TO THE PLASMA MEMBRANE MEDIATE ITS ONCOGENIC ROLE

Cell Culture, Transfection and Cell Fractionation.

Human embryonic kidney (HEK293T) cells and NIH3T3 fibroblasts were cultured on Dulbecco's modified Eagle's medium (DMEM) and harvested as described previously (Pitson *et al.*, 2000, *supra*). Stable and transient transfections were performed using the calcium phosphate precipitation method for HEK293T cells and Lipofectamine 2000 (Invitrogen) for NIH3T3 cells. Stable transfectants were selected for G418-resistance and pooled to avoid the phenotypic artifacts that may arise from the selection and propagation of individual clones from single transfected cells. For subcellular fractionation, post-nuclear supernatants of cell lysates were separated into cytosol and membrane fractions as previously described (Pitson *et al.*, 2003, *EMBO J.* 22:5491–5500).

Antibodies.

The M2 anti-FLAG antibody was from Sigma, anti-H-Ras polyclonal antibody from Santa Cruz Biotechnology, and HRP-conjugated anti-mouse and anti-rabbit IgG were from Pierce. Anti-hSK1 and anti-phospho-hSK1 antibodies have been described previously (Pitson *et al.*, 2003, *supra*).

Sphingosine kinase assays.

Sphingosine kinase activity was determined using D-*erythro*-sphingosine (Biomol, Plymouth Meeting, PA) and [γ^{32}P]ATP (Geneworks, Adelaide, South Australia) as substrates, as previously described (Pitson *et al.*, 2000, *supra*). A unit (U) of sphingosine kinase activity is defined as the amount of enzyme required to produce 1 pmol S1P min^{-1}.

Generation of Lck-hSK1 constructs.

The 10 amino acid N-terminal dual acylation motif of the Lck protein tyrosine kinase (MGCGCSSHPE) has been show to be sufficient to target proteins to the plasma membrane (Zlatkine *et al.*, 1997, *J. Cell Sci.* 110:673–679). Thus, a Lck-hSK1 chimera was generated by PCR with oligonucleotide primers 5'-TAGAATTCGCCACCATGGGCTGTGGCTGCAGCTCACACCCGGAAGATCCAG CGGGCGGCC- 3' (SEQ ID NO:1) and SP6 using pcDNA3-hSK1 (Pitson *et al.*, 2000, *supra*) as template DNA. The resultant product was then cloned into pcDNA3 (Invitrogen) by digestion with Eco*RI*. The orientation was determined by restriction analysis and sequencing verified the integrity of the FLAG tagged Lck-hSK1 cDNA sequence. To generate the FLAG tagged Lck-hSK1^{S225A} chimera, the *Kpn*I-*Pml*I fragment representing the wild-type 5' end of pcDNA3-hSK1^{S225A} (Pitson *et al.*, 2003, *supra*) was replaced with the 150 bp *Kpn*I-*Pml*I pcDNA3-Lck-hSK1 DNA fragment that contains the Lck dual acylation motif.

Immunofluorescence. Stably transfected NIH3T3 cells were plated onto fibronectin coated eight well glass chamber slides (Nalge Nunc International) at 1 x 10^4 cells / well and incubated for 24 h. The cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS, and incubated with M2 anti-FLAG antibody in PBS containing 3% BSA and 0.1% Triton X-100 for 1 h. The immunocomplexes were then detected with FITC-conjugated anti-mouse IgG. Fluorescence microscopy was performed on an Olympus BX-51 microscope equipped with a fluorescein excitation filter (494 nm), acquired to a Cool Snap FX charge-coupled device camera (Photometrics, Phoenix, AZ).

S1P levels.

To determine both intracellular and extracellular S1P levels, cells were incubated for 1 h in phosphate-free DMEM, then metabolically labelled with fresh phosphate-free DMEM containing [^{32}P]orthophosphate (0.2 mCi/ml) and incubated for 4 h at 37°C. To determine

extracellular S1P release the media was then removed, centrifuged at 1,000 x g, and 2.5 ml of the supernatant added to 2.5 ml chloroform, 2.5 ml methanol and 20 µl conc. HCl. The organic phase was then dried under vacuum, resuspended in chloroform and S1P resolved by TLC on silica gel 60 with l-butanol/ethanol/acetic acid/water (8:2:1:2, v/v). Intracellular S1P levels were determined by harvesting the cells into 400 µl methanol containing 25 µl conc. HCl. Lipids were then extracted under alkaline conditions by the addition of 400 µl chloroform, 400 µl KCl and 40 µl 3 M NaOH. The aqueous phase, containing S1P under these conditions, was then acidified through the addition of 50 µl conc. HCl and re-extracted with 400 µl chloroform. The organic phase organic phase was then dried under vacuum, resuspended in chloroform and S1P resolved by TLC as described above.

Cell growth, bromodeoxyuridine incorporation, and staining of apoptotic nuclei.

Assays for cell growth were performed by incubating cells in 48-well plates (2500 cells per well) in medium containing 5% or 1% FCS, or serum free medium (containing 0.1% BSA) as described previously (Xia *et al.*, 2000, *supra*). Cell numbers were determined at the indicated times using the thiazolyl blue (MTT) assay. Bromodeoxyuridine (BrdU) incorporation into nascent DNA were used as a measure of cell proliferation. Cells were plated onto eight well glass chamber slides (Nalge Nunc international) coated with fibronectin at 1 x 10^4 cells / well and grown for 24 h in DMEM with 2% FCS. Cells were then incubated with 10 µM BrdU for 3 h, and then fixed and stained for its incorporation using an anti-BrdU-FLUOS antibody (Roche) following the manufacturers protocol. Cells positive for BrdU incorporation were visualised with an Olympus BX-51 fluorescence microscope, with at least 300 cells were scored per point. Apoptosis was assessed by staining cells with 1 µg/ml DAPI (4',6-diamidino-2-phenylindole) in methanol for 15 min at room temperature. Apoptotic cells were identified by condensation and fragmentation of nuclei using fluorescence microscopy and expressed as a percentage of the total cells counted. A minimum of 300 cells were scored per point.

Results

Phosphorylation of hSK1 is required for enhanced cell proliferation and survival.

Overexpression of wild type hSK1 significantly enhances cell proliferation and survival (Olivera *et al.*, 1999, *supra;* Xia *et al.*, 2000, *supra;* Edsall *et al.*, 2001, *supra;* Sukocheva *et al.*, 2003, *supra*; Olivera *et al.*, 2003, *supra*). The precise molecular mechanisms whereby this occurs, however, are unknown. These effects are dependent on the catalytic activity of hSK1, being blocked by the sphingosine kinase inhibitor, *N,N*-dimethylsphingosine (Olivera *et al.* 1999, *supra*; Xia *et al.*, 2000, *supra;* Edsall *et al.*, 2001, *supra*), and appear independent of G-protein coupled S1P receptors (Olivera *et al.* 1999, *supra;* Olivera *et al.*, 2003, *supra*). Thus, the enhanced growth and survival appear due to the action of S1P on, as yet unidentified, intracellular targets. To date it has been considered that an increase in total S1P levels in the cell, as a consequence of the high intrinsic catalytic activity of the overexpressed hSK1 (Pitson *et al.*, 2000, *supra*), was sufficient to induce these biological effects (Olivera *et al.* 1999, *supra;* Xia *et al.*, 2000, *supra*; Edsall *et al.*, 2001, *supra*; Nava *et al.*, 2002, *supra*; Sukocheva *et al.*, 2003, *supra*). Recent findings have indicated that phosphorylation of hSK1 at Ser225 results in its catalytic activation and translocation to the plasma membrane (Pitson *et al.*, 2003, *supra*).

To test whether phosphorylation of hSK1 is important in proliferation and survival, the ability of a non-activatable $hSK1^{S225A}$ mutant to promote these processes was examined. Overexpression of wild type hSK1 not only markedly enhanced the growth of NIH3T3 cells in media containing either 1% and 5% serum, but also conferred to these cells the ability to survive and grow in the absence of serum (Fig. 1A-C). In contrast, however, cells overexpressing $hSK1^{S225A}$ displayed no such enhanced growth or serum-independence (Fig. 1A-C). This is despite the cells expressing similar levels of the transfected proteins and possessing comparable overall sphingosine kinase activities (Fig. 1D). Similar results were also seen with HEK293T cells.

Previous studies have shown that the increased growth rates from overexpression of wild

type hSK1 result from a combination of both increased cellular proliferation and reduced apoptosis (Olivera *et al.* 1999, *supra;* Edsall *et al.*, 2001, *supra*; Olivera *et al.*, 2003, *supra*). Consistent with these studies, assays for cellular proliferation by BrdU incorporation into nascent DNA showed overexpression of wild type hSK1 had a significant effect in increasing cell proliferation (Fig 1E). In contrast, however, cells overexpressing $hSK1^{S225A}$ displayed no such enhanced proliferation, showing similar incorporation of BrdU as control cells (Fig. 1E). Similarly, overexpression of wild type hSK1 dramatically reduced serum deprivation-induced apoptosis, as measured by condensation and fragmentation of nuclei (Fig. 1F). Again, however, overexpression of $hSK1^{S225A}$ showed markedly different results, providing cells with no such protection against apoptosis (Fig. 1F). Therefore, in stark contrast to wild type hSK1, overexpression of the non-phosphorylatable $hSK1^{S225A}$ mutant neither increases proliferation, nor protects against apoptosis despite both transfected proteins generating similar cellular sphingosine kinase activities. Thus, phosphorylation of hSK1 is essential for the observed effects of enhanced proliferation and survival, suggesting a qualitative change in the enzyme that is important for the signalling processes leading to these effects.

Plasma membrane localisation of hSK1 enhances cell proliferation and survival independent of hSK1 phosphorylation.

It has been well established that hSK1 translocates from the cytosol to the plasma membrane upon exposure of cells to certain agonists (Pitson *et al.*, 2003, *supra*; Rosenfeldt *et al.*, 2001, *supra;* Johnson *et al.*, 2002; Melendez *et al.*, 2002, *supra*; Young et al, 2003, *Cell Calcium* 33:119–128). It has been shown that this translocation is dependent on phosphorylation of hSK1 at Ser225 (Pitson *et al.*, 2003, *supra*). Enhanced proliferation and survival is also dependent on Ser225 phosphorylation of hSK1. The role of hSK1 localization to the plasma membrane on these biological effects was examined. Plasma membrane-localized hSK1 proteins were created through addition of the 10 amino acid Lck protein tyrosine kinase dual acylation motif to the *N*-terminus of wild type hSK1 and $hSK1^{S225A}$, generating Lck-hSK1 and Lck-$hSK1^{S225A}$, respectively. Overexpression of these proteins in NIH3T3 cells generated slightly lower cellular sphingosine kinase

activities to that observed with overexpression of hSK1 and hSK1^{S225A} (Fig 1D). Consistent with previous studies that have shown this motif is sufficient to target proteins to the plasma membrane (Zlatkine *et al.*, 1997, *supra*), a substantial localization of Lck-hSK1 and Lck-hSK1^{S225A} proteins and sphingosine kinase activity to the membrane fraction (Fig 2A) was observed. Further immunofluorescence analysis (Fig. 2B) showed clear localization of Lck-hSK1 and Lck-hSK1^{S225A} to the plasma membrane, while, consistent with earlier reports (Pitson *et al.*, 2003, *supra*), hSK1 and hSK1^{S225A} were present in the cytosol.

Like wild type hSK1, overexpression of Lck-hSK1 markedly enhanced the growth of NIH3T3 cells, and also conferred to these cells the ability to survive and grow in the absence of serum (Fig. 1A-C). In stark contrast to hSK1^{S225A}, however, overexpression of Lck-hSK1^{S225A} also conferred an enhancement of growth, as well as survival in serum deprived conditions (Fig. 1A-C). Further examination of these cells showed that, like wild type hSK1, both Lck-hSK1 and the non-phosphorylatable Lck-hSK1^{S225A} increased cell growth through enhancing cellular proliferation and reducing serum deprivation-induced apoptosis (Fig. 1E, F). Therefore, localization of hSK1 to the plasma membrane is sufficient to enhance cellular proliferation and protect against apoptosis irrespective of the phosphorylation status of the enzyme. Accordingly, phosphorylation of hSK1 mediates these observed biological effects through inducing translocation of hSK1 to the plasma membrane, rather than as a result of the associated increase in catalytic activity.

Phosphorylation-induced plasma membrane localisation of hSK1 is mediates cell transformation.

Since it has been established that Ser225 phosphorylation of hSK1 is essential for its effects in enhancing cell proliferation and survival, its effects on cell transformation were investigated. As described previously (Xia *et al.*, 2000, *supra*), wild type hSK1 exhibited considerable transforming activity when transfected into NIH3T3 cells, as assayed by colony formation in soft agar (Fig. 3). In contrast, however, overexpression of similar levels and catalytic activity of hSK1^{S225A} resulted in remarkably less transformation of

these cells (Fig. 3). Notably, these cells expressing hSK1^{S225A} had considerably higher sphingosine kinase activity than what was previously shown necessary for transformation of NIH3T3 cells by wild type hSK1 (Xia *et al.*, 2000, *supra*). Therefore, like the situation for enhanced proliferation and survival, these experiments demonstrate that it is not simply elevated levels of sphingosine kinase activity that are responsible for cell transformation, but instead indicates another aspect of the phosphorylated, activated state of the protein is responsible for these effects. It has previously been demonstrated that transformation of NIH3T3 cells by oncogenic H-Ras (V12-Ras) is blocked by a catalytically inactive, dominant-negative form of hSK1, indicating that hSK1 is critically involved in Ras-induced cell transformation (Xia *et al.*, 2000, *supra*). Strikingly, non-phosphorylatable hSK1^{S225A} also blocked Ras-induced cell transformation further confirming the requirement of hSK1 activation in this pathway (Fig. 3). In this context, therefore, hSK1^{S225A} is apparently acting as a dominant-negative form of the protein, despite possessing full catalytic activity.

Plasma membrane localisation of hSK1 effects on cell transformation were examined. Like wild type hSK1, the overexpression of both Lck-hSK1 and Lck-hSK1^{S225A} in NIH3T3 cells resulted in the formation of vigorous colonies in soft agar (Fig. 3). Although some background colonies where observed in empty vector control cells, Lck-hSK1 and Lck-hSK1^{S225A} overexpressing cells produced 20– 30-fold greater colonies which were considerably larger in size. Indeed, the colonies generated by Lck-hSK1 and Lck-hSK1^{S225A} overexpression were also larger and more numerous than those observed in cell overexpressing wild type hSK1 (Fig. 3). Thus, localization of hSK1 to the plasma membrane is sufficient to enhance cell transformation irrespective of the phosphorylation status of the enzyme.

Phosphorylation and plasma membrane localisation of hSK1 enhances S1P generation.

While able to diffuse rapidly between cell membranes, sphingosine, the substrate of hSK1, is found largely in the plasma membrane (Slife et al., 1989, *J. Biol. Chem.* 264:10371–10377). Therefore, one possible mechanism for the observed dramatic biological effects of hSK1 localization to the plasma membrane is in enhancing S1P generation. Overexpression of both Lck-hSK1 and the non-phosphorylatable Lck-hSK1S225A resulted in similar increases in intracellular S1P and enhanced S1P release into the media which were substantially greater than that observed with either wild type hSK1 or hSK1S225A (Fig. 4).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

EXAMPLE 2

THE CALMODULIN-BINDING SITE OF SPHINGOSINE KINASE AND ITS ROLE IN AGONIST-DEPENDENT TRANSLOCATION OF SPHINGOSINE KINASE TO THE PLASMA MEMBRANE

Materials and methods

Cell Culture and transfection

Human embryonic kidney cells (HEK293T) were cultured in Dulbecco's modified Eagle's medium (JRH Biosciences, Lenexa, KS) containing 10% bovine calf serum (JRH Biosciences), 2 mM glutamine, 0.2% (w/v) sodium bicarbonate, penicillin (1.2 mg/ml), and streptomycin (1.6 mg/ml). Cells were transiently transfected using the calcium

phosphate precipitation method, harvested and lysed by sonication as described previously (Pitson *et al.*, 2000, *supra*). Protein concentrations in cell homogenates were determined with Coomassie Brilliant Blue reagent (Sigma) using bovine serum albumin as standard.

Sphingosine kinase assays

Sphingosine kinase activity was routinely determined using D-*erythro*-sphingosine (Biomol, Plymouth Meeting, PA) and [γ^{32}P]ATP as substrates as described previously (Roberts *et al.*, *Anal. Biochem.* **331**, 122–129). A unit (U) of sphingosine kinase activity is defined as the amount of enzyme required to produce 1 pmol S1P/min.

Calmodulin binding assays

Assays to assess Ca^{2+}/CaM binding of sphingosine kinase were performed as detailed previously (Pitson *et al.*, 2002, *J. Biol. Chem.* **277**, 49545–49553). Briefly, HEK293T cells overexpressing wild type or mutant hSK1 or hSK2 were harvested and lysed as described above. The cell lysates were then centrifuged (13000 × *g*, 15 min at 4 °C) to remove cell debris. Aliquots of the supernatants were added to tubes containing CaM-Sepharose 4B (Amersham Biosciences) pre-equilibrated with binding buffer composed of 50 mM Tris/HCl (pH 7.4), 5 mM $CaCl_2$, 100 mM NaCl, 10% (w/v) glycerol, 0.05% (w/v) Triton X-100, 1 mM dithiothreitol, 1.5 mM Na_3VO_4, 7.5 mM NaF and protease inhibitors (Complete™, Roche) and incubated for 30 min at 4 °C with continuous mixing. The CaM-Sepharose 4B beads were then pelleted by centrifugation (5000 × *g*, 5 min at 4 °C) and washed twice with binding buffer. Bound hSK1 or hSK2 was then resolved by SDS-PAGE and visualised by Western blotting via the FLAG epitope. Sepharose CL-4B (Amersham Biosciences) was used as a control for non-specific binding to CaM-Sepharose 4B.

Construction of sphingosine kinase mutants

hSK1 cDNA (Genbank accession number AF200328) was FLAG epitope tagged at the 3' end and subcloned into pALTER site-directed mutagenesis vector (Promega Corp., Annandale, Australia), as previously described (Pitson *et al.*, 2000, *supra*). Single-stranded DNA was prepared and used as template for oligonucleotide-directed mutagenesis as detailed in the manufacturer's protocol. The mutagenic oligonucleotides used to generate the point mutant constructs were as follows: for $hSK1^{L134Q}$, 5'-ATGAAGACCAATTGACCAACT-3' (SEQ ID NO:3); $hSK1^{L147Q}$, 5'-GCCGGCTGCAGTCACCCAT-3' (SEQ ID NO:4); $hSK1^{L153Q}$, 5'-TGAACCTGCAAAGCTTGCACACGG-3' (SEQ ID NO:5); $hSK1^{R185A/R186A}$, 5'-GAGAGTGAGAAGTATGCGGCCCTAGGGGAGATGCGCTTC-3' (SEQ ID NO:6); $hSK1^{L187Q}$, 5'-GAAGTATCGTCGACAGGGGGAGAT-3' (SEQ ID NO:7); $hSK1^{L194Q}$, 5'-AGATGCGCTTCACTCAGGGTACCTTCCTGCGTCTGGCA-3' (SEQ ID NO:8); $hSK1^{F197A}$, 5'-GCGCTTCACTCTGGGTACCGCCCTGCGTCTGGCAGC-3' (SEQ ID NO:9); $hSK1^{L198Q}$, 5'-CTCTGGGCACTTTCCAGCGCTTGGCAGCCTTGCGCA-3' (SEQ ID NO:10); $hSK1^{F197A/L198Q}$, 5'-GCGCTTCACTCTGGGTACCGCCCAGCGCTTGGCAGC-3' (SEQ ID NO:11); $hSK1^{L200Q}$, 5'-GCACTTTCCTGCGTCAGGCAGCCTTACGCACTTACCGCGGC-3' (SEQ ID NO:12); $hSK1^{L194Q/L200Q}$, 5'-AGATGCGCTTCACTCAGGGTACCTTCCTGCGTCAGGCAGCCTTACGCACTTACCGCGGC-3' (SEQ ID NO:13); $hSK1^{V290N}$, 5'-CTGTTCTACAACCGGGCCGGCGTGTCTCGT-3' (SEQ ID NO:14); $hSK1^{F303H}$, 5'-CTGCTGCGCCTCCAGCTGGCCATGGAG-3' (SEQ ID NO:15). The mutants were sequenced to verify incorporation of the desired modification and the cDNA subsequently sub-cloned into pcDNA3 (Invitrogen, San Diego CA) for transient transfection into HEK293T cells.

The $hSK1^{F197A/L198Q}$ was tagged at the *N*-terminus with eGFP using methods previously described for wildtype hSK1 (Pitson *et al.*, 2003, *supra*). $hSK2^{V327A/L328Q}$ was generated from hSK2 cDNA in pcDNA3 (Roberts *et al.*, 2004, *supra*) by QuikChange mutagenesis (Stratagene) using the primers, 5'-TTCACACTGGGCACGGCGCAAGGCCTCGCCACACTG-3' (SEQ ID NO:16) and 5'-

CAGTGTGGCGAGGCCTTGCGCCGTGCCCAGTGTGAA-3' (SEQ ID NO:17), and sequenced to verify incorporation of the desired modifications.

Generation of GST-peptides and pull-down analyses

The sequences encoding peptides containing the putative CaM binding (PCB) regions of hSK1 were PCR amplified from the hSK1 cDNA with the following primers: PCB1, 5'-TAGGATCCCCGCCGGTCACCAATGAAGACCTCCT-3' (SEQ ID NO:18) and 5'-TAGAATTCAAGCCGTGTGCAGAGACAGC-3 (SEQ ID NO:19)'; PCB2, 5'-TAGGATCCCCGCCGCTAGAGAGTGAGAAGTATCGG-3' (SEQ ID NO:20) and 5'-TAGAATTCAGCCGCGGTAAGTGCGCAA-3' (SEQ ID NO:21); PCB3, 5'-TAGGATCCCCGCCGGCTGGCGTCATGCATCTGT-3' (SEQ ID NO:22) and 5'-TAGAATTCAATGCCTGCCCTTCTCCATG-3' (SEQ ID NO:23). The products were subsequently digested with *Eco*RI and cloned into pGEX2T. Cultures of *E. coli* JM109 transformed with these pGEX2T vectors were grown overnight in Luira broth containing 100 mg/l ampicillin at 37 °C with shaking. The cultures were then diluted 1 in 10 into the same media and grown at 37 °C for 1 hour with shaking until they reached an OD_{600} of approx 0.6. Expression of the GST-PCB peptides was induced by the addition of IPTG to a final concentration of 1mM, and the cultures incubated for a further 3 hours. The cells were harvested by centrifugation at 6000 × *g* for 15 mins at 4°C and lysed by sonication (3 x 30s pulses of 5 watts) in 50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and protease inhibitors. Following clarification of the lysate by centrifugation 20000 × *g* for 30 min at 4 °C, glutathione-Sepharose (Amersham Biosciences) was added, and the mixture incubated at 4 °C for 1 hour with constant agitation. After this time the glutathione-Sepharose was washed three times with cold PBS, and the GST-peptides eluted with 20 mM glutathione in 50mM Tris/HCl, pH 8.5, for 10 min at 4 °C. Pull-down analyses with CaM-Sepharose and the GST-peptide fusion proteins was performed as described above using approximately 1 µg of each purified GST-peptide or GST alone. Peptide binding to CaM-Sepharose was detected using an anti-GST antibody.

Limited proteolysis and N-terminal sequencing

Recombinant hSK1 was generated and purified from Sf9 cells as described previously (Pitson *et al.*, 2002, *supra*). Limited proteolysis of this hSK1 (1.5 μg in 15 μl) was performed in the presence or absence of a 3-fold molar excess of purified bovine CaM (Sigma) by the addition of 2 ng or 5 ng trypsin (Roche) in 100mM Tris/HCl, pH 8.5. The mixture was then incubated at 37 °C for 60 min, stopped by the addition of 1.5 μl of 100 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Roche), and incubated for a further 5 min at 37 °C. Typtic cleavage products were then resolved by SDS-PAGE and transferred to PVDF membrane. Following Coomassie staining of the membrane, bands that were protected in the presence of CaM were excised and their *N*-terminal sequences determined by 6 cycles of automated Edman degradation using an Applied Biosystems 494 Procise Protein Sequencing System at the Australian Proteome Analysis Facility.

Western Blotting

SDS-PAGE was performed on cell lysates using 12% acrylamide gels. Proteins were blotted to nitrocellulose and the membranes blocked overnight at 4 °C in PBS containing 5% skim milk powder and 0.1% (w/v) Triton X-100. hSK1 expression levels in cell lysates were quantitated over a dilution series of the lysates with the monoclonal M2 anti-FLAG antibody (Sigma), with the immunocomplexes detected with HRP anti-mouse (Pierce) IgG using an enhanced chemiluminescence kit (ECL, Amersham Biosciences).

Yeast two-hybrid screen

Yeast two-hybrid screening was performed using the Matchmaker Gal4 Two-Hybrid System 3 (Clontech) according to the manufacturer's instructions. Full-length hSK1 cDNA (Genbank accession number AF200328) was cloned into pGBKT7 (Clontech) in-frame with the Gal4 DNA-binding domain. This bait construct was then transformed into the yeast strain AH107 together with a human leukocyte cDNA library in pACT2 (Clontech). A total of 1 x 10^6 independent clones were screened.

Generation of GST-calmyrin

The sequence encoding full-length calmyrin was PCR amplified from pACT2-calmyrin obtained from the from the yeast two-hybrid screen with the primers, 5'-CCCGGATCCGCCACCATGGGGGGCTCGGGCAG-3' (SEQ ID NO:24) and 5'-GGGCTCGAGTCACAGGACAATCTTAAAGGA-3' (SEQ ID NO:25). The product was subsequently digested with *Bam*HI and *Xho*I and cloned into pGEX4T2. Generation of GST-calmyrin was performed in *E. coli* BL21 as described above.

Results

Mutagenesis of predicted CaM-binding sites in hSK1

To examine the potential role of Ca^{2+}/CaM binding sites in the interaction of hSK1 with CaM site-directed mutagenesis of hSK1 was performed. In particular, this mutagenesis concentrated on the conserved hydrophobic residues within these motifs, changing them to structurally conservative hydrophilic residues. PCB2 was targeted by a Leu153 to Gln mutation ($hSK1^{L153Q}$), both PCB1 and PCB2 were targeted by a single mutation of Leu147 to Gln ($hSK1^{L147Q}$), PCB3 was targeted by mutations of Leu187 to Gln ($hSK1^{L187Q}$) and Leu200 to Gln ($hSK1^{L200Q}$), and PCB4 was targeted by a Phe303 to His mutation ($hSK1^{F303H}$). These versions of hSK1 were then expressed in HEK293T cells and analysed for both their ability to bind CaM-Sepharose, and their catalytic activity as a measure of retained gross protein folding. While these hSK1 mutants all retained at least some catalytic activity, somewhat surprisingly, all five bound CaM with similar efficiency to wild type hSK1 (Fig. 6). This suggested that these predicted Ca^{2+}/CaM binding regions of hSK1 may not be involved in CaM binding. Further mutagenesis of other conserved hydrophobic residues within these putative Ca^{2+}/CaM binding regions (i.e. Leu134 to Glu and Val290 to Asn) yielded catalytically inactive hSK1 proteins that were, therefore, not further analysed due to the likelihood that the mutations caused disruption to the gross folding of these proteins.

Direct identification of the CaM-binding site in hSK1

Since the mutagenesis experiments suggested that the Ca^{2+}/CaM binding sites of hSK1 predicted from sequence analysis were not responsible for CaM binding, further experiments were undertaken to directly identify the CaM binding region in hSK1. This was initially performed using limited proteolysis of purified recombinant hSK1 and identifying cleavage sites in hSK1 that were protected by the presence of CaM. Limited proteolysis of purified recombinant hSK1 with trypsin generated of several detectable cleavage products ranging in size from approximately 9 kDa to 32 kDa (Fig 7A). Inclusion of CaM during this limited proteolysis, however, resulted in the loss of a number of hSK1-derived products in the 17 to 22 kDa range and the accumulation of larger hSK1-derived polypeptides (Fig. 7A). The two most notable polypeptides not generated during limited proteolysis in the presence of CaM had approximate molecular masses of 21 and 22 kDa (Fig. 7A). These polypeptides represented *C*-terminal fragments of hSK1 since they retained the His-tag that resides at this end of the intact recombinant hSK1 (Fig. 7B), and were presumably not generated due to the presence of bound CaM in close proximity to at least two tryptic cleavage sites within the central region of hSK1. Thus, to identify these cleavage sites protected by CaM, *N*-terminal sequencing of the two peptides was performed. The sequences obtained (FTLGTF (SEQ ID NO:26) and LAALRTYR (SEQ ID NO:27)) indicate the two protected tryptic cleavage sites reside at Arg192/Phe193 and Arg198/Leu199.

To further elucidate the potential CaM binding sites in hSK1, we examined the CaM interaction of 30 amino acid peptides based on the PCB1/2, PCB3 and PCB4 regions of hSK1 that were fused to glutathione *S*-transferase (GST) were examined. The three peptides were fused in frame to GST, expressed in *E. coli*, purified, and then assessed for their ability to bind CaM-Sepharose. Consistent with the results obtained above using limited proteolysis, only the fusion protein containing the PCB3 peptide displayed an association with CaM (Fig. 8). Combined, this data strongly indicates that PCB3 is the CaM recognition region of hSK1.

Generation of a hSK1 mutant deficient in CaM binding

Although mutations of either Leu187 or Leu200 within PCB3 did not alter the binding of hSK1 with CaM (Fig. 6), the results obtained from limited proteolysis and peptide binding studies provided impetus to further examine other residues in this region that may be critical for this association. Thus, we generated versions of hSK1 containing mutations in several individual hydrophobic residues within PCB3, including Leu194 to Gln ($hSK1^{L194Q}$), Phe197 to Ala ($hSK1^{F197A}$), and Leu198 to Gln ($hSK1^{L198Q}$), were generated. Again, these versions of hSK1 were then expressed in HEK293T cells and analysed for their ability to bind CaM. The results (Fig. 9) demonstrate that, while all three appeared to associate with CaM-Sepharose, they did so with somewhat reduced efficiency compared to wild type hSK1 (Fig. 9). This is despite all of the three variant proteins retaining at least some catalytic activity, suggesting the mutations were not affecting gross protein folding. In light of these findings a version of hSK1 containing both the Phe197 to Ala and Leu198 to Gln mutations ($hSK1^{F197A/L198Q}$) was generated and, again, its ability to associate with CaM was examined. These two mutations completely ablated the binding of hSK1 to CaM (Fig. 5). Furthermore, this double mutant of hSK1 retained considerable catalytic activity, again, indicating that this defect in its ability to associate with CaM was not a result of a disruption in gross folding of the protein. Thus, these results firmly establish that the CaM binding region of hSK1 resides within the PCB3 region of hSK1 (residues 191-206) and that Phe197 and Leu198 are critically involved in the interaction of hSK1 with CaM.

To further characterise the CaM-binding site of hSK1, also targeted was a basic region towards the *N*-terminal end of PCB3 since, in addition to the critical involvement of hydrophobic residues in CaM binding, such clusters of basic residues are commonly involved in the electrostatic stabilisation of the interaction of CaM with its targets (Vetter *et al.*, 2003, *supra*). Therefore, a version of hSK1 containing Ala at both Arg185 and Arg186 ($hSK1^{R185A/R186A}$) was generated and its ability to associate with CaM-Sepharose was examined. Somewhat surprising, based on previous analyses and comparison to other

CaM-binding sites (Vetter *et al.*, 2003, *supra*), hSK1$^{R185A/R186A}$ retained the capacity to bind CaM (Fig. 9), indicating that these basic residues are not required for this interaction.

CaM binding is conserved between hSK1 and hSK2

Although no previous studies had examined the ability of hSK2 to bind CaM, sequence analysis demonstrated that the identified CaM binding site of hSK1 is highly conserved in this protein (Fig. 5). Thus, the issue of whether CaM could also bind hSK2 was examined. Indeed, it was found that, like hSK1, hSK2 also associated with CaM (Fig. 10A). While this interaction between hSK2 and CaM was enhanced by the presence of Ca^{2+}, unlike the situation with hSK1, considerable binding of apocalmodulin to hSK2 was observed in absence of Ca^{2+} (Fig. 10A).

While hSK2 interacted with CaM, addition of Ca^{2+}/CaM to enzyme assays of recombinant hSK2 indicated that, like the situation for hSK1 (Pitson *et al.*, 2000, *supra*), Ca^{2+}/CaM does not alter catalytic activity of hSK2 *in vitro* (data not shown). Thus, the physiological role of this interaction of CaM with hSK2 remains to be determined.

To generate a version of hSK2 deficient in CaM binding mutagenesis was performed on the residues in this protein that were conserved with those critical for CaM-binding of hSK1. This version of hSK2 (hSK2$^{V327A/L328Q}$) was then expressed in HEK293T cells and analysed for both its ability to bind CaM-Sepharose, and catalytic activity as a measure of retained gross protein folding. Consistent with the findings with hSK1, this hSK2 mutant retained high catalytic activity, but did not interact with CaM (Fig. 10). Thus, these studies firmly establish that hSK1 and hSK2 not only associate with CaM, but that both enzymes do so via a highly conserved binding site.

Role of CaM-binding site in sphingosine kinase regulation

It has been established that activation of hSK1 through phosphorylation at Ser225 and, in particular, its subsequent translocation to the plasma membrane are critical steps in

oncogenic signalling by this enzyme. The identification of the CaM binding site and generation of a CaM-binding-deficient version of hSK1 in the current study enabled the further examination of the direct role of CaM in the cellular localisation of hSK1. Thus, the involvement of CaM in the well established phorbol ester-induced translocation of hSK1 to the plasma membrane was examined. Wildtype hSK1 and hSK1$^{F197A/L198Q}$ were expressed in HEK293T cells as fusion proteins with eGFP and their localisation examined following cell exposure to phorbol 12-myristate 13-acetate (PMA). A rapid shift in the localisation of wildtype hSK1 from the cytosol to the plasma membrane was observed following this treatment (Fig. 11A). In stark contrast, however, no redistribution of hSK1$^{F197A/L198Q}$ was observed in response to PMA (Fig. 11A) strongly indicating an important role for the CaM-binding site in this process.

Next, the phosphorylation and activation of hSK1$^{F197A/L198Q}$ in response to cell exposure to PMA and tumor necrosis factor-α (TNFα) was examined to ensure that the mutations to the CaM-binding site were not inhibiting translocation via an indirect effect on this process. Indeed, it was found that this was not the case since treatment of cells with both PMA and TNFα resulted in enhanced phosphorylation and catalytic activity of hSK1$^{F197A/L198Q}$ in a comparable manner to that observed with wildtype hSK1 (Fig. 7B). This demonstrates that CaM binding is not involved in the phosphorylation and catalytic activation of hSK1, and suggests that disruption of hSK1 translocation by mutations of the CaM binding site occurs via directly altering protein-protein interactions at this site.

Does CaM mediate translocation of hSK1?

The studies outlined above strongly implicate the involvement of the CaM-binding site of hSK1 in its agonist-induced translocation. The actual role of CaM in this process, however, is less clear since CaM predominantly translocates from the cytosol to the nucleus, rather than the plasma membrane, upon increases in free cellular calcium (Chin *et al.*, 2000, *Trends Cell Biol.* **10**, 322–328).

Using yeast two-hybrid technology, the CaM-related protein, calmyrin (also known as

CIB1, calcium and intergrin binding protein 1), has been identified as a hSK1-interacting protein. This 22 kDa myristoylated, Ca^{2+} binding protein has considerable amino acid sequence similarity to CaM (56%) and calcineurin B (58%). Calmyrin is widely distributed in most tissues and cells examined (Shock *et al.*, 1999, *Biochem. J.* **342**, 729–735). It is known to interact with several other proteins, and appears to have a broad function through these diverse protein interactions, regulating the activity of the protein kinases Plk2 and FAK (Naik *et al.*, 2003, *Blood* **102**, 3629–3636; Ma *et al.*, 2003, *Mol. Cancer Res.* **1**, 376–384), the transcriptional activity of Pax3 (Hollenbach *et al.*, 2002, *Biochim. Biophys. Acta* **1574**, 321–328), and regulating α_{IIb} integrin signalling in platelets (Tsuboi, S., 2002, *J. Biol. Chem.* **277**, 1919–1923). Most importantly in the context of this study, however, is that calmyrin is a member of the Ca^{2+}-myristoyl switch family of proteins that are known to associate with the plasma membrane in a Ca^{2+}-dependent manner (Meyer *et al.*, ,1999, *Nat. Cell Biol.* **1**, E93–E95). In the absence of Ca^{2+}, these myristoylated proteins sequester their fatty acid into a hydrophobic cavity. The binding of Ca^{2+} results in large conformational changes in the protein, leading to extrusion of the myristoyl group so that it is available to interact with membranes.

Recombinant GST-calmyrin has been produced and used to generate anti-calmyrin antibodies. Employing these reagents the interaction between calmyrin and both hSK1 and hSK2 has been confirmed , and it has been shown that these interactions are enhanced by Ca^{2+} in a comparable manner to that observed for CaM (Fig 8). Since calmyrin and CaM share significant sequence similarity, and both bind hSK1 in a Ca^{2+}-dependent manner, analysis was performed as to whether both proteins bind hSK1 at the same site using the CaM-binding-deficient mutant $hSK1^{F197A/L198Q}$. As observed with CaM binding, $hSK1^{F197A/L198Q}$ also failed to bind to calmyrin (Fig. 13), indicating that both CaM and calmyrin interact with hSK1 via a similar mechanism.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of modulating sphingosine kinase mediated signalling, said method comprising contacting sphingosine kinase with an effective amount of an agent for a time and under conditions sufficient to modulate the intracellular localisation of said sphingosine kinase wherein upregulating sphingosine kinase cell membrane localisation upregulates said signalling and downregulating said sphingosine kinase cell membrane localisation downregulates said signalling and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

2. A method of modulating sphingosine kinase mediated cellular activity, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate the intracellular localisation of sphingosine kinase wherein upregulating sphingosine kinase cell membrane localisation upregulates said cellular activity and downregulating said sphingosine kinase cell membrane localisation downregulates said cellular activity and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

3. A method for the treatment and/or prophylaxis of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase mediated cellular activity, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the intracellular localisation of sphingosine kinase wherein upregulating sphingosine kinase cell membrane localisation upregulates said cellular activity and downregulating sphingosine kinase cell membrane localisation downregulates said cellular activity and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

4. A method for the treatment and/or prophylaxis of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase functional activity, said method comprising administering to said mammal an

effective amount of an agent for a time and under conditions sufficient to modulate the intracellular localisation of sphingosine kinase wherein upregulating sphingosine kinase cell membrane localisation upregulates said sphingosine kinase activity and downregulating sphingosine kinase cell membrane localisation downregulates said sphingosine kinase activity and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

5. A method according to any one of claims 1-4 wherein said agent modulates the interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2 wherein inducing or agonising said interaction upregulates sphingosine kinase cell membrane localisation and antagonising said interaction downregulates sphingosine kinase cell membrane localisation.

6. The method according to claim 5 wherein said amino acid is one or both of Phe197 or Leu198.

7. The method according to claim 5 or 6 wherein said sphingosine kinase is human sphingosine kinase 1.

8. The method according to claim 5 or 6 wherein said sphingosine kinase is human sphingosine kinase 2.

9. The method according to any one of claims 1-7 wherein said modulation of intracellular sphingosine kinase localisation is upregulation of cell membrane localisation.

10. The method according to any one of claims 1-8 wherein said modulation of intracellular sphingosine kinase localisation is downregulation of cell membrane localisation.

11. The method according to claim 2 wherein said cellular activity is induced by TNF and said modulation of intracellular sphingosine kinase localisation is downregulation of

cell membrane localisation.

12. The method according to claim 11 wherein TNF-induced cellular activity is TNF-induced proliferation and/or anti-apoptotic characteristic.

13. The method according to claim 2 wherein said cellular activity is the production of inflammatory mediators and said modulation of intracellular sphingosine kinase localisation is downregulation of cell membrane localisation.

14. The method according to claim 13 wherein said inflammatory mediator is adhesion molecule expression.

15. The method according to claim 3 or 4 wherein said condition is unwanted cell growth and said cell membrane localisation of sphingosine kinase is downregulated.

16. The method according to claim 15 wherein said unwanted cell growth is uncontrolled proliferation.

17. The method according to claim 16 wherein said uncontrolled proliferation is a neoplastic condition.

18. The method according to claim 17 wherein said neoplastic condition is a malignant neoplasm.

19. The method according to claim 18 wherein said malignant neoplasm is a solid tumor.

20. The method according to claim 19 wherein said solid tumor is a tumor of the colon, stomach, lung, brain, bone, oesophagus, pancreas, breast, ovary or uterus.

21. The method according to claim 3 or 4 wherein said condition is an inflammatory

condition and said cell membrane localisation of sphingosine kinase is downregulated.

22. The method according to claim 21 wherein said inflammatory condition is rheumatoid arthritis, atherosclerosis, autoimmune disease or inflammatory bowel disease.

23. The method according to any one of claims 1-9 wherein said modulation of intracellular sphingosine kinase localisation is upregulation of cell membrane localisation and said upregulation is achieved by introducing into a cell a sphingosine kinase translocation factor or a nucleic acid encoding a sphingosine kinase translocation factor.

24. The method according to claim 23 wherein said translocation factor is calmodulin.

25. The method according to claim 23 wherein said translocation factor is calmyrin.

26. The method according to any one of claims 1-9 wherein said modulation of intracellular sphingosine kinase localisation is upregulation of cell membrane localisation and said upregulation is achieved by introducing into a cell a proteinaceous or non-proteinaceous molecule which functions as an agonist of the interaction between sphingosine kinase and a translocation factor.

27. The method according to claim 26 wherein said agonist induces elevated calcium levels.

28. The method according to claim 27 wherein said agonist is a calcium ionophore.

29. The method according to claim 28 wherein said agonist is ionomycin.

30. The method according to any one of claims 1-9 wherein said modulation of intracellular localisation is upregulation of cell membrane localisation and said upregulation is achieved by introducing into a cell a molecule which upregulates the transcription and/or translation of either a sphingosine kinase translocation factor or a

molecule which agonises the interaction of sphingosine kinase with a translation factor.

31. The method according to any one of claims 1-8 or 10-20 wherein said modulation of intracellular sphingosine kinase localisation is downregulation of cell membrane localisation and said downregulation is achieved by introducing into a cell a proteinaceous or non-proteinaceous molecule which functions as an antagonist of the interaction between sphingosine kinase and a translocation factor.

32. The method according to claim 31 wherein said antagonist is an antibody.

33. The method according to claim 32 wherein said antibody is directed to the region of sphingosine kinase comprising residues 191-206 of SEQ ID NO:2.

34. The method according to claim 31 wherein said antagonist is a non-functional sphingosine kinase variant which competitively inhibits binding of the translocation factor to wildtype sphingosine kinase.

35. The method according to claim 34 wherein said non-functional variant is a peptide which comprises a region corresponding to residues 191-206 of SEQ ID No:2.

36. The method according to claim 35 wherein said non-functional variant comprises a region corresponding to residues 197 and 198 of SEQ ID No:2.

37. The method according to claim 31 wherein said antagonist acts to decrease levels of intracellular free calcium.

38. The method according to claim 37 wherein said antagonist is a calcium chelator.

39. The method according to claim 38 wherein said calcium chelator is BAPTA or MAPTAM.

40. The method according to any one of claims 1-8 or 10-20 wherein said modulation of intracellular sphingosine kinase localisation is downregulation of cell membrane localisation and said downregulation is achieved by introducing into said cell a nucleic acid molecule encoding an antagonist of the interaction between sphingosine kinase and a translocation factor.

41. The method according to any one of claims 1-8 or 10-20 wherein said modulation of intracellular sphingosine kinase localisation is downregulation of cell membrane localisation and said downregulation is achieved by introducing into said cell a molecule which upregulates the transcription and/or translation of an antagonist of the interaction between sphingosine kinase and a translocation factor.

42. Use of an agent in the manufacture of a medicament for the treatment of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase mediated cellular activity, wherein said agent modulates the intracellular localisation of sphingosine kinase and wherein upregulating sphingosine kinase cell membrane localisation upregulates said cellular activity and downregulating sphingosine kinase cell membrane localisation downregulates said cellular activity and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

43. Use of an agent in the manufacture of a medicament for the treatment of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate sphingosine kinase functional activity, wherein said agent modulates the intracellular localisation of sphingosine kinase wherein upregulating sphingosine kinase cell membrane localisation upregulates said sphingosine kinase activity and downregulating sphingosine kinase cell membrane localisation downregulates said sphingosine kinase activity and wherein said agent functions by a means other than regulating localisation by modulating sphingosine kinase phosphorylation.

44. Use of an agent in the manufacture of a medicament for the treatment wherein said

agent modulates the interaction of a translocation factor with one or more amino acids corresponding to residues 191-206 of SEQ ID NO:2 wherein inducing or agonising said interaction upregulates sphingosine kinase cell membrane localisation and antagonising said interaction downregulates sphingosine kinase cell membrane localisation.

45. Use according to claim 44 wherein said amino acid is one or both of Phe197 or Leu198.

46. Use according to claim 44 or 45 wherein said sphingosine kinase is human sphingosine kinase 1.

47. Use according to claim 44 or 45 wherein said sphingosine kinase is human sphingosine kinase 2.

48. Use according to any one of claims 44-47wherein said modulation of intracellular sphingosine kinase localisation is upregulation of cell membrane localisation.

49. Use according to any one of claims 44-47 wherein said modulation of intracellular sphingosine kinase localisation is downregulation of cell membrane localisation.

50. Use according to claim 43 or 44 wherein said condition is unwanted cell growth and said cell membrane localisation of sphingosine kinase is downregulated.

51. Use according to claim 50 wherein said unwanted cell growth is uncontrolled proliferation.

52. Use according to claim 51 wherein said uncontrolled proliferation is a neoplastic condition.

53. Use according to claim 52 wherein said neoplastic condition is a malignant neoplasm.

54. Use according to claim 53 wherein said malignant neoplasm is a solid tumor.

55. Use according to claim 54 wherein said solid tumor is a tumor of the colon, stomach, lung, brain, bone, oesophagus, pancreas, breast, ovary or uterus.

56. Use according to claim 43 or 44 wherein said condition is an inflammatory condition and said cell membrane localisation of sphingosine kinase is downregulated.

57. Use according to claim 56 wherein said inflammatory condition is rheumatoid arthritis, atherosclerosis, autoimmune disease or inflammatory bowel disease.

58. Use according to any one of claims 42-48 wherein said modulation of intracellular sphingosine kinase localisation is upregulation of cell membrane localisation and said upregulation is achieved by introducing into a cell a sphingosine kinase translocation factor or a nucleic acid encoding a sphingosine kinase translocation factor.

59. Use according to claim 58 wherein said translocation factor is calmodulin.

60. Use according to claim 58 wherein said translocation factor is calmyrin.

61. Use according to any one of claims 42-48 wherein said modulation of intracellular sphingosine kinase localisation is upregulation of cell membrane localisation and said upregulation is achieved by introducing into a cell a proteinaceous or non-proteinaceous molecule which functions as an agonist of the interaction between sphingosine kinase and a translocation factor.

62. The method according to claim 61 wherein said agonist induces elevated calcium levels.

63. Use according to claim 62 wherein said agonist is a calcium ionophore.

64. Use according to claim 63 wherein said agonist is ionomycin.

65. Use according to any one of claims 42-48 wherein said modulation of intracellular localisation is upregulation of cell membrane localisation and said upregulation is achieved by introducing into a cell a molecule which upregulates the transcription and/or translation of either a sphingosine kinase translocation factor or a molecule which agonises the interaction of sphingosine kinase with a translation factor.

66. Use according to any one of claims 42-47 or 49-57 wherein said modulation of intracellular sphingosine kinase localisation is downregulation of cell membrane localisation and said downregulation is achieved by introducing into a cell a proteinaceous or non-proteinaceous molecule which functions as an antagonist of the interaction between sphingosine kinase and a translocation factor.

67. Use according to claim 66 wherein said antagonist is an antibody.

68. The method according to claim 57 wherein said antibody is directed to the region of sphingosine kinase comprising residues 191-206 of SEQ ID NO:2.

69. Use according to claim 67 wherein said antagonist is a non-functional sphingosine kinase variant which competitively inhibits binding of the translocation factor to wildtype sphingosine kinase.

70. Use according to claim 69 wherein said non-functional variant is a peptide which comprises a region corresponding to residues 191-206 of SEQ ID No:2.

71. Use according to claim 70 wherein said non-functional variant comprises a region corresponding to residues 197 and 198 of SEQ ID No:2.

72. Use according to claim 71 wherein said antagonist acts to decrease levels of

intracellular free calcium.

73. Use according to claim 72 wherein said antagonist is a calcium chelator.

74. Use according to claim 73 wherein said calcium chelator is BAPTA or MAPTAM.

75. Use according to any one of claims 42-47 or 49-57 wherein said modulation of intracellular sphingosine kinase localisation is downregulation of cell membrane localisation and said downregulation is achieved by introducing into said cell a nucleic acid molecule encoding an antagonist of the interaction between sphingosine kinase and a translocation factor.

76. Use according to any one of claims 42-47 or 49-57 wherein said modulation of intracellular sphingosine kinase localisation is downregulation of cell membrane localisation and said downregulation is achieved by introducing into said cell a molecule which upregulates the transcription and/or translation of an antagonist of the interaction between sphingosine kinase and a translocation factor.

77. A pharmaceutical composition comprising the modulatory agent hereinbefore defined together with one or more pharmaceutically acceptable carriers and/or diluents.

78. A method for detecting an agent capable of modulating the intracellular localisation of sphingosine kinase or its functional equivalent or derivative thereof said method comprising contacting a cell or extract thereof containing said sphingosine kinase or its functional equivalent or derivative with a putative agent and detecting an altered expression phenotype associated with cell membrane localisation.

79. An isolated sphingosine kinase variant comprising a mutation in a region of said sphingosine kinase which region comprises a translocation mediator binding site, wherein said variant exhibits ablated or reduced translocation capacity relative to wild type sphingosine kinase or a functional derivative, homologue or analogue of said sphingosine

kinase variant.

80. The variant of claim 79 wherein said variant comprises an amino acid sequence with a single or multiple amino acid substitution and/or deletion of amino acids 191-206.

81. The variant of claim 80 wherein said amino acid is amino acid Phe197 and/or Leu198.

82. The variant of claim 81 wherein said substitution is a Phe197Ala and/or Leu198Gln substitution.

83. An isolated nucleic acid molecule encoding the variant of any one of claims 79-82.

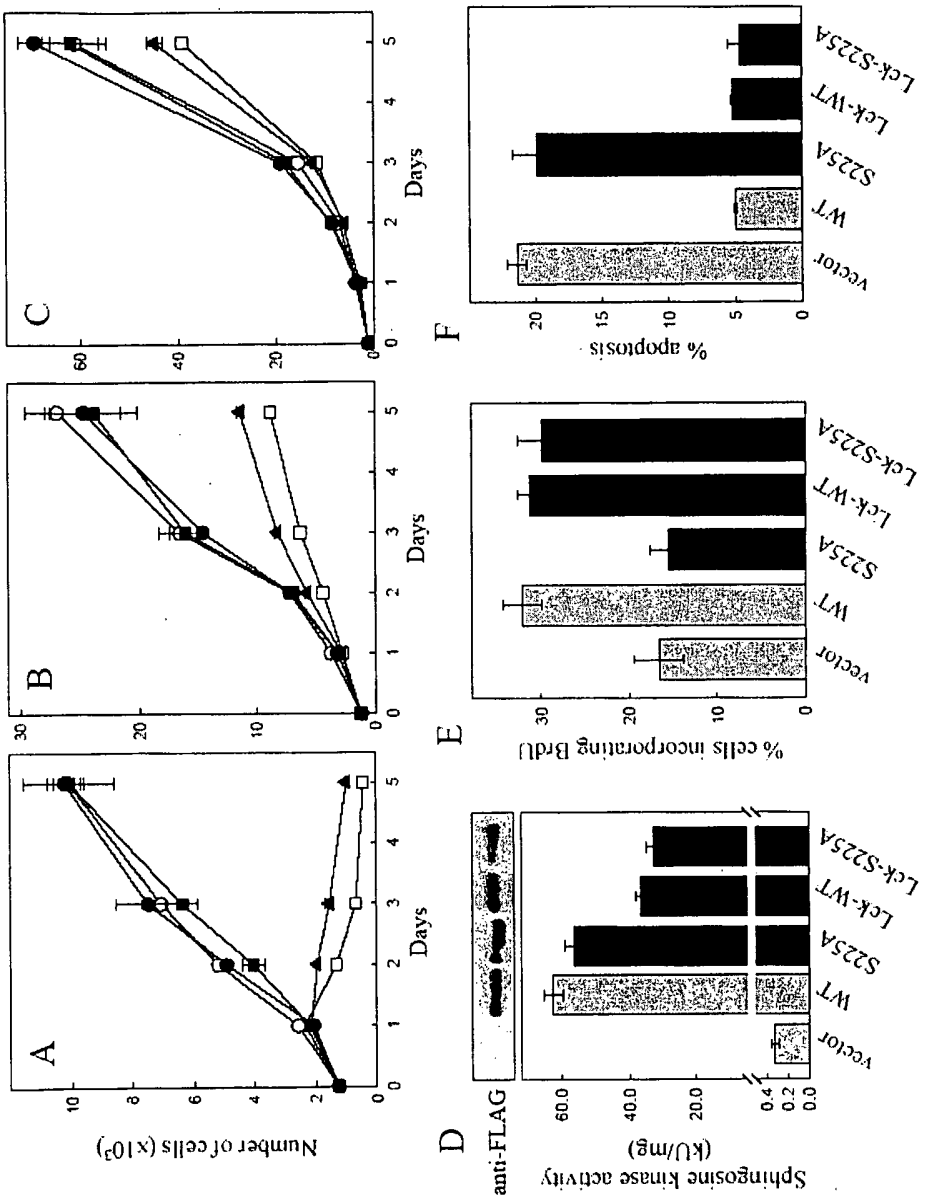
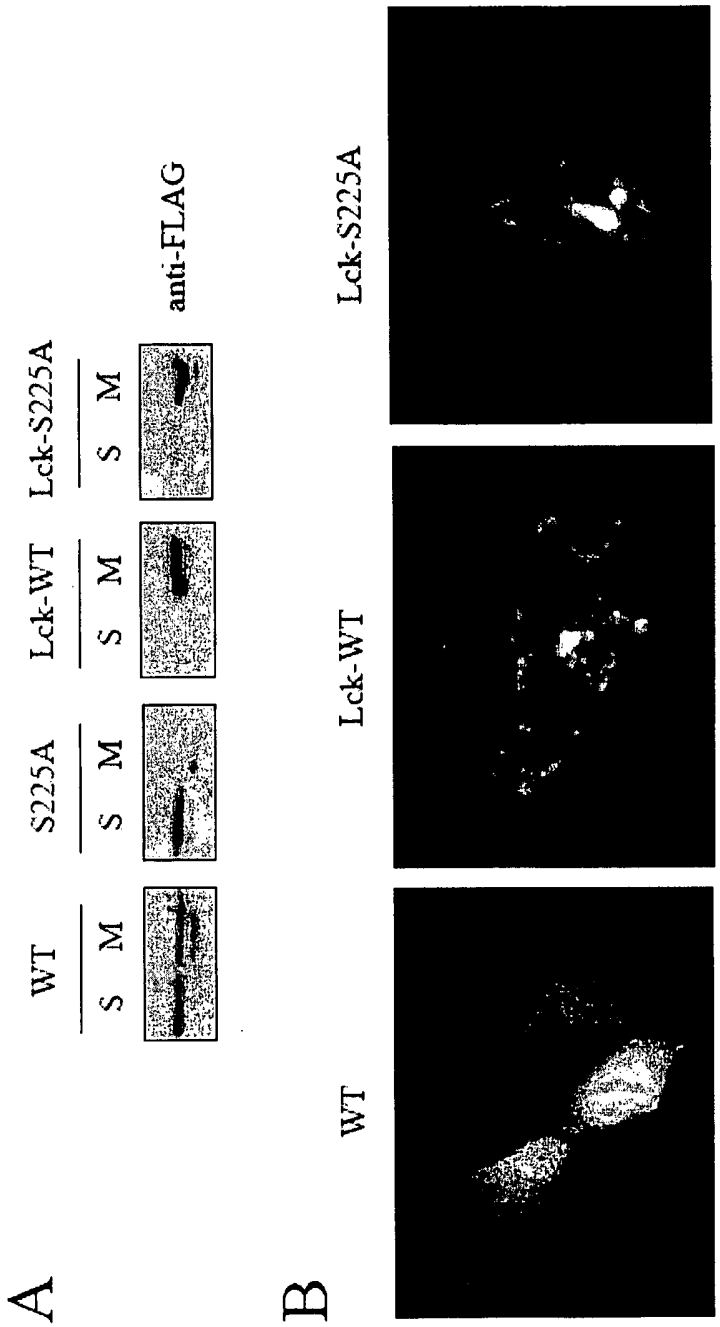

A
B
C
D
E
F
Number of cells (x10³)
Days
anti-FLAG
Sphingosine kinase activity (kU/mg)
% cells incorporating BrdU
% apoptosis
Vector
WT
S225A
Lck-WT
Lck-S225A

Figure 1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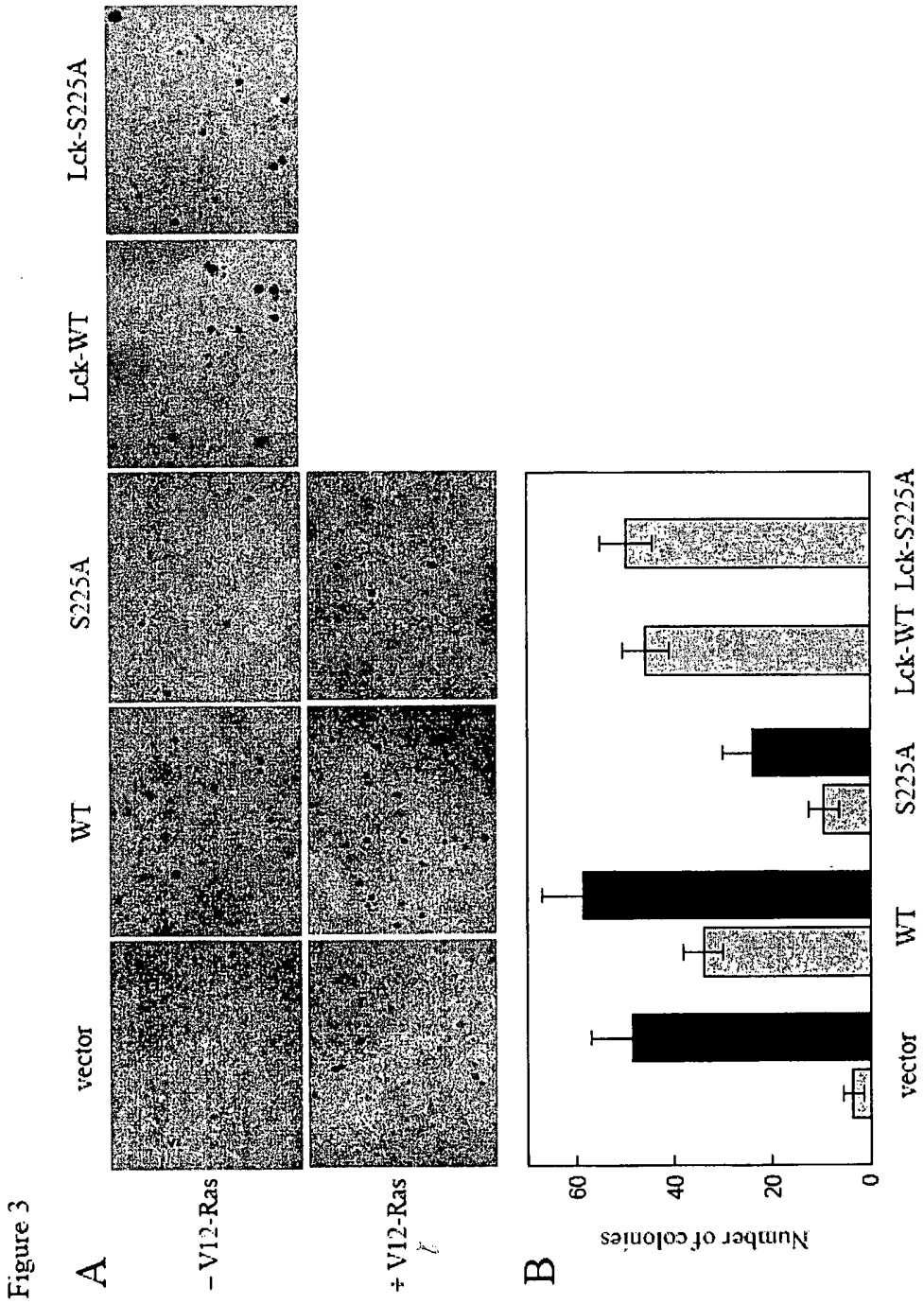
WT
S M
S225A
S M
Lck-WT
S M
Lck-S225A
S M
anti-FLAG
B
WT
Lck-WT
Lck-S225A

Figure 2


A
vector
WT
S225A
Lck-WT
Lck-S225A
– V12-Ras
+ V12-Ras
B
Number of colonies
0
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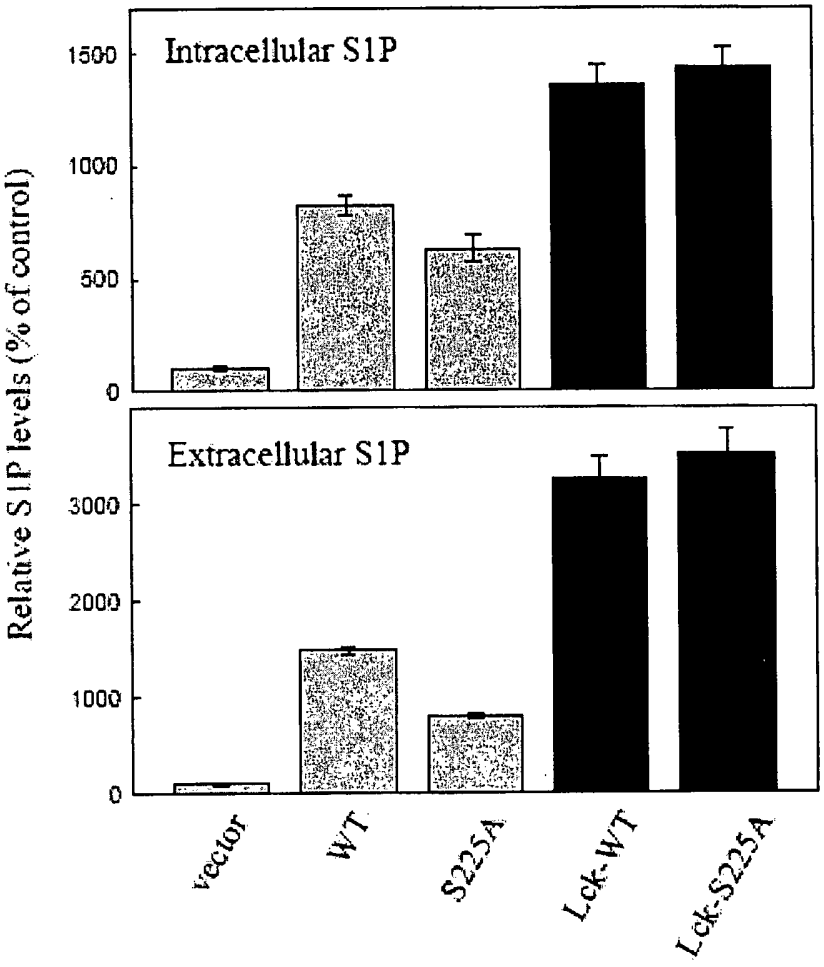
40
60
vector
WT
S225A
Lck-WT
Lck-S225A

Figure 3

Figure 4


Intracellular S1P
Extracellular S1P
Relative S1P levels (% of control)
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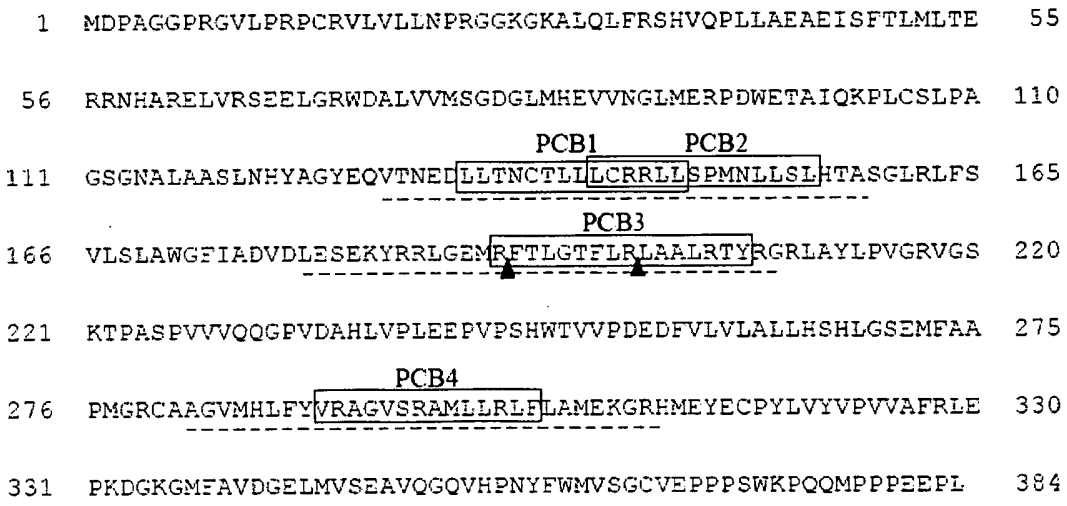
1000
500
0
3000
2000
1000
0
vector
WT
S225A
Lck-WT
Lck-S225A

FIGURE 5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56 RRNHARELVRSEELGRWDALVVMSGDGLMHEVVNGLMERPDWETAIQKPLCSLPA 110
PCB1
PCB2
111 GSGNALAASLNHYAGYEQVTNEDLLTNCTLLLCRRLLSPMNLLSLHTASGLRLFS 165
PCB3
166 VLSLAWGFIADVDLESEKYRRLGEMRFTLGTFLRLAALRTYRGRLAYLPVGRVGS 220
221 KTPASPVVVQQGPVDAHLVPLEEPVPSHWTVVPDEDFVLVLALLHSHLGSEMFAA 275
PCB4
276 PMGRCAAGVMHLFYVRAGVSRAMLLRLFLAMEKGRHMEYECPYLVYVPVVAFRLE 330
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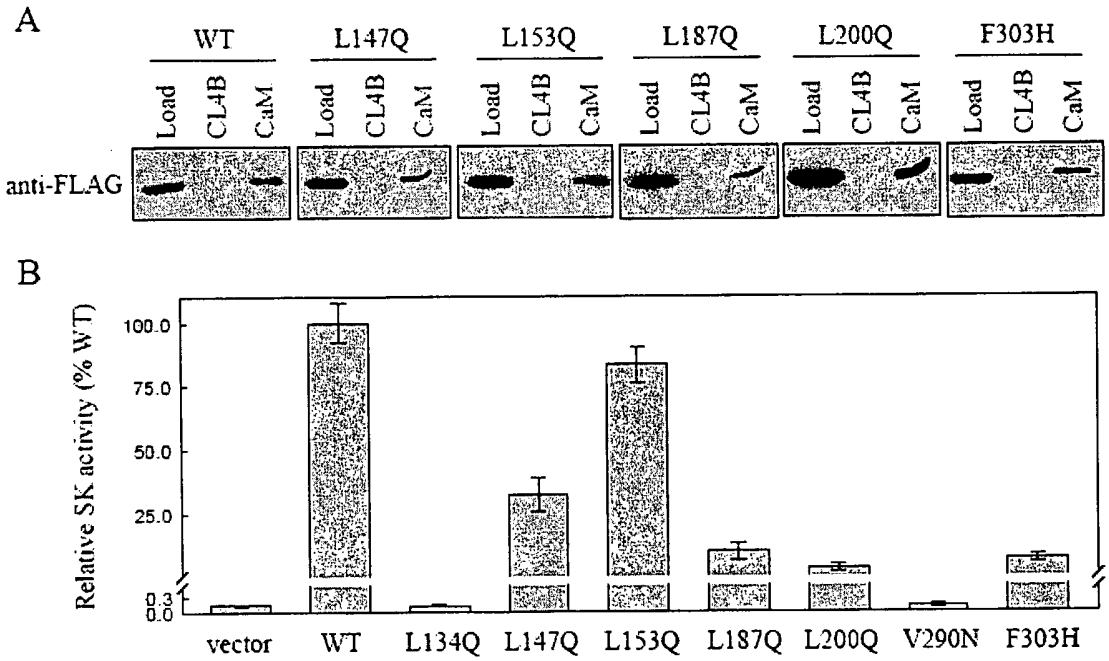
FIGURE 6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L153Q
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L200Q
F303H
Load
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CaM
anti-FLAG
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Relative SK activity (% WT)
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75.0
50.0
25.0
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vector
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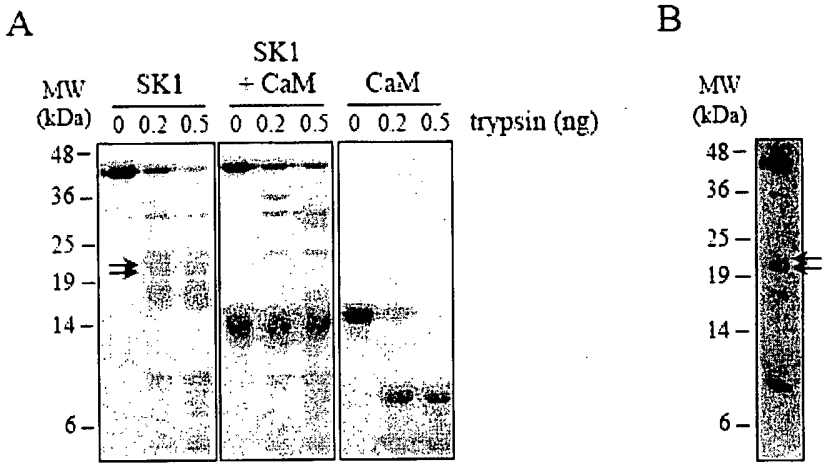
L153Q
L187Q
L200Q
V290N
F303H

FIGURE 7


A
B
MW
(kDa)
SK1
SK1
+ CaM
CaM
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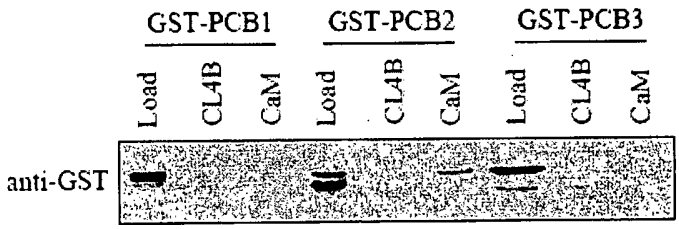
trypsin (ng)
48
36
25
19
14
6
MW
(kDa)
48
36
25
19
14
6

FIGURE 8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GST-PCB2
GST-PCB3
Load
CL4B
CaM
Load
CL4B
CaM
Load
CL4B
CaM
anti-GST

FIGURE 9

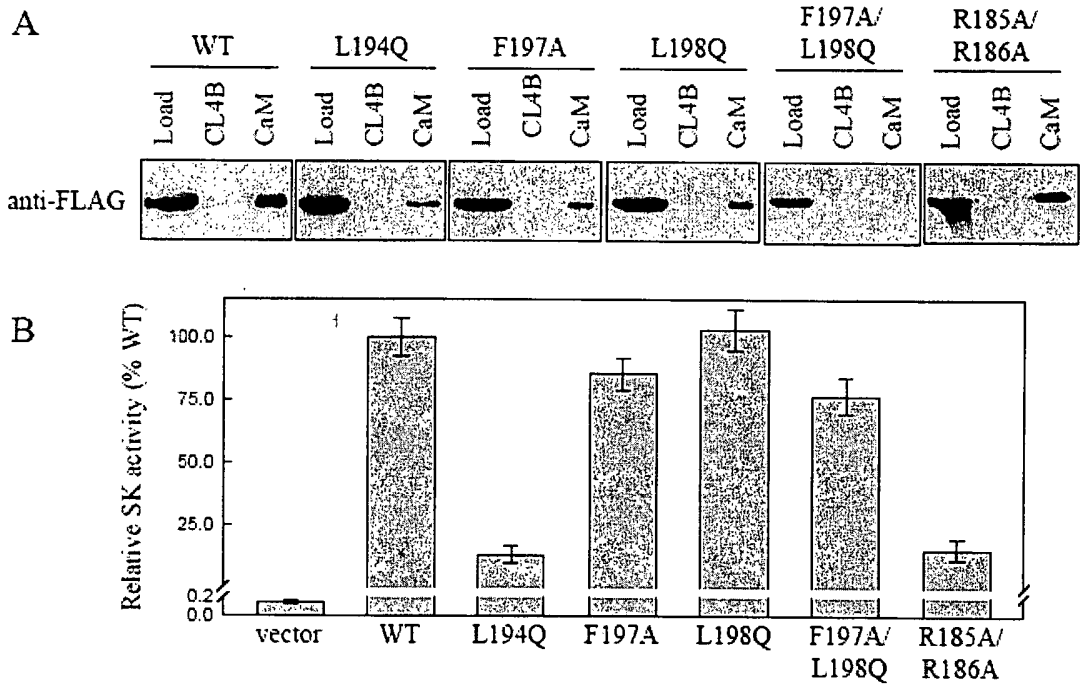
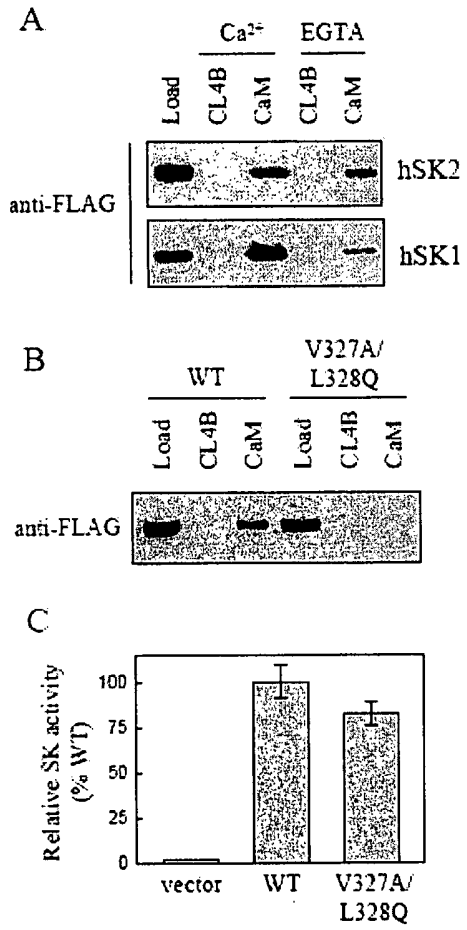

A
WT
L194Q
F197A
L198Q
F197A/ L198Q
R185A/ R186A
Load
CL4B
CaM
anti-FLAG
B
Relative SK activity (% WT)
100.0
75.0
50.0
25.0
0.2
0.0
vector
WT
L194Q
F197A
L198Q
F197A/ L198Q
R185A/ R186A

FIGURE 10


A
Ca2+
EGTA
Load
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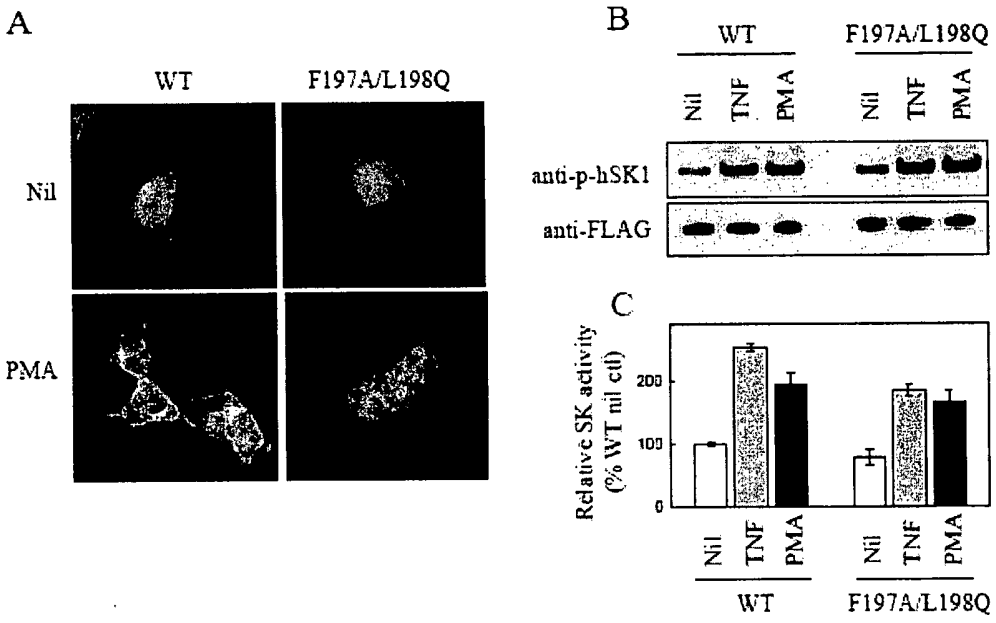
CaM
CL4B
CaM
anti-FLAG
hSK2
hSK1
B
WT
V327A/
L328Q
Load
CL4B
CaM
Load
CL4B
CaM
anti-FLAG
C
Relative SK activity
(% WT)
100
75
50
25
0
vector
WT
V327A/
L328Q

FIGURE 11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F197A/L198Q
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PMA
B
WT
F197A/L198Q
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TNF
PMA
Nil
TNF
PMA
anti-p-hSK1
anti-FLAG
C
Relative SK activity
(% WT nil ctl)
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100
0
Nil
TNF
PMA
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TNF
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WT
F197A/L198Q

FIGURE 12

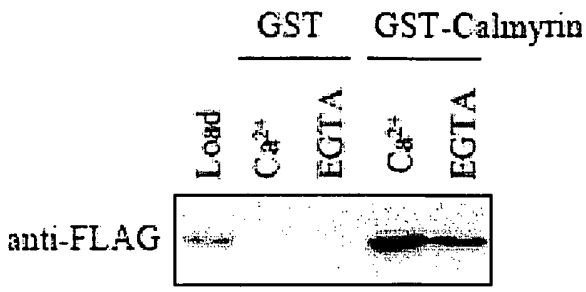
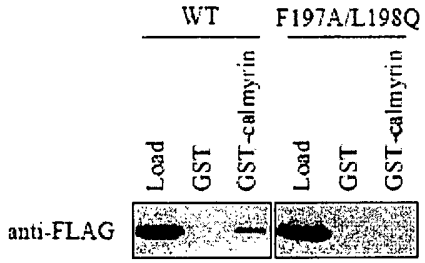


FIGURE 13



SEQUENCE LISTING

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<150> 2004903249

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35 40 45

Phe Thr Leu Met Leu Thr Glu Arg Arg Asn His Ala Arg Glu Leu Val
50 55 60

Arg Ser Glu Glu Leu Gly Arg Trp Asp Ala Leu Val Val Met Ser Gly
65 70 75 80

Asp Gly Leu Met His Glu Val Val Asn Gly Leu Met Glu Arg Pro Asp
85 90 95

Trp Glu Thr Ala Ile Gln Lys Pro Leu Cys Ser Leu Pro Ala Gly Ser
100 105 110

Gly Asn Ala Leu Ala Ala Ser Leu Asn His Tyr Ala Gly Tyr Glu Gln
115 120 125

Val Thr Asn Glu Asp Leu Leu Thr Asn Cys Thr Leu Leu Leu Cys Arg
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Arg Leu Leu Ser Pro Met Asn Leu Leu Ser Leu His Thr Ala Ser Gly
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165 170 175

Val Asp Leu Glu Ser Glu Lys Tyr Arg Arg Leu Gly Glu Met Arg Phe
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Thr Leu Gly Thr Phe Leu Arg Leu Ala Ala Leu Arg Thr Tyr Arg Gly
195 200 205

Arg Leu Ala Tyr Leu Pro Val Gly Arg Val Gly Ser Lys Thr Pro Ala
210 215 220

Ser Pro Val Val Val Gln Gln Gly Pro Val Asp Ala His Leu Val Pro
225 230 235 240

Leu Glu Glu Pro Val Pro Ser His Trp Thr Val Val Pro Asp Glu Asp
245 250 255

Phe Val Leu Val Leu Ala Leu Leu His Ser His Leu Gly Ser Glu Met
260 265 270

Phe Ala Ala Pro Met Gly Arg Cys Ala Ala Gly Val Met His Leu Phe
275 280 285

Tyr Val Arg Ala Gly Val Ser Arg Ala Met Leu Leu Arg Leu Phe Leu
290 295 300

Ala Met Glu Lys Gly Arg His Met Glu Tyr Glu Cys Pro Tyr Leu Val
305 310 315 320

Tyr Val Pro Val Val Ala Phe Arg Leu Glu Pro Lys Asp Gly Lys Gly
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Met Phe Ala Val Asp Gly Glu Leu Met Val Ser Glu Ala Val Gln Gly
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1 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/000856

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61K 38/43, 38/18, 38/19, 38/00, A61P 29/00, 35/00, C12N 9/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
GenBank, EMBL SWISSPROT: SEQ ID 2., & WPIDS, Medline, Chemical Abstracts : Keywords: sphingosine kinase, calmodulin, translocation, transmodulation, calmyrin,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2003/082322 A1 (MEDVET SCIENCE PTY.LTD.) 9 October 2003.	1–76,78-83
X	YOUNG, K. W. et. al., (2003) Ca^{2+}/calmodulin-dependent translocation of sphingosine kinase: role in plasma membrane relocation but not activation. *Cell Calcium*. 33:119-28. (see Abstract and part 3.4)	1,2
X	FUKUDA, Y., et. al. (27 February 2004) Identification of PECAM-1 association with sphingosine kinase 1 and its regulation by agonist-induced phosphorylation. *Biochimica et Biophysica Acta* 1636(1):12-21.	1,2,79
X	TOLAN, D, et. al. (1999) Assessment of the Extracellular and Intracellular Actions of Sphingosine 1-Phosphate by Using the p42/p44 Mitogen-Activated Protein Kinase Cascade as a Model. *Cellular signalling*. 11(5):349-54.	1,2

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 August 2005	11 AUG 2005
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **ALISTAIR BESTOW** Telephone No : (02) 6283 2450

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/000856

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 1999/012533 A1 (MEDVET SCIENCE PTY. LTD.) 18 March 1999. See particularly Examples, claims 1, 2, 9, 10, 15, 17, 23,, 27, 35, 36)	1–76,78-83
X	BLAUKET, A., et. al. (2001) Activation of Sphingosine Kinase by the Bradykinin B2 Receptor and Its Implication in Regulation of the ERK/MAP Kinase Pathway. *Biological Chemistry*. 382:135-9.	1,2
X	MACHWATE, M., et. al. (1998) Sphingosine Kinase Mediates Cyclic AMP Suppression of Apoptosis in Rat Periosteal Cells. *Molecular Pharmacology*. 54:70-7. (see Abstract)	1,2
X	CUVILLIER, O., et. al. (2001) Sphingosine 1-phosphate antagonizes apoptosis of human leukemia cells by inhibiting release of cytochrome *c* and Smac/DIABLO from mitochondria. *Blood* 98(9):2828-36.	1,2
X	WO 2002/098458 A1 (MEDVET SCIENCE PTY LTD) 12 December 2002. (see Claims)	1-76,78-83.
X	GenBank Accession Number : AB049575. 16 March 2002. (see particularly amino acid portion 191-206.)	79-83.
X	GenBank Accession Number : AF068749. 29 September 1998. (see particularly amino acid portion 191-206.)	79-83.
P,X	GenBank Accession Number : BC037710. 30 June 2004. (see particularly amino acid portion 191-206.)	79-83.
A	WO 2001/085953 A1 (MEDVET SCIENCE PTY. LTD.) 15 November 2001	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/000856

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **77.**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The claimed pharmaceutical composition comprises a 'modulatory agent' the scope of which is unknown. It cannot be searched.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2005/000856

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	03082322	AU	2003215426	CA	2480661	EP	1499343
WO	9912533	AU	89658/98	CA	2302838	EP	1011654
		US	6649362	US	2002051777	US	2005074830
WO	02098458	CA	2449487	EP	1404364	US	2005100547

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX