Functional Characterisation of the Human

S1P₄ G Protein-Coupled Receptor

Gillian Holdsworth BSc (Hons)

A thesis submitted in partial fulfilment of the
requirements of the Open University for the degree of
Doctor of Philosophy

September 2003

Department of NCE Biology
Celltech R&D Ltd., 216 Bath Road, Slough,
Berkshire SL1 4EN, U. K.

Molecular Pharmacology Group
Division of Biochemistry and Molecular Biology,
Institute of Biomedical and Life Sciences, University of Glasgow,
Glasgow G12 8QQ, Scotland, U.K.
"...man will occasionally stumble over the truth, but usually manages to pick himself up, walk over or around it, and carry on."

Winston S. Churchill (1874-1965)
Abstract

The human G protein-coupled receptor (GPCR) S1P$_4$ (formerly named Edg6) was identified as an orphan receptor and assigned to the Edg family on the basis of sequence similarity with other Edg receptors. S1P$_4$ was recently shown to couple to G$\alpha_i$ and G$\alpha_{12/13}$ G proteins in response to stimulation by the lysophospholipid, sphingosine-1-phosphate (SIP). This thesis describes functional characterisation of the S1P$_4$ receptor using a modified $[^{35}\text{S}]$GTP$\gamma$S binding assay.

CHO-K1 cells were stably transfected to express S1P$_4$, or a fusion protein between S1P$_4$ and a pertussis toxin-resistant form of G$\alpha_i$. S1P$_4$ exhibited significant constitutive activity and treatment with SIP or phytoSIP further stimulated the receptor in a dose-dependent manner. Pertussis toxin treatment demonstrated that both S1P$_4$ constitutive activity and the effects of SIP were transduced via endogenous G$\alpha_i$ G proteins, whilst the S1P$_4$-G$\alpha_i$ fusion signalled via the tethered, pertussis toxin-insensitive G protein. Residue$^{122}$ was shown to be important in governing S1P$_4$ ligand selectivity; mutation of the naturally occurring glutamic acid to glutamine attenuated the ability of the receptor to respond to SIP and conferred sensitivity to the related compound, lysophosphatidic acid.

Since most cell types express endogenous SIP and LPA family receptors, the profile of Edg family receptor expression in CHO-K1 cells was investigated. Transcripts for S1P$_{1,2,4}$ and LPA$_1$ receptors were detected using RT-PCR with species-specific oligonucleotides.
Further investigations into S1P4 constitutive activity, which were undertaken using an inducible expression system, demonstrated direct correlation between S1P4 expression and constitutive activity. This suggested that S1P4 constitutive activity represents an inherent property of this receptor. In common with other naturally constitutively active GPCRs, S1P4 lacks a generally conserved cysteine residue within the first extracellular loop. It is proposed that this may account for S1P4 constitutive activity and may be relevant to the *in vivo* function of this receptor.
Acknowledgements

Firstly I would like to thank my supervisors for their insight, support, guidance and humour during this project: Professor Graeme Milligan at the University of Glasgow and Dr’s Gill Hutchinson and Martin Perry at Celltech R&D. Dr Ray Owens also supervised the initial phase of this work. I must extend my thanks to Dr Martin Perry for initially suggesting that I do my PhD at Celltech, and to Dr Melanie Lee for agreeing with him.

As is the nature of such a project, there are many people whose willingness to share their considerable expertise must be acknowledged. Mr Craig Carr at the University of Glasgow introduced me to GPCR functional assays and was a general fountain of knowledge. Thanks to Dr Pat Slocombe at Celltech for assistance with all things DNA, being prepared to debate the literature and never being at all grumpy. Dr Derek Brown helped with the fluorescence microscopy and Mr James Snowdon provided assistance with bioinformatics. Dr Jim Turner made the space-filling models and Mr Stuart Bailey co-ordinated synthesis of the FTY720 and AAL analogues (and is probably still trying to work out what he did to deserve this.) Dr Lloyd King and Miss Arjinder Rayet performed LC-MS analysis of in vitro phosphorylation reactions. My colleagues in labs L242 and L236 discussed ideas, helped sort out technical problems and generally put up with me, for which I’m grateful.

I’d like to thank my friends, who showed an interest in what I was doing and wondered why I answered through gritted teeth when they asked if I’d finished yet. Anna, Sarah and Krystyna did a fantastic job of humouring me, talking shop and then reminding me that there was life beyond the lab – cheers guys. I understand the most effective means of thanking you all is by buying the beers. I’m also very grateful to my Mum for proof-reading my thesis.

I must express my gratitude to all of my family for being having faith in me, it means a lot. Finally, a huge thank you to Nigel for always being there and never taking it personally when I was a little preoccupied with work. I couldn’t have done it without you, and I promise I won’t do another one!
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT&lt;sub&gt;1A&lt;/sub&gt;</td>
<td>5-hydroxytryptamine 1A receptor</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;2B&lt;/sub&gt;</td>
<td>5-hydroxytryptamine 2B receptor</td>
</tr>
<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>A (aa)</td>
<td>alanine</td>
</tr>
<tr>
<td>A (nt)</td>
<td>adenine</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AAL</td>
<td>2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol</td>
</tr>
<tr>
<td>α&lt;sub&gt;1A&lt;/sub&gt;AR</td>
<td>α&lt;sub&gt;1A&lt;/sub&gt; adrenergic receptor</td>
</tr>
<tr>
<td>α&lt;sub&gt;2A&lt;/sub&gt;AR</td>
<td>α&lt;sub&gt;2A&lt;/sub&gt; adrenergic receptor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>β&lt;sub&gt;1&lt;/sub&gt;AR</td>
<td>β&lt;sub&gt;1&lt;/sub&gt; adrenergic receptor</td>
</tr>
<tr>
<td>β&lt;sub&gt;2&lt;/sub&gt;AR</td>
<td>β&lt;sub&gt;2&lt;/sub&gt; adrenergic receptor</td>
</tr>
<tr>
<td>β-Arr</td>
<td>β-arrestin</td>
</tr>
<tr>
<td>BGH</td>
<td>bovine growth hormone</td>
</tr>
<tr>
<td>BH3</td>
<td>Bcl-2 homology 3 domain</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>Bq</td>
<td>Becquerel</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>C (aa)</td>
<td>cysteine</td>
</tr>
<tr>
<td>C (nt)</td>
<td>cytosine</td>
</tr>
<tr>
<td>CAM</td>
<td>constitutively active mutant</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CB</td>
<td>cannabinoid</td>
</tr>
<tr>
<td>CCR1</td>
<td>CC chemokine receptor 1</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRLR</td>
<td>calcitonin receptor-like receptor</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>CTR-2</td>
<td>calcitonin receptor 2</td>
</tr>
<tr>
<td>CXCR1</td>
<td>CXC chemokine receptor 1</td>
</tr>
<tr>
<td>D (aa)</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>D&lt;sub&gt;2&lt;/sub&gt;long</td>
<td>dopamine D2 long isoform receptor</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6'-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>dhS1P</td>
<td>dihydrosphingosine-1-phosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMS</td>
<td>dimethylsphingosine</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
</tbody>
</table>
DNA  deoxyribonucleic acid
DNase I  deoxyribonuclease I
dNTP  deoxyribonucleoside triphosphate
dpm  disintegrations per minute
DTT  dithiothreitol
E (aa)  glutamic acid
E. Coli  Escherichia Coli
E/GRE  ecdysone/glucocorticoid receptor elements
EBV  Epstein-Barr virus
EC50  half maximal concentration
ECMV  encephalomyocarditis virus
EcR  ecdysone receptor
Edg  endothelial differentiation gene
EGF  epithelial growth factor
ELC  EBV-induced molecule 1 ligand chemokine
ELISA  enzyme linked immunosorbent assay
eNOS  endothelial nitric oxide synthase
Erk  extracellular signal related kinase
F (aa)  phenylalanine
FACS  fluorescence activated cell sorter
FCS  foetal calf serum
FITC  fluorescein 5(6)-isothiocyanate
FMLP  formyl-methionine-leucine-phenylalanine
FTY720  2-amino-2-[2-(4-octylphenyl)ethyl]-propane-1,3-diol
g  gram
g  gravity
G (nt)  guanine
G protein  guanine nucleotide binding protein
G2A  G2 accumulation G protein-coupled receptor
GAPDH  glyceraldehyde-3-phosphate dehydrogenase
GDP  guanosine 5’-diphosphate
GFP  green fluorescent protein
GPCR  G protein-coupled receptor
GRK  G protein-coupled receptor kinase
GTP  guanosine 5’-triphosphate
GTPyS  guanosine 5’-[γ-thio]triphosphate
HA  haemagglutinin
HEK  human embryonic kidney
HEPES  4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid
HPLC  high performance liquid chromatography
HRP  horseradish peroxidase
HUVEC  human umbilical vein endothelial cell
I (aa)  isoleucine
i.e.  id est (that is)
Ig  immunoglobulin
IPTG  isopropyl β-D-thiogalactopyranoside
IRES  internal ribosome entry site
IUPHAR  International Union of Pharmacology
IVS  intervening sequence
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>R (aa)</td>
<td>arginine</td>
</tr>
<tr>
<td>R (nt)</td>
<td>adenine or guanine</td>
</tr>
<tr>
<td>RAMP</td>
<td>receptor activity modifying protein</td>
</tr>
<tr>
<td>RGS</td>
<td>regulators of G protein signalling</td>
</tr>
<tr>
<td>RH</td>
<td>rat hepatoma</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RSV</td>
<td>rous sarcoma virus</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>S (aa)</td>
<td>serine</td>
</tr>
<tr>
<td>S (nt)</td>
<td>guanine or cytosine</td>
</tr>
<tr>
<td>S1P</td>
<td>sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>SK</td>
<td>sphingosine kinase</td>
</tr>
<tr>
<td>SLC</td>
<td>secondary lymphoid tissue chemokine</td>
</tr>
<tr>
<td>SLC-1</td>
<td>somatostatin-like receptor 1</td>
</tr>
<tr>
<td>SPC</td>
<td>sphingosylphosphorylcholine</td>
</tr>
<tr>
<td>SPP</td>
<td>sphingosine-1-phosphate phosphatase</td>
</tr>
<tr>
<td>SST₁</td>
<td>somatostatin type 1 receptor</td>
</tr>
<tr>
<td>SST₂</td>
<td>somatostatin type 2 receptor</td>
</tr>
<tr>
<td>T</td>
<td>tera</td>
</tr>
<tr>
<td>T (aa)</td>
<td>threonine</td>
</tr>
<tr>
<td>T (nt)</td>
<td>thymine</td>
</tr>
<tr>
<td>TDAG8</td>
<td>T-cell death-associated gene 8</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor α</td>
</tr>
<tr>
<td>TRAF2</td>
<td>TNF receptor-associated factor 2</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V2</td>
<td>vasopressin 2 receptor</td>
</tr>
<tr>
<td>vzg-1</td>
<td>ventricular zone gene 1</td>
</tr>
<tr>
<td>Y (aa)</td>
<td>tyrosine</td>
</tr>
<tr>
<td>Y (nt)</td>
<td>cytosine or thymine</td>
</tr>
<tr>
<td>μ</td>
<td>micro</td>
</tr>
</tbody>
</table>
Chapter 1 – Introduction

1.1 General introduction .................................................................................... 1

1.2 G protein-coupled receptors ........................................................................ 1
   1.2.1 Introduction ........................................................................................... 1
   1.2.2 Structural features of GPCRs ................................................................. 2
   1.2.3 Classification of GPCRs ......................................................................... 4
   1.2.4 Activation of GPCRs ............................................................................. 7
       1.2.4.1 Basic receptor theory ........................................................................ 7
       1.2.4.2 Conformational changes associated with GPCR activation ............. 8
   1.2.5 Heterotrimeric G proteins .................................................................... 12
   1.2.6 Termination of signalling at GPCRs .................................................... 16

1.3 The Edg family of GPCRs ........................................................................... 18
   1.3.1 Introduction to Edg receptors ................................................................ 18
   1.3.2 Classification of Edg receptors ............................................................... 21
   1.3.3 The S1P receptor family ........................................................................ 22
   1.3.4 The LPA receptor family ....................................................................... 29
   1.3.5 S1P4 (Edg6) .......................................................................................... 33
   1.3.6 Other lipid GPCRs ................................................................................ 40

1.4 Sphingolipid signalling ................................................................................ 42
   1.4.1 Sphingosine and ceramide .................................................................... 42
   1.4.2 Sphingosine-1-phosphate ...................................................................... 43
   1.4.3 Phytosphingosine-1-phosphate ............................................................... 52

1.5 FTY720 and SIP receptors .......................................................................... 54
Chapter 2 – Materials and Methods

2.1 General chemicals and materials ......................................................... 61
   2.1.1 Media for bacterial growth .............................................................. 63
   2.1.2 Buffer formulations ....................................................................... 63
   2.1.3 Sources of cDNA ........................................................................... 66
   2.1.4 Bacterial strains ............................................................................. 66
   2.1.5 Antibodies ...................................................................................... 66
   2.1.6 Oligonucleotides ............................................................................ 67
   2.1.7 Mammalian cell lines ..................................................................... 72

2.2 DNA methods ........................................................................................ 72
   2.2.1 PCR amplification of DNA fragments ........................................... 72
   2.2.2 Analysis of DNA fragments by gel electrophoresis ....................... 74
   2.2.3 Spin column purification of PCR products .................................... 75
   2.2.4 Purification of DNA fragments from agarose gels ....................... 75
   2.2.5 Analysis of DNA fragments by restriction enzyme digest .......... 76
   2.2.6 Alkaline phosphatase treatment of digested vector ..................... 76
   2.2.7 Phenol-chloroform extraction of DNA ......................................... 77
   2.2.8 Ligation of DNA fragments ........................................................... 77
   2.2.9 Transformation of DNA into competent *E. Coli* cells ............... 78
   2.2.10 TOPO cloning of PCR products ................................................. 78
   2.2.11 Isolation of plasmid DNA using QIAGEN Miniprep ................. 79
       columns
   2.2.12 Isolation of plasmid DNA using QIAGEN Maxiprep kit ............. 80
   2.2.13 Optical density measurement of DNA ......................................... 80
   2.2.14 DNA sequencing ......................................................................... 81
   2.2.15 Isolation of genomic DNA from mammalian cells .................... 82
   2.2.16 Isolation of total RNA from mammalian cells ......................... 82
   2.2.17 Synthesis of first strand cDNA by RT-PCR ............................... 83

2.3 Cell culture and cell biology methods ................................................ 84
   2.3.1 General cell culture ....................................................................... 84
   2.3.2 Transient transfection of HEK-293 cells ...................................... 85
2.3.3 Generation of stable cell lines ............................................................... 86
2.3.4 Induction of HA-S1P\textsubscript{4} expression in ecdysone-inducible ....... 87
CHO-EcR cell line
2.3.5 Detection of cell-surface proteins (FACS analysis) ............................... 88
2.4.6 Fluorescence microscopy ...................................................................... 89
2.4 Protein methods ........................................................................................ 89
2.4.1 Preparation of cell membranes ............................................................. 89
2.4.2 Determination of protein concentration ............................................... 90
2.4.3 Immunological detection of protein (Western blotting) ........................ 90
2.5 Receptor activity assays .......................................................................... 91
2.5.1 Handling of receptor agonists ............................................................... 91
2.5.2 [\textsuperscript{35}S]GTP\textsubscript{y}S binding assay ........................................ 93
2.5.3 Data analysis ...................................................................................... 94

Chapter 3 - Cloning of Human S1P\textsubscript{4} and Production of
Expression Constructs
3.1 Introduction ............................................................................................. 96
3.2 Results ..................................................................................................... 98
3.2.1 Cloning of HA-tagged S1P\textsubscript{4} ......................................................... 98
3.2.2 Generation of HA-tagged S1P\textsubscript{4}-\textit{G}\textsubscript{\alpha}\textsubscript{11}(C\textsuperscript{351l}) fusion construct .......... 102
3.2.3 Generation of HA-tagged S1P\textsubscript{4} construct for inducible .......... 105
expression
3.2.4 Generation of HA-tagged S1P\textsubscript{4}(E\textsuperscript{122Q}) mutant fused........ 107
with pertussis toxin-insensitive \textit{G}\textsubscript{\alpha}\textsubscript{11}(C\textsuperscript{351l})
3.2.5 Determination of expression of endogenous S1P and ....................... 110
LPA receptors in CHO-K1 cells by RT-PCR
3.3 Discussion .................................................................................................. 125

Chapter 4 – Expression and functional responses of S1P\textsubscript{4}
4.1 Introduction ............................................................................................. 131
4.2 Results ..................................................................................................... 135
4.2.1 Transient expression of \textit{\alpha}_2\textsubscript{\textit{AR}}-\textit{G}\textsubscript{\alpha}\textsubscript{11}(C\textsuperscript{351l}) fusion in HEK-293 .... 135
cells
Chapter 5 – Use of an Inducible Expression System to Examine the Constitutive Activity of S1P4

5.1 Introduction........................................................................................................156
5.2 Results...............................................................................................................161
  5.2.1 Generation of HA-S1P4 inducible expression stable cell line.............161
  5.2.2 Characterisation of the effect of the ecdysone-receptor............... 161
  hormone analogues, ponasterone A and muristerone A,
on cell-surface expression of HA-S1P4
  5.2.3 The effect of cell-surface receptor expression level on .......... 171
  HA-S1P4 basal activity in [35S]GTPγS binding assay
  5.2.4 The effect of cell-surface receptor expression level on S1P-........ 175
  stimulated HA-S1P4 activity in [35S]GTPγS binding assay
5.2.5 Correlation analysis of HA-SIP constitutive and S1P-................. 177
stimulated [35S]GTPγS binding activity with the level of
HA-SIP cell-surface expression

5.3 Discussion ........................................................................................................... 179

Chapter 6 – Investigation into S1P4 Ligand Preference

6.1 Introduction ......................................................................................................... 191
6.2 Results ..............................................................................................................196
  6.2.1 Activation of HA-S1P4 by phS1P in [35S]GTPγS binding......................... 196
  assay
  6.2.2 Inactivity of FTY720 and AAL compounds as HA-S1P4................. 197
  receptor agonists in [35S]GTPγS binding assay
  6.2.3 In vitro phosphorylation of FTY720 and AAL compounds................. 197
  via incubation with rabbit blood
  6.2.4 Stable expression of HA-S1P4(E122Q)-Gα1i(C351I) in CHO-K1 ....... 201
  cells
  6.2.5 Investigation of HA-S1P4(E122Q)-Gα1i(C351I) ligand preference ..... 202
  in [35S]GTPγS binding assay
  6.2.6 The effect of LPA hydrocarbon chain length on activation.......... 205
  of HA-S1P4(E122Q)-Gα1i(C351I) in [35S]GTPγS binding assay
  6.2.7 Dose-dependent stimulation of HA-S1P4(E122Q)-Gα1i(C351I)....... 207
  by 14:0 LPA in [35S]GTPγS binding assay
  6.3 Discussion .......................................................................................................210

Chapter 7 – Final Discussion

7.1 Introduction .....................................................................................................219
7.2 The in vivo role of S1P4 ............................................................................. 223
  7.2.1 S1P4 constitutive activity ................................................................. 224
  7.2.2 Regulation of S1P4 ........................................................................... 225
  7.2.3 Sphingosine-1-phosphate signalling .............................................. 227
7.3 Summary .........................................................................................................229

References .......................................................................................................230

XII
**List of Figures**

1.1 Schematic representation of a GPCR residing in the plasma membrane ........................................... 2

1.2 Phylogenetic tree of selected family A GPCRs ........................................................................... 5

1.3 Schematic representation of the extended ternary complex model ........................................... 10

1.4 GPCRs exist in an ensemble of microstates ........................................................................... 11

1.5 Structures of Edg family ligands ................................................................................... 20

1.6 Phylogenetic tree of Edg family receptors ........................................................................... 22

1.7 Signal transduction by S1P family receptors ........................................................................... 23

1.8 Expression of S1P4 detected in tissue sections ........................................................................... 37

1.9 Synthesis and degradation of S1P ................................................................................... 44

1.10 The ceramide-S1P rheostat ................................................................................... 47

1.11 Structure of phS1P ........................................................................................................ 53

1.12 Structure of FTY720 ........................................................................................................ 55

1.13 Structure of FTY720-P ........................................................................................................ 57

1.14 Structure of (R)-AAL-P ........................................................................................................ 58

3.1 Schematic diagram of the pIRESpuro mammalian expression vector ................................................................. 96

3.2 Schematic diagram of the pIND ecdysone-inducible mammalian expression vector ......................... 97

3.3 Construction of S1P4/pIRES .......................................................................................... 99

3.4 Construction of HA-S1P4/pIRES .................................................................................. 101

3.5 Construction of HA-S1P4(No Stop)/pIRES .......................................................................... 103

3.6 Construction of Gα11(C351)/pIRES .................................................................................. 104

3.7 Construction of HA-S1P4-Gα11(C351)/pIRES .......................................................................... 106

3.8 Construction of HA-S1P4/pIND .................................................................................. 107

3.9 Construction of HA-S1P4(E122Q)-Gα11(C351)/pIRES ..................................................... 109

3.10 PCR amplification of partial S1P and LPA receptor sequences from Chinese hamster genomic DNA .................................................................................................................. 112

3.11 Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA .................................................................................................................. 113
3.12 Expression of S1P and LPA receptor mRNA in CHO-K1 cells by RT-PCR

4.1 Schematic diagram showing guanine nucleotide exchange cycle on Gαβγ G proteins

4.2 Expression of α2AAR-Gα1(C351I) in HEK-293 cells

4.3 Comparison of different anti-G protein antisera in [35S]GTPγS binding assay

4.4 Expression of HA-S1P4 in CHO-K1 cells

4.5 Determination of optimal GDP concentration using HA-S1P4 in [35S]GTPγS binding assay

4.6 Optimisation of response of HA-S1P4 in [35S]GTPγS binding assay

4.7 Constitutive activity of HA-S1P4 after expression in CHO-K1 cells

4.8 Concentration dependent stimulation of HA-S1P4-mediated [35S]GTPγS binding by S1P

4.9 Expression of HA-S1P4-Gα1(C351I) in CHO-K1 cells

4.10 Determination of optimal GDP concentration using HA-S1P4-Gα1(C351I) in [35S]GTPγS binding assay

4.11 Optimisation of response of HA-S1P4-Gα1(C351I) in [35S]GTPγS binding assay

4.12 Constitutive activity of HA-S1P4-Gα1(C351I) expressed in CHO-K1 cells

4.13 Concentration dependent stimulation of HA-S1P4-Gα1(C351I)-mediated [35S]GTPγS binding by S1P

5.1 Schematic diagram describing the ecdysone-inducible mammalian expression system

5.2 Structures of the ecdysone receptor hormone analogues, ponasterone A and muristerone A

5.3 Dot plot analysis of parental CHO-EcR cells

5.4 Concentration dependent induction of cell-surface receptor expression by ponasterone A treatment of ecdysone-inducible HA-S1P4-transfected CHO-EcR cells
5.5 Concentration dependent induction of cell-surface receptor expression by muristerone A treatment of ecdysone-inducible HA-S1P₄-transfected CHO-EcR cells

5.6 Induction of cell-surface receptor expression by ponasterone A treatment of ecdysone-inducible HA-S1P₄-transfected CHO-EcR cells detected by fluorescence microscopy

5.7 Time dependent induction of cell-surface receptor expression by ponasterone A and muristerone A treatment of ecdysone-inducible HA-S1P₄-transfected cells

5.8 Analysis of receptor expression in ecdysone-inducible HA-S1P₄-transfected CHO-EcR cells using Western blotting

5.9 Constitutive activity of HA-S1P₄ expressed in ecdysone-inducible CHO-EcR cells

5.10 Constitutive activity of HA-S1P₄ is not influenced by the presence of ponasterone A

5.11 S1P-stimulated activity of HA-S1P₄ expressed in ecdysone-inducible CHO-EcR cells

5.12 Correlation of HA-S1P₄ constitutive- and S1P-stimulated activity from membranes prepared from ecdysone-inducible CHO-EcR cells with percentage of cells deemed to express HA-S1P₄

5.13 Model to represent the effect of ponasterone A concentration on cell-surface HA-S1P₄ expression in ecdysone-inducible CHO-EcR cells

6.1 Structures of three naturally occurring forms of LPA

6.2 Models of LPA analogues and S1P

6.3 Concentration dependent stimulation of HA-S1P₄-mediated [³⁵S]GTPγS binding by phS1P

6.4 FTY720 and AAL compounds are unable to stimulate HA-S1P₄-mediated [³⁵S]GTPγS binding

6.5 Conversion of FTY720 and AAL compounds to phosphate metabolites via in vitro incubation with rabbit blood

6.6 Snake plot of S1P

6.7 Expression of HA-S1P₄(E¹²²Q)-Gα₁₁(C³⁵¹I) in CHO-K1 cells
6.8 Ligand preference of HA-S1P₄(E₁²²Q)-Gα₁₁(C³⁵¹I) in [³⁵S]GTPγS binding assay
6.9 The effect of acyl chain length on stimulation of HA- S1P₄(E₁²²Q)- Gα₁₁(C³⁵¹I)
6.10 Concentration dependent stimulation of HA-S1P₄(E₁²²Q)- Gα₁₁(C³⁵¹I)-mediated [³⁵S]GTPγS binding by 14:0 LPA
List of Tables

1.1 Summary of G protein $\alpha$ subunit actions ...................................................... 13
1.2 Summary of G protein activation by Edg family members .............................. 20
1.3 Summary of Edg family receptor classification consistent with IUPHAR guidelines 21
1.4 Sequence identity between S1P$\gamma_4$ and other S1P and LPA receptors .......... 34
1.5 Summary of tissues and cells reported to express S1P$\gamma_4$............................... 36
2.1 Oligonucleotides and PCR programs used to amplify S1P and LPA receptor gene fragments from Chinese hamster genomic DNA or first strand cDNA 74
3.1 Oligonucleotides used to clone fragments of S1P and LPA receptor genes from Chinese hamster genomic DNA ................................................................. 111
3.2 Summary of similarities between cloned partial Chinese hamster S1P and LPA receptor sequences and those from mouse, rat and human 121
3.3 Oligonucleotides used for RT-PCR analysis of CHO-K1 mRNA to identify endogenously expressed S1P and LPA receptor genes .................. 122
3.4 Summary of S1P and LPA expression data in ovarian tissues and CHO-K1 cells 129
5.1 Summary of ecdysone analogue potency for induction of HA-S1P$\gamma_4$ expression in HA-S1P$\gamma_4$-transfected ecdysone-inducible CHO-EcR cells .......... 166
5.2 Summary of statistical analyses performed on data describing the relationship between the percentage of cells expressing HA-S1P$\gamma_4$ and [$^{35}$S]GTP$\gamma$S binding attributable to the presence of this receptor 177
Chapter 1 – Introduction

1.1 General introduction

The activity of virtually every cell in the body is regulated by extracellular stimuli, which provide signals that are transmitted via a complex array of plasma membrane-associated receptors and intracellular proteins. A large proportion of these cell-surface receptors, including the SIP₄ receptor which is the topic of this thesis, belong to the G protein-coupled receptor (GPCR) superfamily. In this chapter, the GPCR family will be introduced before the SIP₄ receptor is considered within the context of the Edg receptor family, of which it is a member. The nature of the ligands which act at SIP₄ and their routes of production in vivo will be reviewed and the aims of this thesis will be outlined.

1.2 G protein-coupled receptors

1.2.1 Introduction

G protein-coupled receptors are integral membrane proteins which are found in a wide variety of organisms, including yeast [Marsh and Herskowitz, 1988] and the slime mould Dictyostelium discoideum [Ueda et al., 2001], the plant Arabidopsis thaliana [Josefsson and Rask, 1997] and higher mammals such as man. The GPCR superfamily represents one of the largest protein families in the human genome. The first human GPCR was cloned in 1986 [Dixon et al., 1986] and since this event, research in the field has continued and the range of techniques which can be employed in the analysis of the functions and properties of receptors has expanded. GPCRs are the subject of much industrial research since they are involved in many normal physiological and pathophysiological
Chapter 1

processes and it has been estimated that 50 % of marketed drugs exert their actions via interactions with GPCRs [Howard et al., 2001].

1.2.2  **Structural features of GPCRs**

GPCRs share common structural elements which may be illustrated by analysis of their amino acid sequences. Each GPCR sequence contains seven distinct regions of hydrophobic residues, which are believed to form α-helices and span the cellular membrane. These helices are connected by intracellular and extracellular loops comprised of hydrophilic amino acids. The amino-terminal region of a GPCR is located outside of the cell, whilst the carboxy-terminal tail resides intracellularly. Figure 1.1 shows a schematic representation of a GPCR located within the plasma membrane.

**Figure 1.1**

**Schematic representation of a GPCR residing in the plasma membrane.**

This diagram illustrates the seven membrane-spanning α-helices of a GPCR. These are linked via intra- and extracellular loop regions. The amino-terminal tail of the receptor is located extracellularly whilst the carboxy-terminal tail is situated intracellularly.

The highest degree of sequence homology between GPCRs is observed within the transmembrane (TM) regions and each TM domain is comprised of between approximately 20-27 amino acids. The sequences of the loops and tails of
different GPCRs tend to be far more divergent. The size of the amino-terminal region varies and can contain between ten and 500 residues, whilst the loop sections can consist of as few as five amino acids to greater than 200 residues and the carboxy-terminal tail between 12 and 400 residues. In spite of their common structural elements, GPCRs are activated by myriad structurally diverse molecules including photons, odourants, neurotransmitters, nucleotides, lipids and amines, to polypeptides and proteins.

Direct study of the three-dimensional structures of GPCRs has not been as straightforward as it has been for cytosolic proteins such as non-receptor tyrosine kinase enzymes, due to the transmembrane spanning nature of GPCRs. Much of what was known about GPCR structure was inferred from studies of bacteriorhodopsin (reviewed by Khorana, 1988; Gether, 2000), a light activated proton pump expressed in the salt-loving archaebacterium *Halobacterium halobium*. This protein possesses seven α-helices, which span the membrane and, like vertebrate rhodopsin, uses the molecule retinal as a chromophore. In contrast, however, to mammalian GPCRs, bacteriorhodopsin is not coupled to G proteins and shares little sequence similarity with known GPCRs.

In 2000, the structure of rhodopsin, the first GPCR to be crystallised, was recorded at a resolution of 2.8 Å [Palczewski et al., 2000]. The crystal structure of this inactive receptor was a highly organised heptahelical transmembrane bundle, with the seven α-helices arranged in an anticlockwise manner when viewed from the extracellular perspective. This structure has provided an improved template for the analysis of GPCR structure-function studies and computational modelling of receptors for use in drug discovery activities.
1.2.3 Classification of GPCRs

Over recent years, estimates of the number of GPCRs in the human genome have ranged from several hundred to greater than 1000. It is possible to categorise GPCRs into “endoGPCRs”, which are activated by endogenous molecules, and “chemosensory GPCRs”, which respond to exogenous stimuli such as odours and pheromones. A recent survey calculated there to be 367 endoGPCRs in the human genome, of which the majority have known ligands [Vassilatis et al., 2003]. The remaining GPCRs have no known ligand and are therefore classified as orphan receptors. There has been much interest in the identification of ligands for these orphan receptors, since many may represent novel therapeutic targets (reviewed by Stadel et al., 1997; Murphy et al., 1998; Wilson et al., 1998; Howard et al., 2001).

GPCRs may be classified into four distinct categories using phylogenetic analysis. Family A (rhodopsin-like) contains the majority (284) of endoGPCRs, which are activated by peptides (e.g. chemokine, bradykinin and orexin receptors) and small molecules such as neurotransmitters (e.g. dopamine, serotonin and acetylcholine receptors) and lipids (e.g. S1P, LPA and cannabinoid receptors) or were classed as orphan receptors (98). Family B (secretin/glucagon) comprises 50 GPCRs, of which 16 are activated by peptides; the remainder were orphans. Family C (metabotropic glutamate) receptors number 17, including 6 orphans, whilst family D receptors numbered 10 with no orphans [Vassilatis et al., 2003].

Of the four families, family A, to which the S1P receptor family belongs, is the
largest and best characterised. A phylogenetic tree of some family A receptors is shown in Figure 1.2.

![Phylogenetic tree of selected family A GPCRs.](image)

**Figure 1.2**
**Phylogenetic tree of selected family A GPCRs.**

A number of highly conserved motifs are found within family A GPCRs, even though the overall homology between family members is quite low. This receptor family contains several proline residues present in TM domains IV, V, VI and VII, which probably introduce kinks into the helices. The conserved
D/ERY motif is located at the intracellular end of TM III and appears to be important in receptor activation. The arginine residue of this amino acid sequence is the only residue to be absolutely conserved throughout family A. Many family A receptors possess two cysteine residues: one present within the second extracellular loop and the other located at the top of TM III. The disulphide bridge which forms between these two residues serves to subdivide this extracellular loop into two smaller loops and may function to constrain the transmembrane bundle.

The extracellular amino-terminal tail of family A receptors varies in length and frequently contains a number of N-linked glycosylation motifs (N-X-T/S). The role of this post-translational modification remains unclear but may influence transportation of protein to the cell membrane after its synthesis in the endoplasmic reticulum as well as stabilising the protein on the cell-surface [Fiedler and Simons, 1995]. The length of the carboxy-terminal intracellular tail is also highly variable but it is typically rich in serine and threonine residues, which are sites for phosphorylation by intracellular kinases. The intracellular tail of family A GPCRs also contains one or more cysteine residues: these are sites of palmitoylation which causes tethering of the C-terminal tail to the cytoplasmic face of the plasma membrane to create a fourth intracellular loop [Wess, 1998].

The site of ligand binding for family A receptors is dependent on the nature of the ligand. Small ligands such as biogenic amines, purines, eicosanoids and lipids are believed to bind within the transmembrane crevice whilst peptide and protein ligands appear to interact with the extracellular loop and tail regions of the receptor.
1.2.4 Activation of GPCRs

1.2.4.1 Basic receptor theory

Ligands of GPCRs possess two fundamental properties: affinity and efficacy. The term affinity describes how well that ligand binds to the receptor, whilst the property of efficacy depicts the extent of the change in activation status of the receptor induced by that ligand. It is possible to determine the affinity of a ligand for a receptor using a technique such as radioligand binding, which allows calculation of the apparent dissociation constant ($K_d$) of that ligand for that receptor. If that ligand induces a functional response after binding, it is considered to display positive efficacy and is therefore defined as an agonist. In the current study, the term efficacy is used in the context of relative efficacy, and provides a description of the maximal response induced by that agonist, expressed as a percentage of that achieved by a full agonist (in this case S1P for the S1P$_4$ receptor). An agonist that exhibits an relative efficacy of less than 100% is termed a partial agonist. Functional assays such as $[^{35}S]$GTP$_\gamma$S binding, adenylyl cyclase activation and receptor internalisation provide a means to measure the consequences of a ligand binding to a receptor and are used to determine the potency of a ligand. Agonist potency is described by the EC$_{50}$ value, which represents the concentration of agonist required to elicit 50% of the maximal response in that system. If a ligand merely binds a receptor and in so doing, prevents activation of that receptor by an agonist, it has zero efficacy and is therefore classed as an antagonist. In recent years, many molecules that were thought to be antagonists have been reclassified as inverse agonists. This reflects the ability of a receptor system to display activity in the absence of agonist stimulation (the phenomenon of constitutive activity, discussed in this chapter,
section 2.4.3). An inverse agonist possesses negative efficacy and is able to reduce receptor constitutive activity (reviewed by Milligan et al., 1995; de Ligt et al., 2000).

1.2.4.2 Conformational changes associated with GPCR activation

Despite the diversity amongst ligands which activate members of the GPCR superfamily, the underlying fundamental mechanism of activation of these proteins appears to have been conserved throughout evolution. This allows a receptor activated by a small molecule to stimulate the same intracellular signalling pathways via the same class of G proteins, as a receptor activated by a protein. Regardless of the location of the ligand binding pocket, GPCR activation is achieved via conformational change.

GPCRs appear to preferentially exist in an inactive conformation, which is likely to be the result of evolutionary pressure and is mediated by constraining intramolecular interactions. This concept resulted from a landmark study of the α1β-adrenergic receptor [Kjelsberg et al., 1992] in which mutation of A293 to any other amino acid conferred a constitutively active phenotype to this receptor. It was subsequently shown that a constitutively active mutant of the β2-adrenergic receptor was structurally unstable. Hence it seemed probable that receptor activation as a consequence of agonist binding or the introduction of a constitutively activating mutation reflected the release of inactivating constraints.

The highly conserved D/ERY motif present within TM III of family A receptors may be involved in initiating the conformational change which occurs as a consequence of GPCR activation. Protonation of the aspartic or glutamic acid residue within this motif promotes rotation of TM VI [Gether, 2000]. Movement
of this transmembrane domain, along with TM III and possibly TM VII seems to be critical for the transition of a GPCR to the activated state (reviewed by Wess, 1998; Gether, 2000).

1.2.4.3 Constitutive activity of GPCRs

Traditional receptor theory proposed the existence of a single quiescent receptor state in which binding of an agonist promoted a conformational change in the receptor to yield the activated, or functional state. Recent evidence suggests that GPCRs have the potential to be active in the absence of agonist stimulation; this phenomenon is known as constitutive activity (reviewed by Leurs et al., 1998; de Ligt et al., 2000; Gether, 2000).

The discovery of agonists which possessed negative efficacy (defined in this chapter, section 2.4.1) implied the existence of a pre-existing equilibrium between two receptor states, in which one receptor state was associated with G proteins (and hence may be thought of as being active) and the second state was as a free (or inactive) receptor. Based on this assumption, the mode of action of the inverse agonist was to inhibit the spontaneous interaction of the receptor with G protein.

In a landmark study of receptor constitutive activity, replacement of residues 266-272 of the \( \beta_2 \)-AR with the homologous region of the \( \alpha_{1b} \)-AR resulted in agonist-independent receptor signalling [Samama et al., 1993]. The authors determined that the established ternary complex model of receptor activation was unable to mathematically describe the experimental results obtained with the constitutively active \( \beta_2 \)-AR. The ternary complex model was therefore extended so that it could account for constitutive activity. This model (shown in Figure
1.3) assumes that the receptor exists in an equilibrium between two states, the uncoupled inactive receptor (R) and the active state (R*). Only the active R* form is capable of binding G protein and so the state AR*G represents the sole ternary complex which may be formed. The extent of spontaneous isomerisation between R and R* is described by the isomerisation constant (J). An increase in the term J therefore accounts for enhanced basal activity observed with constitutively active receptors.

\[
\begin{align*}
    A + R + G & \xrightleftharpoons{\beta J} AR + G \\
    A + R^* + G & \xrightleftharpoons{\beta K} AR^* + G \\
    A + R^*G & \xrightleftharpoons{\alpha M} ARG
\end{align*}
\]

**Figure 1.3**

**Schematic representation of the extended ternary complex model.**

This theoretical model describes the interactions of receptor which is considered to exist in equilibrium between the resting state (R) and the active state (R*), with agonist (A) and G protein (G). Equilibria are described by the various constants shown. Reproduced from [Samama *et al.*, 1993].

Contrary to the traditional concepts, where receptors were thought to be “on” or “off”, it seems likely that GPCRs are mobile proteins which are subject to constant local unfolding and refolding reactions. Each of these reactions has the capacity to subtly change the receptor conformation and hence a GPCR may be considered to exist in an ensemble of conformational states [Kenakin, 2002].
This range of conformations may be represented by a Gaussian distribution. In the absence of agonist, this population may be considered to exist in the "inactive" state. After agonist stimulation, the population shifts and is in the "G protein bound state" which allows activation of G protein and hence propagation of the signal. These two states may intersect (Figure 1.4) and the population of receptors within this intersection may spontaneously activate G protein without the need for agonist activation and hence represent the property of GPCR constitutive activity [Kenakin, 2002].

![Figure 1.4](image)

**Figure 1.4**
**GPCRs exist in an ensemble of microstates.**
The inactive (non-signalling) and G protein bound (signalling) states are shown as two normal distributions. The shaded intersection between the two ensembles represents the conformations common to the two states. Receptors nominally in the inactive state that fall within this intersection can spontaneously activate G protein and hence represent GPCR constitutive activity.

Even though only a small proportion of the population of states of a given receptor may be in the constitutively active conformation, it is often possible to detect agonist-independent signalling simply by over-expressing that receptor. It has been suggested that many GPCRs possess a degree of basal activity and that receptor density is directly related to constitutive activity. Many studies of this nature have been reported (for example see Casarosa et al., [2001]). The
physiological relevance of constitutively active receptors is unclear, but it may be related to the concept of tonic regulation of physiological systems by receptors. Tonic regulation had previously been explained by the presence of an endogenous agonist and its continued interaction with that receptor system; however, the realisation that receptors possess constitutive activity sheds new light on this idea. It is tempting to speculate that physiological tone mediated by GPCRs may be mediated by receptor basal activity as an addition to, or instead of, the action of endogenous agonists.

1.2.5 Heterotrimeric G proteins

Guanine nucleotide binding proteins (G proteins) couple membrane bound GPCRs to their cellular effector systems. GPCR-mediated signal transduction is considered to progress via activation of G proteins. Recently however, it was reported that a mutant of the angiotensin II type 1 (AT1a) receptor which lacked the ability to couple to G proteins was able to activate the ERK pathway [Seta et al., 2002]; so it is possible that a GPCR can still transmit information to cells even if interaction with their cognate G protein is prevented.

Heterotrimeric G proteins have been said to be “involved in everything from sex in yeast to cognition in humans” [Marx, 1994]. The G protein heterotrimer comprises α, β and γ subunits, which are the products of distinct genes. When stimulated, G proteins undergo a cycle of activation and deactivation in which the α subunit and βγ heterodimer are able to interact with their reaction partners (such as the GPCR itself, guanine nucleotides and effector molecules). During this cycle, the inactive, GDP bound G protein heterotrimer interacts with an activated receptor. This causes dissociation of bound GDP from the Gα subunit,
which is replaced by GTP present within the cellular cytosol. Binding of GTP promotes a conformational change within the \(\mathrm{G}\alpha\) subunit, causing dissociation of this subunit from the \(\beta\gamma\) heterodimer and allowing interaction of the GTP-bound \(\mathrm{G}\alpha\) and \(\beta\gamma\) subunits with effector molecules. Intrinsic GTPase activity of the \(\mathrm{G}\alpha\) subunit cleaves the terminal phosphate group from the bound GTP, converting it to GDP and permitting reassociation of the \(\mathrm{G}\alpha\) \(\mathrm{G}\) protein with the \(\beta\gamma\) heterodimer, which causes deactivation of the \(\mathrm{G}\) protein subunits.

At least 20 \(\mathrm{G}\) protein \(\alpha\) subunits, encoded by 17 different genes, have been identified. These may be divided into the four families: \(\mathrm{G}\alpha_5\), \(\mathrm{G}\alpha_i/o\), \(\mathrm{G}\alpha_q/11\), \(\mathrm{G}\alpha_{12/13}\), which couple to different effector systems (summarised in Table 1.1).

<table>
<thead>
<tr>
<th>Family</th>
<th>Effector systems regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mathrm{G}\alpha_5)</td>
<td>Stimulates adenylyl cyclase isoforms</td>
</tr>
<tr>
<td>(\mathrm{G}\alpha_{i/o})</td>
<td>Further divided into (\mathrm{G}\alpha_{i/o/z}) and (\mathrm{G}\alpha_{i/o/g}) sub groups</td>
</tr>
<tr>
<td>(\mathrm{G}\alpha_{i/o/g})</td>
<td>(\mathrm{G}\alpha_{i/o/g}) subunits inhibit some adenylyl cyclase isoforms</td>
</tr>
<tr>
<td>(\mathrm{G}\alpha_q)</td>
<td>(\mathrm{G}\alpha_q) activates (\beta)-isoenzymes of phospholipase C and non-receptor</td>
</tr>
<tr>
<td>(\mathrm{G}\alpha_{12/13})</td>
<td>Regulates low molecular weight Rho family (\mathrm{G}) proteins</td>
</tr>
</tbody>
</table>

Table 1.1
Summary of G protein \(\alpha\) subunit actions.

Activation of adenylyl cyclase and formation of cAMP is the most well established paradigm for second messenger generation in response to GPCR activation and is mediated by the \(\mathrm{G}\alpha_5\) class of \(\mathrm{G}\) protein. Activation of a \(\mathrm{G}\alpha_{i/o}\) subunit inhibits cAMP formation via inhibition of adenylyl cyclase. There are three distinct \(\mathrm{G}\alpha_4\) species: \(\mathrm{G}\alpha_{41}\), \(\mathrm{G}\alpha_{42}\) and \(\mathrm{G}\alpha_{43}\). Subunits of the \(\mathrm{G}\alpha_q/11\) class couple to various isoforms of phospholipase C (PLC) and include \(\mathrm{G}\alpha_q\), \(\mathrm{G}\alpha_{11}\),
Chapter 1

The Ga family appear to be involved in activation of the low molecular weight G protein, Rho (reviewed by Sah et al., [2000]).

 Activation of Ga and Gβγ subunits can affect the activity of a whole host of effector molecules and/or ion channels. Thus, the pattern of effects which result from receptor activation are complex. Additionally, cells may express multiple receptors that respond to the same ligand by activating opposing signalling pathways. It has also been suggested that the actions of different agonists at the same receptor can activate different G proteins (the phenomenon of agonist trafficking, [Kenakin, 2002; Hermans, 2003]).

Clearly, the ability of a given GPCR to propagate a signalling response via activation of a particular G protein requires interaction between receptor and G protein. Investigations into the regions of the receptor involved in mediating such interactions have been obtained through the analysis of hybrid receptors constructed between functionally distinct GPCRs. Amino acids present within the second and third intracellular loops appear to be essential to confer selectivity of G protein recognition [Wess, 1998; Hamm, 2001]. Similarly, efforts have been made to understand the areas of the Ga subunit which form contacts with the active GPCR. The five carboxy-terminal amino acids of Ga subunits are key in controlling the specificity of receptor-G protein coupling [Wess, 1998]. Chimeric Ga G proteins in which these five amino acids are replaced with those from a different class of G protein have been used to stimulate pathways that would not normally be activated by that particular receptor [Milligan and Rees, 1999; Kowal et al., 2002]. For example, expression of a Gaq/i chimera (which is a Gaq backbone containing the final five amino acids from Ga) permits
stimulation of PLC\(\beta\) by the dopamine D2 or adenosine A1 receptors, which normally couple exclusively to G\(\alpha\)i G proteins [Wess, 1998].

Many G\(\alpha\) subunits have been shown to be expressed almost ubiquitously in mammalian cells, with exceptions such as G\(\alpha\)o, which is expressed in brain, and G\(\alpha\)16, expression of which is restricted to B and T cells [Offermanns and Simon, 1995; Grant et al., 1997]. The murine G protein, G\(\alpha\)15 and its human orthologue, G\(\alpha\)16, are promiscuous G proteins which are thought to be able to couple to most GPCRs [Stables et al., 1997] and hence are often employed when attempts are made to assign ligands to orphan receptors. The likelihood of the orphan receptor coupling to the promiscuous G protein is high and any activation of the receptor may be conveniently measured using calcium flux assays.

Various exogenous agents can interfere with the interaction between G proteins and GPCRs, or can modulate the activity of the G protein itself. Such reagents may be useful in the elucidation of the nature of G proteins activated by a particular receptor. The bacterium Bordetella pertussis, the causative agent of whooping cough, produces a number of toxins of which one, pertussis toxin (also named islet activating protein), inactivates G proteins of the G\(\alpha\)i class via ADP-ribosylation. This covalent modification serves to prevent interaction of an agonist- (or constitutively-) activated receptor with GDP-bound G protein. However, pertussis toxin treatment does not affect the intrinsic ability of the G\(\alpha\) subunit to exchange guanine nucleotides or to hydrolyse GTP (reviewed by Milligan [1988]). The location of G\(\alpha\)i G protein subunit ADP-ribosylation by pertussis toxin is a conserved cysteine residue present near the carboxy-terminus.
of the protein. Mutation of this residue to any other amino acid has been shown to confer resistance to the actions of this toxin [Bahia et al., 1998].

Another exotoxin, in this case isolated from culture supernatants of *Vibrio cholerae*, modulates Go\textsubscript{s} G protein activity. Like pertussis toxin, cholera toxin also possess ADP-ribosyltransferase activity which catalyses the transfer of ADP-ribose to an arginine residue present in the Go\textsubscript{s} subunit. This modification stabilises the GTP-bound form of the \( \alpha \) subunit of G\textsubscript{s}, and as the intrinsic GTPase activity of the subunit is inhibited, the Go\textsubscript{s} subunit is in a permanently activated state.

1.2.6 Termination of signalling at GPCRs

Activation of a GPCR leads to its interaction with G protein, which becomes stimulated as a result of this interaction. In its active conformation, a GPCR may stimulate many G protein molecules resulting in amplification of the initial stimulus. Negative regulation of responses to agonists is clearly necessary to allow control of such systems.

The lifetime of the signalling event is dictated by the longevity of the G\( \alpha \)-GTP species, which is dependent on the intrinsic GTPase activity of the G\( \alpha \) subunit. This activity promotes hydrolysis of GTP back to GDP and allows reassociation of the G\( \alpha \) subunit with the \( \beta \gamma \) heterodimer to form the inactive, GDP-bound heterotrimeric G protein. However, this is not normally sufficient to account for the rapid termination of signalling which is observed in most systems. Recently, the actions of a family of RGS (regulators of G protein signalling) proteins have been shown to further attenuate signalling mediated via G\( \alpha \)\textsubscript{i} or G\( \alpha \)\textsubscript{q} G proteins.
Interaction between RGS proteins and the GTP-bound Go subunit causes a significant increase (100-1000 fold) in the GTPase activity of the target Go subunit and therefore silences the G protein-signalling pathway (reviewed by Hepler, 1999; Zhong and Neubig, 2001).

Signalling is also commonly regulated at the level of the GPCR itself via the phenomenon of receptor desensitisation. There are two types of desensitisation: homologous and heterologous, each of which will be briefly considered.

Homologous desensitisation describes the specific attenuation of signalling arising from an activated receptor. The active receptor conformation is a target for phosphorylation by members of the G protein-coupled receptor kinase (GRK) family (reviewed by Buenemann and Hosey, 1999; Penn et al., 2000; Ferguson, 2001). These kinases phosphorylate serine and threonine residues present in the third intracellular loop and carboxy-terminal tail of the active receptor, which permits binding of β-arrestin proteins [Ferguson, 2001]. β-arrestin appears to function as an adapter protein to link the receptor with components of the clathrin internalisation machinery. Subsequent to GPCR phosphorylation and binding of β-arrestin, receptors may be sequestered from the plasma membrane. Once internalised, the GPCR may be resensitised, dephosphorylated and recycled back to the plasma membrane or targeted to lysosomes for degradation. The fate of the internalised GPCR appears to depend on the arrangement of serine residues in the carboxy-terminal tail of the receptor. Receptors which possess a serine cluster within this region are internalised and recycle slowly (e.g. vasopressin V2 receptor) whereas receptors which lack such a cluster are rapidly dephosphorylated and recycled back to the cell-surface (e.g. β2AR).
There is some evidence that β-arrestins can also act as scaffold proteins and may facilitate GPCR-mediated activation of the mitogen activated protein (MAP) kinase pathway [McDonald et al., 2000]. It has also been suggested that agonist-dependent phosphorylation of GPCRs on serine/threonine residues may be mediated by casein kinase 1α in addition to the actions of GRKs (reviewed by Tobin [2002]).

The process of heterologous desensitisation is independent of stimulation of that receptor by an agonist and is achieved via receptor phosphorylation by the serine/threonine kinases, protein kinase A (PKA) and protein kinase C (PKC), which leads to loss of receptor function.

1.3 The Edg family of GPCRs

1.3.1 Introduction to Edg receptors

In 1990, an abundant transcript induced in phorbol ester-treated differentiating endothelial cells was cloned and found to encode a 381 amino acid orphan GPCR [Hla and Maciag, 1990]. The gene product was named Endothelial differentiation gene 1 (Edg1) and was the first member of this new receptor subfamily. Even before the ligand for this receptor had been identified, it was suggested that Edg1 may be involved in angiogenesis since it was an immediate-early gene induced in cultured human umbilical vein endothelial cells (HUVEC). An elegant study used the yeast two-hybrid system to demonstrate the ability of the third intracellular loop of Edg1 to associate with Goαi G proteins hence suggesting the pathway that may be stimulated by this orphan receptor. [Lee et al., 1996]. The subsequent observation that Edg1-transfected HEK-293 cells
formed a network of cell-cell aggregates when grown in FCS-containing medium, whilst untransfected cells maintained normal morphology under the same conditions led to the identification of sphingosine-1-phosphate (S1P) as the putative endogenous agonist for this receptor [Lee et al., 1998]. The related Edg2 receptor, which had been cloned from a neuronal cell cDNA library, had already been identified as a receptor for lysophosphatidic acid (LPA) [Hecht et al., 1996]. These findings suggested that the remaining orphans within this receptor family would be activated by these ligands and that the Edg receptors represented a new receptor family, which were activated by the lysophospholipids S1P and LPA. Further Edg family members were cloned and studies of these GPCRs conducted by many groups have confirmed their identity as high affinity receptors for S1P or LPA (reviewed by Fukushima et al., 2001; Kluk and Hla, 2002; Takuwa et al., 2002). A total of eight family members have been identified and phylogenetic analysis of GPCRs from the human genome predicted that there are no further Edg receptors [Vassilatis et al., 2003].

The ligands for the Edg receptor family are structurally similar. S1P and LPA (shown in Figure 1.5) both contain a polar head group with a long chain fatty acid tail. The Edg receptors are most closely related to the cannabinoid (CB) receptor family (shown in the phylogenetic tree in Figure 1.2), and share approximately 25 % sequence identity with CB receptors. The endogenous ligands of the cannabinoid receptors, anandamide and 2-arachidonoylglycerol, have structures which are similar to S1P and LPA since they also comprise a polar head group and long aliphatic tail. In common with the CB receptors, Edg family GPCRs lack the commonly conserved cysteine residue present within the first extracellular loop of most family A receptors (previously discussed in this
chapter, section 2.3). These common features might suggest that Edg and CB receptors evolved from a common ancestral gene.

![Figure 1.5](image)

**Figure 1.5**
**Structures of Edg family ligands.**
A  Sphingosine-1-phosphate (SIP)
B  Lysophosphatidic acid (LPA)  
(shown here is 18:1 LPA)

The signalling characteristics of the Edg receptor family have been extensively studied and the G proteins to which each receptor couples has been determined.

Each Edg receptor couples to $G_\alpha$, G proteins and with the exception of Edg1, they couple to additional G protein families (summarised in Table 1.2).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>G protein coupling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edg1</td>
<td>SIP</td>
<td>$G_i$</td>
</tr>
<tr>
<td>Edg2</td>
<td>LPA</td>
<td>$G_i, G_{q, 12/13}$</td>
</tr>
<tr>
<td>Edg3</td>
<td>SIP</td>
<td>$G_i, G_{q, 12/13}$</td>
</tr>
<tr>
<td>Edg4</td>
<td>LPA</td>
<td>$G_i, G_{q, 12/13}$</td>
</tr>
<tr>
<td>Edg5</td>
<td>SIP</td>
<td>$G_i, G_{q, 12/13}$</td>
</tr>
<tr>
<td>Edg6</td>
<td>SIP</td>
<td>$G_i, G_{12/13}$</td>
</tr>
<tr>
<td>Edg7</td>
<td>LPA</td>
<td>$G_i, G_{q}$</td>
</tr>
<tr>
<td>Edg8</td>
<td>SIP</td>
<td>$G_i, G_{12/13}$</td>
</tr>
</tbody>
</table>

**Table 1.2**
**Summary of G protein activation by Edg family receptors.**

-20-
1.3.2 Classification of Edg receptors

The colloquial name “Edg”, which has been used to describe this family of receptors was originally used to describe the immediate early response gene product cloned from HUVEC, Edg1 [Hla and Maciag, 1990]. Other orphan receptors were cloned and given various trivial names (e.g. vzg-1 for Edg2) and it became clear that the term Edg bore no relevance to the other seven receptors. The Edg family members were renamed in 2002, in accordance with IUPHAR guidelines, whereby a receptor is named using an abbreviation for the natural agonist with the highest potency, followed by a subscript Arabic numeral [Chun et al., 2002]. The order of the numbering of the reclassified Edg receptors was also chosen to reflect the chronology of publication of the novel receptor sequences. Table 1.3 shows the new names of the Edg receptors.

<table>
<thead>
<tr>
<th>IUPHAR name</th>
<th>Edg name</th>
<th>Previous names</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1P₁</td>
<td>Edg₁</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1P₂</td>
<td>Edg₅</td>
<td>H218, AGR16</td>
<td>S1P</td>
</tr>
<tr>
<td>S1P₃</td>
<td>Edg₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1P₄</td>
<td>Edg₆</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1P₅</td>
<td>Edg₈</td>
<td>nrg-1</td>
<td></td>
</tr>
<tr>
<td>LPA₁</td>
<td>Edg₂</td>
<td>vzg-1, rec1.3</td>
<td>LPA</td>
</tr>
<tr>
<td>LPA₂</td>
<td>Edg₄</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPA₃</td>
<td>Edg₇</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.3 Summary of Edg family receptor classification consistent with IUPHAR guidelines.

Phylogenetic analysis of the Edg receptor family reveals that the receptors form two clusters, which correspond to the ligands by which they are activated (Figure 1.6).
Figure 1.6
Phylogenetic tree of Edg family receptors.
Comparison of sequences of Edg family GPCRs reveal the receptors form two clusters, which correspond with ligand preference. The Edg family was renamed to reflect ligand preference exhibited by the receptors (in accordance with IUPHAR guidelines) and the new nomenclature is used here.

1.3.3 The S1P receptor family
The S1P receptor family has been the subject of several comprehensive reviews (for example see, Pyne and Pyne, 2000a; Takuwa et al., 2001; Takuwa et al., 2002; Kluk and Hla, 2002; Siehler and Manning, 2002). S1P receptors are found in many organisms, including the puffer fish Takifugu rubripes [Yamaguchi et al., 1999] and the sea squirt Ciona intestinalis, which indicates the highly conserved nature of GPCR-mediated S1P signalling. Characterisation of the members of the S1P receptor has revealed that these highly specific S1P receptors mediate different responses to S1P and that cells may express multiple S1P receptors which activate opposing pathways (summarised in Figure 1.7). Dissection of the pharmacology of the individual S1P receptors has been hampered by the expression of endogenous S1P receptors on most cell types and the lack of receptor selective agonists or antagonists. Progress is being made in this area however, and a recent publication described the identification of an S1P$_{1/3}$ selective agonist [Im et al., 2001a].
Figure 1.7

Signal transduction by S1P family receptors.

S1P, present in serum binds to members of the S1P receptor family to activate the various G proteins shown. Different S1P receptors couple to different G protein subfamilies (e.g. S1P₁ activates G₅ whilst S1P₂ is linked to G₁, G₉ and G₁₂/₁₃). G protein activation leads to stimulation or inhibition of various downstream intracellular pathways, some of which are shown here. Reproduced and updated from Spiegel and Milstien [2003].

Since it is the prototypical S1P receptor, S1P₁ has been the most extensively studied member of this receptor family. The S1P₁ receptor, which was previously named Edg1 and has been introduced in this chapter, section 3.1, is a 381 amino acid protein, the mRNA of which is widely expressed. The human S1P₁ gene is located on chromosome 1p21. As described previously in this chapter, overexpression studies defined S1P₁ as a high affinity S1P receptor (Kₐ = 8.1 nM) [Lee et al., 1998]. The related molecule, dihydrosphingosine-1-phosphosphate, which lacks the double bond present within the S1P backbone, was also able to bind and activate this receptor. High concentrations of the related molecule sphingosylphosphorylcholine (SPC) were able to displace S1P, in common with its effects as a low affinity ligand for other S1P receptors. Rapid internalisation of the receptor occurs after stimulation with S1P which is
followed by recycling of the receptor back to the plasma membrane [Liu et al., 1999]. Additionally, Kohno et al., [2002] showed that internalisation of this receptor was dependent on receptor glycosylation since mutant S1P1 which could not be glycosylated was poorly internalised in response to S1P. Agonist-dependent phosphorylation of S1P1 has been found to be mediated by GRK-2 and the extreme carboxy-terminus of the receptor was shown to be the target for the actions of this kinase. Phosphorylation of residues within this region was required for receptor internalisation [Watterson et al., 2002].

Many different cellular responses to S1P have been shown to depend on S1P1. S1P1 couples to Goi G proteins and stimulation of the receptor with S1P promotes activation of ERK, inhibition of adenylyl cyclase and Rac-dependent chemotaxis. Reports describing the ability of S1P1 to stimulate mobilisation of calcium are conflicting and may suggest that this response is cell type dependent [Siehler and Manning, 2002]. Activation of S1P1 positively regulates proliferative/survival and migratory signalling pathways in many cell types. Endothelial cells migrate towards S1P in a manner which requires phosphorylation of S1P1 by Akt [Lee et al., 2001], although it is unlikely that other S1P receptors would be Akt substrates as the residue of S1P1 which is phosphorylated is not conserved throughout this receptor family. Binding of S1P to S1P1 expressed on endothelial cells has been shown to stimulate endothelial nitric oxide synthase (eNOS), resulting in production of the potent vasodilator, nitric oxide (NO) [Igarashi and Michel, 2001].

Disruption of the S1P1 gene in mice caused embryonic lethality between E12.5 and E14.5. This was due to the lack of vascular smooth muscle cell recruitment
to the developing vasculature and demonstrated a clear role for S1P₁ in vascular maturation [Liu et al., 2000]. Specific deletion of the S1P₁ gene in endothelial cells of mice using the conditional Cre/LoxP system produced a highly similar phenotype to that of the S1P₁ knockout. It was suggested the phenotype of the S1P₁⁻/⁻ mice was due to the loss of function of this receptor in endothelial cells, rather than vascular smooth muscle cells, since deletion of S1P₁ in vascular smooth muscle cells did not affect vascular maturation [Allende et al., 2003].

Interestingly, the phenotype of the S1P₁⁻/⁻ mice was similar to that caused by deletion of platelet-derived growth factor (PDGF-β) or its receptor (PDGFR-β) in mice. This suggested that vascular maturation may involve cross-talk between S1P₁ and the PDGF-β receptor. This concept was supported by the observations of Hobson et al., [2001] who showed that PDGF-stimulated cell motility in HEK-293 cells was dependent on S1P₁ expression. The authors proposed a sequential model in which PDGF activates sphingosine kinase promoting release of S1P, which acts as an autocrine mediator to stimulate S1P₁ thereby activating the small G protein Rac which is required for cell motility. Studies by Alderton et al., [2001] reported that PDGFR-β forms a tethered complex with S1P₁ in HEK-293 cells and is involved in cell proliferation. Within this complex, efficient stimulation of the ERK pathway by S1P and PDGF was reported to occur through a mechanism that involves PDGR-β tyrosine phosphorylation of Gα₅, which is released upon S1P₁ stimulation.

S1P₂ (Edg5) was independently cloned from rat by two groups and named AGR16 or H218 [Fukushima et al., 2001]. The human gene, located on chromosome 19p13.2, encodes a 352 amino acid protein and S1P was shown to
be a high affinity agonist ($K_d = 27 \text{ nM}$) of this receptor [Van Brocklyn et al., 1999]. The S1P$_2$ transcript is abundantly expressed in heart and lung, is detected at lower levels in brain and is expressed more strongly in embryonic brain. Transfection of cells with S1P$_2$ induces cell rounding and neurite retraction in response to S1P [Van Brocklyn et al., 1999]. S1P$_2$ couples to multiple ($G_\alpha_i$, $G_\alpha_q$, $G_\alpha_{12/13}$) G proteins and can also activate Rho and induce stress fibre formation. In contrast to S1P$_1$, which stimulates Rac and promotes cell movement, S1P$_2$ is chemorepellant, inhibiting Rac and blocking migration to S1P [Takuwa, 2002].

Targeted deletion of the S1P$_2$ gene was reported to lead to neuronal dysfunction, although surprisingly there were no apparent anatomical or physiological abnormalities [MacLennan et al., 2001]. Interestingly, the zebrafish gene Mil (Miles Apart), which closely resembles the mammalian S1P$_2$ gene, appears to be involved in cardiac development [Kupperman et al., 2000].

The S1P$_3$ receptor (formerly called Edg3) was initially isolated from human DNA and mapped to chromosome 9q22.1-22.2 [Yamaguchi et al., 1996]. The gene encodes a 378 amino acid protein which was shown to bind S1P with high affinity ($K_d = 26 \text{ nM}$) [Kon et al., 1999]. S1P$_3$ mRNA is expressed in many tissues including heart, brain, liver, lung, kidney, pancreas, thymus and spleen [Kluk and Hla, 2002]. S1P$_3$ couples to multiple G proteins to exert its effects and the ability of this receptor to stimulate $G_\alpha_i$, $G_\alpha_q$ and $G_\alpha_{12/13}$, but not $G_\alpha_s$, has been demonstrated using $[^{35}\text{S}]$GTP$\gamma$S binding [Windh et al., 1999]. Activation of S1P$_3$ by S1P promotes mobilisation of intracellular calcium via PLC activation, stimulation of ERK, cell migration, cytoskeletal organisation and
morphogenetic differentiation [reviewed by Kluk and Hla, 2002; Siehler and Manning, 2002]. In common with S1P$_2$, S1P$_3$ has been shown to activate nuclear factor κB (NF-κB) in response to S1P stimulation and this appears to reflect activation of G$_q$ G proteins [Siehler et al., 2001].

Deletion of the S1P$_3$ gene in mice did not produce any obvious phenotypic abnormality [Ishii et al., 2001]. This suggested a non essential role for S1P$_3$ in murine development, however activation of PLC by S1P was significantly decreased in embryonic fibroblasts derived from S1P$_3$ / mouse. Generation of mice null for both S1P$_2$ and S1P$_3$ caused reduced litter sizes and significant infant mortality; although those pups which survived lacked any obvious phenotype [Ishii et al., 2002]. *In vitro* analysis of S1P signalling in S1P$_2$S1P$_3$ / embryonic fibroblasts showed severe defects in activation of Rho, PLC and calcium mobilisation. S1P receptor signalling therefore appears to be involved in perinatal survival and the signalling of S1P$_2$ and S1P$_3$ receptors is non-redundant.

The S1P$_4$ gene was cloned from *in vitro* differentiated dendritic cells and was originally named Edg6 [Graeler et al., 1998]. This receptor will be discussed in more detail in this chapter, section 3.5.

The final S1P receptor, S1P$_5$ (previously named Edg8/nrg-1) was cloned from rat PC12 cells and encodes a 400 amino acid protein. S1P$_5$ was shown to be another high affinity S1P receptor (K$_d$ = 2 nM [Im et al., 2000a]) and has differential expression patterns in rat and human. The human S1P$_5$ gene is located on chromosome 19p13.2 and its mRNA has been detected in skeletal muscle, heart, and kidney with lower expression in liver and placenta; in contrast, rat S1P$_5$
mRNA was predominantly expressed in brain [Niedemberg et al., 2002]. Activation of S1P₅ causes cell rounding, mobilisation of calcium, cell survival and inhibition of both adenylyl cyclase and ERK activation [Im et al., 2000a; Niedemberg et al., 2003a]. Differential effects on cell proliferation were observed between human and rat S1P₅: the rat receptor is antiproliferative whilst the human receptor is not [Niedemberg et al., 2002]. S1P₅ can couple to Gᵢ and G₁₂/₁₃ G proteins and was reported by Niedemberg et al., [2003a] to be constitutively active. Interestingly, constitutive inhibition of adenylyl cyclase was enhanced by S1P-induced receptor activation but the ability of S1P₅ to inhibit basal ERK activity was not affected by S1P. These data were interpreted as suggesting that different signal transduction pathways are not equally activated through a constitutively active GPCR that couples to multiple G proteins. It is not clear whether this scenario is applicable to other S1P receptors.

The effect of deleting the S1P₅ gene in mice has not been reported, but given the reported differences in expression patterns between human and rodent forms of S1P₅ [Niedernberg et al., 2002], it is unclear how useful analysis of S1P₅ null mice would be in understanding the function of the human receptor.

Significant progress has been made in elucidating the in vivo function of the various S1P receptors and the generation of mice null for S1P₁ demonstrated the critical involvement of the receptor in vascular maturation [Liu et al., 2000]. Conditional knockout experiments and production of double knockout mice have been used to further address the question of the role of these receptors in physiology and disease. Additional work is required to fully unravel the significance of S1P receptor activation and to understand precisely how the
different receptor isoforms, which can have similar or opposing functions, control the diverse effects of SIP in vivo.

1.3.4 The LPA receptor family

The LPA receptor family comprises three closely related GPCRs, which bind LPA with nM affinity. A fourth GPCR has recently been designated as LPA\(_4\) but this receptor is only distantly related and shares approximately 20\% sequence identity with the other LPA receptors (shown in Figure 1.2). This receptor (previously named P\(_2\)Y\(_9\)/GPR\(_{23}\)) was found to bind LPA with high affinity (45 nM \(K_d\)) and couples to mobilisation of intracellular calcium and stimulation of adenylyl cyclase activity [Noguchi et al., 2003]. This LPA receptor has evolved from a different ancestor than that of the LPA\(_{1-3}\) receptors and the role of LPA\(_4\) in vivo is currently unclear.

Unlike the SIP receptors, the LPA\(_{1-3}\) receptors contain an intron located within the middle of TM VI, the location of which is absolutely conserved between human and mouse [Contos and Chun, 1998; Contos and Chun, 2000; Contos and Chun, 2001]. This suggests that these LPA receptors were derived from a common ancestral gene. Significant progress has been made in this area since the identification of the first LPA receptor. Most notably, synthetic LPA mimetics have been developed, some of which show receptor subtype-selective activities [Im et al., 2000b], whilst others act as competitive antagonists [Sardar et al., 2002].

The ligand, LPA can be produced by several distinct enzymatic mechanisms. Intracellularly, the sequential actions of phospholipase D (which converts phosphatidylcholine to phosphatidic acid) and phospholipase A1 and A2
enzymes (which deacylate phosphatidic acid) yield LPA. Phospholipase A2 may also be secreted from cells to produce extracellular LPA. An alternative route of production of extracellular LPA is via the lysophospholipase D enzyme, autotaxin, which acts within plasma to hydrolyse lysophosphatidylcholine and is believed to represent the principal route of production of extracellular LPA. Bioactive LPA is dephosphorylated by enzymes of the lipid phosphate phosphohydrolase family, some of which may possess ecto-activity. Finally, LPA may be produced from PA (and vice versa) by the action of the LPA acyl transferase enzyme.

LPA is released from activated platelets and is present in serum at low μM concentrations. Although the term LPA is used to refer to the single entity 1-oleoyl LPA, it actually describes a family of molecules which can contain different fatty acyl chains with different levels of saturation. LPA is a potent signalling molecule which is involved in many physiological and pathophysiological process such as cell differentiation and proliferation, induction of apoptosis, cytoskeletal arrangements and cell invasion [reviewed by Ye et al., 2002]. LPA affects a wide variety of cell types and reflects the widespread expression of the various LPA receptors. The LPA receptor family has been recently reviewed [Fukushima et al., 2001; Fukushima and Chun, 2001]; each receptor will now be briefly discussed.

LPA₁ was identified as a result of studies designed to identify novel GPCRs associated with neuron production in mouse cerebral cortex. It was named ventricular zone gene (vzg-1) and assigned as the second Edg family member since it was similar to the orphan Edg1 (S1P₁) receptor. The human LPA₁ gene
is located on chromosome 9p31.3-32 and encodes a protein of 364 amino acids [An et al., 1997; Fukushima and Chun, 2001]. Analysis of heterologously expressed LPA₁ revealed that stimulation of the receptor caused cell proliferation, Rho-mediated actin rearrangement and stimulation of serum response elements via activation of Gα₁, Gα₄ and Gα₁₂/13 G proteins. The receptor was found to be widely expressed in the murine embryonic nervous system where it may play a role in neurogenesis and is also expressed in adult central nervous system (CNS) where it may be involved in the biology of myelinating cells. Receptor mRNA is also expressed outside the CNS, predominantly in testis and intestine, and moderately in heart, lung, kidney, spleen, thymus, muscle and stomach, as well as in several cancers. Disruption of the LPA₁ gene in mice was expected to cause nervous system defects. Targeted deletion of this gene resulted in approximately 50% neonatal lethality due to decreased suckling, possibly caused by olfaction defects [Contos et al., 2000]. In addition, LPA₁⁻/⁻ mice were smaller and exhibited craniofacial abnormalities and increased apoptosis in peripheral nerves. This suggests a role for LPA₁ in nervous system development and survival of myelinating cells.

Functional LPA₁ has recently been detected at the cell nucleus [Gobeil et al., 2003]. Stimulation of nuclear LPA₁ caused activation of G₁/o G proteins, mobilisation of nuclear calcium and induction of pro-inflammatory gene expression. This observation suggests that the nucleus may be a potential organelle for intracrine signalling by lysophospholipids. It will be interesting to discover whether other LPA or S1P receptors are also active at the cell nucleus.
The LPA<sub>2</sub> receptor, which is located on human chromosome 19p12, is a 351 amino acid GPCR that shares approximately 46% sequence identity with LPA<sub>1</sub>. Its sequence was discovered from database searches of human genomic DNA during attempts to identify LPA<sub>1</sub> receptor homologues [An et al., 1998]. Several variants of this receptor have been detected in human cancer cells and in fact the original report describing the LPA<sub>2</sub> (Edg4) receptor had cloned and characterised a mutant from an ovarian cancer cell line [An et al., 1998]. This clone contained a base deletion which caused a frame shift and resulted in a mutant that contained an extended carboxy-terminal tail [Contos and Chun, 2000]. Subsequent comparison with the wild type receptor did not reveal any differences in G protein coupling; both wild type and mutant forms of the receptor couple to G<sub>α</sub>13, G<sub>α</sub>q and G<sub>α</sub>12/13 G proteins [An et al., 1998; Ishii et al., 2000] and the role of mutant LPA<sub>2</sub> receptors in cancer is unclear. Activation of LPA<sub>2</sub> results in activation of PLC, stimulation of ERK, cell rounding and inhibition of adenylyl cyclase [Ishii et al., 2000]. The gene is expressed in peripheral leukocytes, thymus, spleen, prostate and pancreas [An et al., 1998]. Expression of LPA<sub>1</sub> and LPA<sub>2</sub> has been shown to change after T cell activation and these receptors transduce opposing effects on T cell motility which may be relevant in vivo [Zheng et al., 2001]. LPA<sub>2</sub> knockout mice exhibited no obvious phenotypic abnormalities and failed to reveal any obvious behavioural, anatomical or histological defects [Contos et al., 2002a]. Indeed, the same report describes analysis of mice null for both LPA<sub>1</sub> and LPA<sub>2</sub>, which displayed essentially the same phenotype as LPA<sub>1</sub> /<sup>−</sup>/ mice. This suggests that the LPA<sub>1</sub> and LPA<sub>2</sub> receptors have redundant functions in mediating multiple LPA responses.
The discovery of a third LPA receptor was independently reported by two groups [Bandoh et al., 1999; Im et al., 2000b]. The LPA\textsubscript{3} gene is present on human chromosome 1p22.3-31.1 [Contos and Chun, 2001] and the resultant 353 amino acid receptor shares approximately 50\% sequence identity with LPA\textsubscript{1} and LPA\textsubscript{2}. LPA\textsubscript{3} mRNA was found to be expressed in heart, pancreas, kidney, brain, prostate and testis, and less abundantly in ovary and lung [Bandoh et al., 1999; Im et al., 2000b]. An LPA\textsubscript{3} variant (HOFNH30) which is highly expressed in placenta has also been identified and may be a splice variant of LPA\textsubscript{3} [Fitzgerald et al., 2000]. LPA-stimulation of LPA\textsubscript{3} promoted mobilisation of calcium from intracellular stores which was mediated via activation of G\textalpha\textsubscript{q} G proteins and was dependent on activation of PLC [Im et al., 2000b]. The receptor has also been reported to couple to G\textalpha\textsubscript{i} G proteins although other groups have contradicted these findings [Fukushima et al., 2001]. In marked contrast to the effects of LPA\textsubscript{1} and LPA\textsubscript{2} stimulation, activation of LPA\textsubscript{3} was found to promote neurite elongation and inhibited LPA-induced cell rounding in LPA\textsubscript{1/2} expressing cells [Ishii et al., 2000]. Analysis of \textit{in vivo} LPA\textsubscript{3} function by deletion in mouse has not yet been reported and the functional role of this receptor remains unclear.

1.3.5 S1P\textsubscript{4} (Edg6)

S1P\textsubscript{4} (formerly named Edg6) was cloned in 1998 from \textit{in vitro} differentiated dendritic cells, in an effort to discover novel chemokine receptors [Graeler et al., 1998]. It was categorised as the sixth member of the then named Edg receptor family, on the basis of sequence similarity with known members of this family. The sequence of this gene coded a 384 amino acid protein, which bore the hallmarks of being a GPCR since it possessed seven putative transmembrane domains. The translated S1P\textsubscript{4} sequence also contained a putative N-
glycosylation site in the amino-terminal tail as well as several sites for potential modification by PKC phosphorylation and palmitoylation within the carboxy-terminal region [Graeler et al., 1998]. The S1P₄ receptor shares greatest sequence identity with other S1P receptors and is less closely related to the LPA receptors, summarised in Table 1.4. It is also closely related to the cannabinoid receptors, sharing 20 % and 34 % sequence identity with CB1 and CB2, respectively.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Sequence identity with S1P₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1P₁</td>
<td>39 %</td>
</tr>
<tr>
<td>S1P₂</td>
<td>36 %</td>
</tr>
<tr>
<td>S1P₃</td>
<td>40 %</td>
</tr>
<tr>
<td>S1P₅</td>
<td>37 %</td>
</tr>
<tr>
<td>LPA₁</td>
<td>30 %</td>
</tr>
<tr>
<td>LPA₂</td>
<td>35 %</td>
</tr>
<tr>
<td>LPA₃</td>
<td>30 %</td>
</tr>
</tbody>
</table>

Table 1.4
Sequence identity between S1P₄ and other S1P and LPA receptors.

S1P₄ has a very distinct pattern of mRNA expression and is principally detected within the immune system. This is in marked contrast to that of the other S1P receptors (detailed in this chapter, section 3.3) and suggested that S1P₄ may play a role in immune function. More recent studies of S1P₄ expression have added to the initial findings, and are summarised in Table 1.5. In addition, the study of [Vassilatis et al., 2003] detected low levels of S1P₄ mRNA in murine cerebral tissues (e.g. brain stem, hippocampus, striatum, thalamus). Since no other studies have detected this transcript in these tissues, the significance of these findings is unclear.
The pattern of S1P₄ expression in various human tissues has been investigated using a polyclonal anti-S1P₄ antibody in an immunohistochemistry study. The findings are summarised in Table 1.5 and Figure 1.8 shows some examples of S1P₄-expressing cells present within tissue sections.

Whilst significant advances were made with the other S1P receptors, progress with S1P₄ was slow. Based on its similarity with other S1P receptors S1P was a candidate S1P₄ ligand, but attempts to demonstrate this experimentally were frustrated by reported difficulties in expressing functional receptor [Im et al., 2001a]. However, in 2000, two independent groups showed that S1P bound to S1P₄ with an apparent Kₐ of 12-63 nM [Van Brocklyn et al., 2000; Yamazaki et al., 2000]. Van Brocklyn et al., [2000] expressed S1P₄ in HEK293 cells and detected specific binding of S1P to the recombinant receptor. Bound S1P could be displaced by competition with the related lipids dihydroS1P (dhS1P) and sphingosylphosphorylcholine (SPC), although SPC could only compete for S1P binding when used at high (µM) concentrations. This was in broad agreement with the ligand preference exhibited by other S1P receptors, except for the relatively low affinity with which S1P₄ bound S1P. S1P₄ expressed in CHO-K₁ cells demonstrated pertussis toxin-sensitive activation of ERK in response to S1P, indicating that S1P₄ coupled to Gα₄ G proteins. The study of Yamazaki et al., [2000] also detected specific binding of S1P which could be displaced by high concentrations of SPC. Additionally, this group reported that stimulation of S1P₄ with S1P induced mobilisation of intracellular calcium via activation of the phospholipase C (PLC) enzyme. This response was partially sensitive to pertussis toxin treatment which suggested coupling of the receptor to Gα₄ and
<table>
<thead>
<tr>
<th>Tissue/Cell Type</th>
<th>Relative level</th>
<th>Method</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>spleen</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thymus</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>peripheral leukocyte</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lymph node</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bone marrow</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>foetal spleen</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>foetal liver</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>foetal thymus</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>foetal lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>naïve CD4 T cell</td>
<td>N.A.</td>
<td>RT-PCR, Western blotting</td>
<td>mouse</td>
<td>2</td>
</tr>
<tr>
<td>kidney</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lung</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ovary</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>peripheral leukocyte</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>prostate</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>skin</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>spleen</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stomach</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thymus</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thyroid</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lung</td>
<td>+++</td>
<td>Northern blot</td>
<td>mouse</td>
<td>4</td>
</tr>
<tr>
<td>spleen</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thymus</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>peripheral monocytes</td>
<td>++</td>
<td>RT-PCR</td>
<td>mouse</td>
<td>3</td>
</tr>
<tr>
<td><em>in vitro</em> differentiated macrophages</td>
<td>++</td>
<td>RT-PCR</td>
<td>human</td>
<td>5</td>
</tr>
<tr>
<td>platelet</td>
<td>++</td>
<td>RT-PCR</td>
<td>human</td>
<td>6</td>
</tr>
<tr>
<td>polymorphonuclear leukocytes</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lymphocytes (subsets of)</td>
<td>+ to +++</td>
<td>immunohistochemistry</td>
<td>human</td>
<td>7</td>
</tr>
<tr>
<td>mast cells</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>granulocytes</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Langerhans cells</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lymph node interdigitating cells</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>neutrophils</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eosinophils</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.5
Summary of tissues and cells reported to express S1P4.
Note: “relative level” refers to the level of signal obtained within that experiment and it would not be appropriate to compare relative S1P4 expression levels taken from different studies/publications.
+ faint signal; ++ medium signal; +++ strong signal; N.A. not appropriate.
References:
1 Graeler et al., 1998
2 Graeler and Goetzl, 2002
3 Vassilatis et al., 2003
4 Contos et al., 2002b
5 Fueller et al., 2003
6 Motohashi et al., 2000
7 In house studies
Figure 1.8
Expression of S1P4 detected in tissue sections.
Immunohistochemical studies have been performed using a rabbit polyclonal antibody, which recognises the human S1P4 protein. Affinity purified antiserum was used at a dilution of 1:250 on paraffin-embedded, formalin-fixed tissues. Cells positive for S1P4 expression stained fuschia-red. Tissue sections were also stained with haematoxylin and eosin. The identity of S1P4 positive cells was defined by histological analysis. This study was performed by LifeSpan Biosciences, Inc. (Seattle, WA., U.S.A.).

A Normal lymph node showing strong staining of interdigitating cells (40x)
B Inflamed lymph node showing strong staining of mast cells (40x)
C Inflamed skin showing strong staining of Langerhans cells (60x)
D Normal lymph node showing staining of lymphocytes within interfollicular zone (40x)
E Inflamed lymph node showing staining of neutrophils (60x)
F Small intestine with Peyer’s patches showing staining of lymphocytes (40x)
possibly $\text{G}_{\alpha q}$ G proteins. However, untransfected cells were also observed to mobilise calcium in response to SIP stimulation, although the magnitude of response was smaller than for the SIP$_4$-transfected cells. The ability of this receptor to promote a calcium flux has not been confirmed by other laboratories and therefore remains unclear.

Analysis of the SIP$_4$ gene in mouse, found it to be present as a single copy comprising two exons with an intronless coding region, located on central chromosome 10, which is syntenic with human chromosome 19. The presence of a single, uninterrupted coding region is similar to that observed for the other SIP receptor genes and supports the theory that an ancestral SIP gene diverged to yield SIP$_4$ and the other SIP receptor genes [Contos et al., 2002b]. Within the same study, it was reported that the SIP$_4$ gene is arranged in tandem with the murine $\text{G}_{\alpha16}$ orthologue, $\text{G}_{\alpha15}$ on mouse chromosome 10. Expression of the $\text{G}_{\alpha15}$ and SIP$_4$ genes primarily in haematopoietic tissues and their chromosomal proximity suggests that these transcripts may be co-expressed in the same cells. The authors therefore suggested that co-expression of $\text{G}_{\alpha15/16}$ and SIP$_4$ proteins might imply this G protein to be the native coupling partner of SIP$_4$ and that stimulation of SIP$_4$ would result in increased concentration of intracellular calcium [Contos et al., 2002b]. Direct analysis of the G proteins activated by SIP$_4$ was performed by Graeler et al., [2003] and revealed that this receptor coupled to $\text{G}_{\alpha i}$ and $\text{G}_{\alpha12/13}$ subunits, but not $\text{G}_{\alpha q}$ or $\text{G}_{\alpha15/16}$. These observations were interpreted as suggesting that the previously reported tandem genomic arrangement of the SIP$_4$ and $\text{G}_{\alpha15/16}$ genes may not be functionally relevant. In agreement with the well documented ability of this receptor to couple to $\text{G}_{\alpha i}$ G
proteins, the same study observed pertussis toxin-sensitive activation of PLC by S1P$_4$. Activation of S1P$_4$ was also shown to potently activate Rho and promote stress fibre formation. The authors suggested this reflected the ability of stimulated S1P$_4$ to cause pronounced activation of Go$_{12/13}$ which could be relevant to the *in vivo* functions of this receptor [Graeler *et al.*, 2003]. S1P$_4$ has also recently been shown to promote cell migration via activation of Cdc42 [Kohno *et al.*, 2003].

There is some uncertainty as to whether S1P represents the true endogenous ligand for S1P$_4$. This lipid binds to the S1P$_4$ receptor with significantly lower affinity than that with which it binds other S1P receptors and is a relatively poor agonist of S1P$_4$. In addition, phylogenetic analysis of the S1P receptor family (Figure 1.6) reveals that although this receptor family forms a discrete cluster, S1P$_4$ is on the edge of this cluster, suggesting that it may be activated by a structurally distinct lipid. The molecule, phytosphingosine-1-phosphate (phS1P) was recently identified as a high affinity ligand for the S1P$_4$ receptor, and is discussed further in this chapter, section 4.3.

There is still little data regarding the physiological role of the S1P$_4$ receptor within the immune system. This is partly a consequence of the lack of receptor selective agonists, combined with the expression of multiple S1P receptor subtypes on haematopoietic and lymphoid cells. Disruption of the S1P$_4$ gene in mice may indicate the processes in which the function of this receptor is required.
1.3.6 Other lipid GPCRs

LPA and S1P receptors are not the only GPCRs to respond to lipid agonists. In addition to the well characterised GPCRs for platelet activating factor (PAF), cannabinoids and prostaglandins, several orphan receptors have recently been reported to respond to lipids which are structurally similar to S1P and LPA.

The ovarian cancer GPCR 1 (OGR1) receptor (previously named GPR68) is closely related to the PAF receptor and \( P_2Y \) purinergic receptors but shares little homology with Edg family GPCRs. The gene was cloned from an ovarian cancer cell line and is expressed in placenta, lung, liver, testis, spleen, small intestine and peripheral leukocytes [Xu et al., 2000]. OGR1 is activated by nM concentrations of the lipid SPC, which had previously been identified as a low affinity S1P receptor agonist. Binding of SPC to OGR1 stimulated activation of \( G_i \) and \( G_q \) G proteins, resulting in mobilisation of intracellular calcium, activation of ERK and inhibition of cell proliferation [Xu et al., 2000]. The related GPR65 receptor, named T cell death-associated gene 8 (TDAG8) is expressed in spleen, lymph node and peripheral leukocytes [Im et al., 2001b]. This receptor shares 41% identity with OGR1 and has been shown to respond to \( \mu \)M concentrations of the glycosphingolipid, psychosine. Stimulation of TDAG8 with psychosine promoted pertussis toxin-insensitive inhibition of cAMP, which was suggested to reflect activation of \( G_{o\gamma} \) G proteins [Im et al., 2001b]. The transcriptionally regulated gene, G2A (G2 accumulation G protein-coupled receptor), is predominantly expressed in lymphocytes and is also closely related to OGR1 and TDAG8. Genetic studies of this then orphan receptor suggested a role in peripheral lymphocyte homeostasis, since mice null for G2A developed secondary lymphoid organ enlargement and late-onset autoimmune syndrome.
[Le et al., 2001]. Subsequently, the serum lipids lysophosphatidylcholine (LPC) and SPC were identified as G2A receptor agonists (Kd values of 65 nM and 230 nM, respectively). The phenotype of the G2A knockout was particularly interesting given that the G2A receptor ligand, LPC is implicated in the pathogenesis of the autoimmune disease systemic lupus erythematosus. Stimulation of the receptor with LPC promoted mobilisation of intracellular calcium, activation of ERK and cell migration via G\(\alpha_i\) G proteins [Kabarowski et al., 2001]. Another GPCR closely related to the OGR1, TDAG8 and G2A receptors, GPR4, also responds to SPC and LPC. In contrast to the profile of ligand preference exhibited by G2A, GPR4 binds SPC with higher affinity (36 nM) than it does LPC (159 nM) [Zhu et al., 2001]. This receptor was found to couple to G\(\alpha_i\) G proteins and stimulate activation of ERK, mobilisation of calcium from intracellular stores and DNA synthesis. These four lipid receptors form a distinct cluster (shown in Figure 1.2) and may represent a new lysophospholipid subfamily.

S1P has recently been shown to activate several non Edg family GPCRs. The closely related GPR3, 6 and 12 orphan receptors are predominantly expressed in cerebral tissues and are most similar to Edg and cannabinoid receptors (shown in Figure 1.2), sharing approximately 42 % sequence identity with these receptors. GPR3, 6 and 12 are constitutively active receptors and stimulate opposing G\(\alpha_s\) and G\(\alpha_i\) G proteins. Treatment with low concentrations of S1P was found to further stimulate these receptors and although their physiological role is not currently understood, they have been identified as three additional S1P receptors [Uhlenbrock et al., 2002].
The mammalian orthologue of the *Xenopus laevis* PSP24 receptor, GPR63 was recently reported to be a low affinity S1P receptor [Niedernberg *et al.*, 2003b]. This receptor is expressed in stomach, small intestine and central nervous system and does not share significant sequence homology with Edg or other S1P receptors (Figure 1.2).

The existence of multiple receptors which respond to lysophospholipids and elicit diverse effects reinforces the importance of lipid mediators in biology.

### 1.4 Sphingolipid signalling

Sphingolipids are a major component of cell membranes and the sphingolipid metabolites ceramide, sphingosine and S1P play important roles in the regulation of cell proliferation, survival and death. The organisms in which these processes occur are diverse and include mammals, yeast and plants.

#### 1.4.1 Sphingosine and ceramide

The bioactive lipids sphingosine and ceramide generally inhibit cell proliferation and promote apoptosis. The molecule sphingosine was named in 1884 after the Sphinx to reflect “the many enigmas which it has presented to the enquirer” [Smith and Merrill, 2002]. Ceramide may be produced via *de novo* synthesis or from the precursor sphingomyelin whilst sphingosine is produced via deacylation of ceramide by the family of ceramidase enzymes. The levels of ceramide and sphingosine found in resting cells are generally low since their production is tightly regulated.
1.4.2 Sphingosine-1-phosphate

SIP (D-erythro-sphingosine-1-phosphate) is the principal phosphorylated sphingoid base in mammalian cells. In contrast to the pro-apoptotic actions of its precursors, ceramide and sphingosine, SIP is a cell survival factor. This bioactive lipid regulates a range of cellular responses, including cytoskeletal changes, cell movement, cell proliferation, angiogenesis and vascular maturation. SIP was originally thought to be an intracellular second messenger but the discovery of the SIP family of GPCRs, which bound this lipid with high affinity, redefined SIP as an extracellular receptor agonist. Despite the clear role for cell-surface SIP receptors in mediating many effects of SIP, the suggestion that this lysophospholipid also functions as an intracellular signalling molecule remains. Whilst some of the evidence to support this is compelling, the absence of identified intracellular targets has led to considerable debate in this area. The actions of SIP have been the subject of numerous reviews (for example see Pyne and Pyne, 2000a; Pyne and Pyne, 2000b; Maceyka et al., 2002; Spiegel et al., 2002; Pyne and Pyne 2002; Hla, 2003; Spiegel and Milstien, 2003). Many of the micro-effects of SIP have already been discussed in the context of the GPCRs which respond to SIP (this chapter, section 3). The current section will discuss the mechanisms controlling SIP production and breakdown as well as the macro-effects induced by this bioactive lipid and the concept of SIP as a dual intracellular and extracellular signalling molecule.

As is common with many signalling molecules, SIP levels in cells tend to be low and are balanced by an equilibrium between its synthesis and degradation (Figure 1.9). SIP is generated via the phosphorylation of sphingosine, which is catalysed by the sphingosine kinase (SK) enzyme. There are two different routes
for S1P degradation: reversible dephosphorylation by S1P phosphatase, or irreversible degradation into hexadecenal and phosphoethanolamine via the actions of S1P lyase. Platelets are an exception and store high concentrations of S1P, which is not subject to degradation by S1P lyase since platelets do not express this enzyme. This stored S1P is released on cell activation and aggregation and represents the major source of S1P in serum and plasma.

**Figure 1.9**

**Synthesis and degradation of S1P.**
Sphingosine is converted to S1P by the actions of sphingosine kinase. Conversely, two different enzymes may degrade S1P: S1P phosphatase removes the phosphate to yield sphingosine; S1P lyase cleaves the C2-C3 bond of S1P to produce hexadecenal and phosphoethanolamine.

Extracellular S1P is albumin bound which is believed to prevent it from binding non-specifically to plasma membrane surfaces and is metabolically stable in this form [Yatomi et al., 1997]. The concentrations of S1P in normal human plasma and serum have been reported to be approximately 200 nM and 485 nM, respectively [Yatomi et al., 1997]. Release of S1P from activated platelets will significantly increase the concentration of circulating S1P. These concentrations are significantly higher than the dissociation constants for S1P binding to each isolated S1P-family receptor.
Most cells produce S1P intracellularly which reflects the widespread expression of sphingosine kinase. It is not clear exactly how S1P is transported out of cells and the presence of the polar headgroup suggests S1P will not readily traverse the plasma membrane; candidate transporters include the P-glycoprotein lipid transporter Abcbl [Honig et al., 2003]. S1P released from cells acts as an autocrine or paracrine mediator, stimulating cell-surface receptors on the cell from which it was released (autocrine) or on nearby cells (paracrine). In its putative intracellular role, S1P is proposed to function as a second messenger for growth factors and alters the activity of specific intracellular target proteins (discussed by [Pyne and Pyne, 2000b; Spiegel and Milstein, 2003]).

Sphingosine kinase catalyses formation of S1P and is a highly conserved enzyme present in organisms as diverse as plants, yeast, worms and mammals. It is activated in response to external stimuli such as growth and survival factors. Two human genes encoding sphingosine kinase enzymes have been cloned (SK1 and SK2), which are the products of distinct genes. SK1 and SK2 are cytosolic proteins of 384 and 618 amino acids, respectively, which contain five conserved catalytic domains and are most closely related to the diacylglycerol kinase family.

The SK1 enzyme was originally from isolated from rat kidney [Olivera et al., 1998]. The importance of S1P as a signalling molecule is underscored by the existence of SK orthologues in many organisms ranging from mammals to yeast, plants and flies. The sequence of SK1 contains putative calcium/calmodulin binding domains and consensus PKA and PKC phosphorylation sites, which may be involved in regulation of enzyme activity [Pyne and Pyne, 2000b]. Precise
studies of the role of these enzymes in cellular processes have been hampered by the lack of specific inhibitors. Whilst compounds such as dimethylsphingosine (DMS) competitively inhibit SK1, DMS also inhibits PKC, which complicates interpretation of results since PKC may modify the activity of SK1.

Although this enzyme is believed to be cytosolic and contains no signal sequence or TM domains, constitutive export of SK1 from endothelial cells has been observed [Ancellin et al., 2002]. Many molecules have been reported to increase SK1 activity, including ligands for GPCRs, agonists of growth factor receptor tyrosine kinases such as PDGF, epithelial growth factor (EGF) and nerve growth factor (NGF), as well as tumour necrosis factor-α (TNF-α), phorbol esters and even S1P itself [Maceyka et al., 2002; Pyne and Pyne, 2002]. The activity of SK1 is reported to be bimodal: prior to activation by exogenous stimuli the enzyme exhibits relatively high basal turnover which may be involved in housekeeping functions; after activation the catalytic activity of the enzyme increases approximately 2-fold resulting in a concomitant increase in S1P concentration [Pitson et al., 2000]. In the same study, expression of a catalytically inactive dominant negative SK1 mutant prevented activation of SK1 in response to exogenous stimuli such as TNF-α and phorbol esters but did not affect basal SK1 activity in these cells. Activation of SK1 in response to TNF-α has been shown to involve a direct interaction between the TNF receptor-associated factor 2 (TRAF2) and SK1 [Xia et al., 2002]. SK1 protects against apoptosis induced by ceramide, TNF-α, Fas ligand and other toxic agents via production of S1P, which inhibits the cytochrome-c release-dependent mitochondrial death pathway induced by these stimuli [Hla, 2003]. Production of S1P as a consequence of SK activation is accompanied by a decrease in
ceramide levels and the fate of a cell appears to depend on the relative levels of these molecules, rather than their absolute concentrations. This system is a conserved stress mechanism that has been referred to as the “sphingolipid rheostat”, shown in Figure 1.10.

![Figure 1.10](image)

**Figure 1.10**
The ceramide-S1P rheostat.
The sphingolipids ceramide, sphingosine and S1P are interconvertible and the balance between them determines cell fate. Ceramide and sphingosine are associated with arrest of cell growth and apoptosis whilst S1P promotes cell growth and survival.

The related SK2 enzyme has been less extensively studied, but in contrast to the pro-survival actions of SK1, this enzyme has been shown to enhance apoptosis via activation of caspase-3 [Liu et al., 2003]. Induction of cell death by this enzyme was reported to be independent of S1P receptor activation but the presence of the Bcl-2 homology 3 (BH3) domain within SK2 was required. The BH3 domain is a nine amino acid sequence present in the group of “BH3 domain only” proteins, which are pro-apoptotic members of the Bcl-2 family. Interestingly, the SK1 enzyme does not contain a BH3 domain and this
difference may explain the opposing effects of these enzyme isoforms on cell fate.

Cellular effects of S1P may be divided into two broad categories: growth related functions or cytoskeletal functions. Effects on growth are often cell type dependent and include inhibition or stimulation of proliferation, altered differentiation, inhibition or activation of apoptosis and promotion of cell survival. Cytoskeletal S1P effects include chemotaxis, changes in cell morphology and aggregation. These varied responses are in part controlled by S1P receptor activation and the fate of a given cell is likely to depend on the profile of S1P receptors expressed and the presence or absence of costimulators such as PDGF.

Given its importance in regulation of cell signalling, S1P is liable to be involved in the pathology of many disease states. As was demonstrated by deletion of the S1P₁ gene in mice [Liu et al., 2000], S1P is a key molecule in the progression of angiogenesis. This process is likely to be of key importance in tumour growth and other diseases where blood vessel formation is important, such as rheumatoid arthritis, diabetic retinopathy and psoriasis [reviewed by Pyne and Pyne, 2000a; Toman et al., 2001]. S1P promotes cell survival and motility and may therefore be involved in tumour proliferation and invasion. Since S1P affects neuronal cells to promote survival, it has been suggested that the role of S1P in neurological disease states which are associated with deregulated apoptosis, such as Alzheimer’s and Parkinson’s diseases should be examined [Pyne and Pyne, 2000a].
The presence of S1P in serum and its release from blood-borne cells suggests that this molecule may be a vasoactive and cardioactive mediator. Indeed, the S1P₁ receptor was identified as an abundant transcript induced in endothelial cell differentiation [Hla and Maciag, 1990] and S1P₁ and related receptors are expressed in cardiovascular tissues. There is a role for S1P in cardiac development, as demonstrated by the phenotype of the zebrafish Mil knockout [Kupperman et al., 2000] and the observation that S1P activation of S1P₁ promotes activation of eNOS and production of the potent vasodilator NO [Igarashi and Michel, 2001]. S1P induces well defined effects in vascular smooth muscle cells, particularly chemotaxis and proliferation, that are mediated by the S1P₁ receptor [Hla, 2003].

S1P may also function as an immune system regulator. This lipid is important in allergic responses and the balance between sphingosine and S1P was shown to be decisive for mast cell activation after triggering of the immunoglobulin (Ig) E receptor [Prieschl et al., 1999]. This effect reflects activation of SK activity in response to IgE receptor activation. S1P secretion into the lung is increased after allergen challenge and mediates airway smooth muscle cell contraction and proliferation which leads to the characteristic hypercontractility and remodelling seen in asthma (reviewed by Jolly et al., [2002]). Graeler and Goetzl, [2002] reported that ligation of the T cell receptor on resting T cells regulates the expression of S1P₁ and S1P₄ receptors. This suggests that S1P may modulate the function of naïve and activated T cells. The mode of action of the sphingosine analogue immunomodulatory drug, FTY720 implies a role of S1P as a regulator of immune cell trafficking (discussed further in this chapter, section 5).
Of the numerous reports describing the effects of S1P on a given cell type, those concerning cells which express the S1P$_4$ receptor are of particular interest since they may suggest a role for this receptor. Fueller et al., [2003] observed S1P-induced mobilisation of intracellular calcium in human monocytes and macrophages. These cells express mRNA for S1P$_{1,2,4}$ receptors although it is not clear which of these receptors accounts for this response. The effects of S1P on migration of murine splenic T cells have also been investigated [Graeler and Goetzl, 2002]. Resting T cells express S1P$_1$ and S1P$_4$ receptors and activation of these receptors was proposed to account for the observed stimulation of T cell chemotaxis by S1P. Activated T cells did not respond to S1P in this manner and this was thought to be a consequence of the marked decrease in expression of S1P$_{1,4}$ receptors after T cell receptor activation. The effects of S1P on T cell mobility were believed to be mediated via the S1P$_1$ receptor, since transfection of this receptor into the HTC4 cell line (which does not respond to S1P) enhanced chemokinesis of these cells, whilst expression of recombinant S1P$_4$ did not. Another recent study described the ability of S1P to dose-dependently inhibit T cell proliferation [Jin et al., 2003]. S1P has also been shown to stimulate chemotaxis of natural killer (NK) cells. The receptor which mediated this effect was not identified, however sensitivity of this response to pertussis toxin suggests involvement of S1P$_1$ [Kveberg et al., 2002]. In contrast to the effects of chemokines, which recruit and activate NK cells, S1P recruits NK cells but inhibits their cytolytic activity against tumour cells [Maghazachi, 2003]. The effects of S1P on cells of the immune system suggest that this molecule is an important immunoregulator, which acts to maintain immune system homeostasis.
In contrast to the clear role of S1P as an activator of specific cell-surface GPCRs, evidence to support the concept that S1P is a second messenger is less clear-cut (reviewed by [Pyne and Pyne, 2000b; Hla, 2003; Young and Nahorski, 2002; Spiegel and Milstien, 2003]). The idea that S1P was a second messenger arose from its ability to mobilise calcium from intracellular stores in a manner that was dependent on the activation of sphingosine kinase. Many cell stimuli elicit S1P production, although it is likely that many of the functions previously attributed to the “second messenger” actions of S1P actually reflect activation of Edg family receptors (discussed by Pyne and Pyne [2000b]). Much of the evidence supporting an intracellular role for S1P arose from agonist stimulation of SK and the use of SK inhibitors in mammalian systems. In support of the proposed dual effects of S1P, dhS1P, which binds to and activates S1P receptors with comparable affinity and potency to S1P itself, does not mimic all the effects of S1P, particularly those related to cell survival (reviewed by Pyne and Pyne [2000b]).

Calcium mobilisation occurs after stimulation of HEK293 cells with S1P. However, this response was found to be independent of PLC activation and correlated with activation of SK and the transient production of S1P [Meyer zu Heringdorf et al., 2001]. This suggested the existence of an alternative pathway for calcium mobilisation in mammalian cells. It has been proposed that intracellular S1P has a general role in calcium mobilisation for many GPCRs since other GPCRs have been shown to mobilise calcium in a manner which was dependent on SK activation [Meyer zu Heringdorf et al., 1998]. Although several other reports support this idea, the intracellular target of S1P, which is likely to be located in the endoplasmic reticulum, has not yet been identified.
Sphingosine kinase orthologues exist in lower organisms that do not express lipid-responsive GPCRs. In yeast, deletion of the two SK genes was associated with decreased survival after heat stress [Hla, 2003]. S1P is also produced in plants and regulation of stomatal function was found to be dependent on SK activation [Coursol et al., 2003]. These responses in yeast and plants, which occur after environmental stress, also involve calcium regulation, suggesting that S1P might have a universal role in calcium regulation [Spiegel and Milstien, 2003]. This is an interesting concept since calcium itself has been suggested as a regulator of SK activity [Pyne and Pyne, 2000b]. Identification of the intracellular S1P targets in plants or yeast will be valuable in understanding the role of S1P as a second messenger in mammalian cells.

S1P is a bioactive lipid, which mediates myriad effects in diverse cell types. These effects are influenced by the repertoire of cell-surface-expressed S1P receptors and the presence or absence of co-stimulatory signals. It seems likely that the site of action of intracellular S1P will be identified, confirming S1P as an extracellular agonist of S1P receptors and an intracellular second messenger involved in calcium mobilisation. This is not unprecedented since the lipid mediators leukotriene B4 (LTB4) and LPA have been shown to activate both cell-surface and nuclear receptors [Yokomizo et al., 1997; Gobeil et al., 2003].

1.4.3 Phytosphingosine-1-phosphate

The related compound, phS1P (D-ribo-phytosphingosine-1-phosphate) shown in Figure 1.11, was recently found to be a high affinity ligand for the S1P4 receptor [Rios Candelore et al., 2002]. In phS1P, the double bond present in S1P has been reduced to yield an extra hydroxyl group on the C4 carbon atom.
Although the exact route of synthesis of phS1P has not been shown in vivo, the likely precursor (phytosphingosine) is produced in mammalian cells by the recently identified alkaline ceramidase aPHC [Mao et al., 2001]. The activity of certain alkaline and neutral ceramidases may be regulated by growth factors and cytokines, which is suggestive of a role in mediation of cell signalling. It is probable that phytosphingosine is a substrate for the sphingosine kinase enzymes and it is through this route that phS1P is produced. Traditionally, phytoceramide (and phS1P) have been thought of as only being present in high concentrations in lower eukaryotes, such as Sacchromyces cerevisiae, however the recent detection of trace amounts of phytoceramide in mammalian tissues such as skin, liver and kidney coupled with the expression of aPHC mRNA in these tissues suggests that phS1P may have an important physiological role to play in these environments [Mao et al., 2001]. These researchers suggested that the relevance of phS1P as a ligand for S1P₄ might be linked with the restricted expression pattern of aPHC. The presence of aPHC mRNA and its product (phytosphingosine) in skin has been highlighted as particularly interesting since Langerhans cells are skin-specific dendritic cells which strongly express S1P₄.

PhS1P binds to human S1P₄ with a Kₐ value of approximately 2 nM and is displaced by competition with dhS1P, S1P and FTY720-P, suggesting a shared binding pocket. The ability of phS1P to act as an agonist of S1P₄ was shown
using microphysiometer analysis. In this system, S1P was defined as a full agonist and had an EC\textsubscript{50} of approximately 12 nM, whilst phS1P was a partial agonist with an EC\textsubscript{50} of approximately 30 nM.

The physiological relevance of phS1P has yet to be investigated and the possibility that this compound represents the true \textit{in vivo} ligand for S1P\textsubscript{4} is speculation. It has not been reported whether phS1P is a high affinity ligand solely for S1P\textsubscript{4}, or whether it can bind strongly to the other S1P receptors, although given the structural similarity with S1P, it seems likely that phS1P would bind to other S1P receptors.

1.5 \textbf{FTY720 and S1P receptors}

Immunosuppressant drugs, such as cyclosporin A (CsA), are used in the prevention of organ transplant rejection and for the treatment of autoimmune diseases including rheumatoid arthritis and psoriasis. The mode of action of this class of compound is to suppress immune responses by inhibiting production of the cytokine interleukin-2 (IL-2) by antigen-stimulated T cells. However, their use is associated with severe side effects, including hepatic and renal toxicity.

Recently, the compound FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-propane-1,3-diol) has been identified as a potent immunomodulatory compound (shown in Figure 1.12). This compound has a highly novel mechanism of action and is believed to exert its effects via activation of S1P receptors. FTY720 is a synthetic structural analogue of a fungal metabolite derived from \textit{Isaria sinclairii}, which has been used as a traditional Chinese herbal medicine for thousands of years and is considered as one of the three oriental medicines that bestow eternal youth [Im, 2003]. The development of FTY720 is a classic
example of ethnopharmacology, wherein the biological mechanism for a traditional therapeutic herbal agent has been elucidated and has resulted in the chemical optimisation of a novel class of compound.

**Figure 1.12**
Structure of FTY720.

FTY720 functions *in vivo* to promote reversible lymphopenia via retention of circulating B and T cells in secondary lymphoid organs [Chiba *et al.*, 1998]. The effect of T cell redistribution is to reduce the numbers of these cells at sites of inflammation and hence to attenuate the immune response. The compound has been extensively profiled in animal transplantation models, including skin, islet and solid organ allograft (reviewed by Brinkmann and Lynch [2002]) and synergises with traditional immunosuppressive agents, allowing combination therapy of FTY720 with subclinical doses of compounds such as CsA. FTY720 monotherapy in models of adjuvant- and collagen-induced arthritis and systemic lupus erythmatosus has also been successfully applied. Low doses of FTY720 can reportedly be used as a prophylactic or a therapeutic in the model of multiple sclerosis, experimental autoimmune encephalomyelitis. Efficacy of this compound has been shown in human phase II trials for the prevention of acute rejection in *de novo* renal patients on baseline immunosuppression [Brinkmann and Lynch, 2002].
Chapter 1

Administration of FTY720 promotes sequestration of naïve and activated CD4 and CD8 T cells and B cells from the circulation into lymph nodes and Peyer's patches [Xie et al., 2003]. Although the effects of this compound had been known for some years, its mode of action was unclear. Sensitivity of the lymphocyte homing effect to pertussis toxin suggested the involvement of a Gα4 coupled GPCR and the chemokine receptor CCR7 was proposed as a potential candidate. CCR7 and its chemokine ligands, ELC (EBV-induced molecule 1 ligand chemokine) and SLC (secondary lymphoid tissue chemokine) are known to play a critical role in the migration of B and T cells into secondary lymphoid organs. It was subsequently shown that FTY720 did not act via this chemokine receptor, since the compound was still able to promote lymphopenia in both CCR7 deficient and plt mice, which do not express either ligand for CCR7 [Henning et al., 2001], although the kinetics of FTY720-induced lymphocyte homing in these mice were delayed compared to wild type control animals.

The breakthrough in determining the way in which FTY720 elicits its response came with the observation that FTY720 is rapidly metabolised *in vivo* by addition of phosphate to the terminal alcohol group to yield the compound FTY720-P (shown in Figure 1.13). The structure of this metabolite bears noticeable resemblance to that of S1P and it was proposed that FTY720-P may act at S1P receptors. Two independent groups recently identified this compound as a high affinity (Kd's 0.2-6 nM), potent agonist at four of the five S1P receptors (S1P1,3,4,5) [Mandala et al., 2002; Brinkmann et al., 2002], but not the S1P2 receptor. FTY720 was shown to be rapidly converted *in vivo* to the phosphate, and this conversion could also be mediated via the isolated sphingosine kinase enzyme, albeit more slowly. Since T cells express mRNA for S1P1,4,5 receptors,
the implication of these studies was that activation of one or more S1P receptors
present on T cells promoted sequestration of circulating T cells to secondary
lymphoid organs. FTY720 is the first example of the application of a
sphingomimetic compound as a therapeutic agent (reviewed by Kester and
Kolesnick [2003]).

\[
\text{Figure 1.13} \\
\text{Structure of FTY720-P.}
\]

In a subsequent study, the effect of FTY720 on T cell chemotaxis \textit{in vitro} was
investigated [Graeler and Goetzl, 2002]. FTY720 did not stimulate movement of
murine T cells when tested at concentrations of 1-100 nM, but could dose-
dependently inhibit S1P-induced T cell chemotaxis \textit{in vitro}. These results were
interpreted as suggesting that FTY720 has no direct chemotactic activity but can
suppress the stimulatory effects of S1P on T cell migration. The apparent
contradiction between these results and the previous findings that the
lymphopenic action of FTY720 was a consequence of its actions as a high
affinity S1P receptor agonist highlight the need for further research in this area.

The report of Brinkmann \textit{et al.}, [2002] identified the phosphorylated form of the
(R)-isomer of the related compound, AAL-P (2-amino-4-(4-heptyloxyphenyl)-2-
methylbutanol) [Figure 1.14] to be another potent agonist of S1P\textsubscript{1,3,4,5} receptors.
This compound was a more potent agonist of S1P\textsubscript{4} than FTY720-P (EC\textsubscript{50} = 4 nM
compared to 63 nM for FTY720-P [Brinkmann and Lynch, 2002]).
unphosphorylated AAL compound exhibited similar *in vivo* lymphopenic effects to those of FTY720.

![Figure 1.14 Structure of (R)-AAL-P.](image)

Although it seems highly likely that the lymphopenic effect of FTY720 administration occurs, at least in part, as a result of activation of S1P receptors expressed on T cells, the precise manner in which the T cells are recruited to, and held in lymph nodes and Peyer’s patches is unclear. Recent advances have suggested that FTY720 has dual effects to promote increased intrinsic mobility of peripheral B and T cells and to increase endothelial integrity, thereby preventing egress from secondary lymphoid organs.

These exciting observations have significantly increased interest in the S1P receptors as potential therapeutic targets and have also identified highly potent agonists against four of the five receptors. The actions of FTY720-P have suggested an important role for S1P in lymphocyte trafficking and control of the immune response.

### 1.6 Project aims

When the work described in this thesis was started, S1P₄ was the orphan receptor named Edg6. From sequence similarities with other Edg receptors, it seemed likely that the Edg6 orphan would be activated by the lysophospholipid S1P and would couple to Gα₁ G proteins. Therefore, the first aim of this work was to
express S1P4 in mammalian cells and investigate whether the receptor coupled to this class of G proteins and could be activated by S1P. It was known that other members of the S1P and LPA receptor families were widely expressed in mammalian cells and that their presence could lead to the production of interfering signals in functional assays. A fusion protein between S1P4 and a mutated, pertussis toxin-insensitive variant of the Go1i G protein was also used in these investigations so that potential interference due to activation of endogenous Edg receptors present in host cells could be eliminated via treatment with pertussis toxin.

Since it was expected that the host cells used for expression of the recombinant S1P4 and S1P4-Go1i fusion proteins would express endogenous Edg family receptors, the second aim of this work was to use the technique of RT-PCR to determine the profile of LPA and S1P receptors endogenously expressed within these cells.

Many GPCRs exhibit the phenomenon of constitutive activity, and results generated in the early stages of this thesis using a [35S]GTPyS binding assay to detect S1P4 activation suggested this receptor to be constitutively active. At the time of these investigations, no inverse agonists were available for this class of GPCRs and so it was necessary to use an alternative approach to further characterise agonist-independent signalling by this receptor. Therefore, the relationship between the expression level of S1P4 and the level of basal signalling was investigated using an mammalian inducible expression system.

As would be expected, many laboratories are working on the S1P receptor family and significant findings were reported during the course of the investigations.
described in this thesis. As a consequence, the aims of this project developed as
new observations concerning this receptor family were published. The final aim
of the work presented in this thesis was to examine the nature of ligand
preference exhibited S1P₄, in the light of published observations. Firstly, the
activity of newly identified agonists such as phS1P was determined using the
[^35S]GTPγS binding assay. Novel information regarding the role of the
conserved E¹²¹ residue present in the S1P receptor family was then used to
further understand the nature of S1P₄ ligand preference. The role of the this
residue in S1P receptors has been investigated using the S1P₁ receptor to
exemplify this receptor family. Mutation of this residue to glutamine (present at
this position in the LPA receptor family) was found to influence ligand
selectivity and allowed activation of S1P₁ by LPA, whilst preventing its response
to S1P [Wang et al., 2001]. The second part of this final aim was to further test
the hypothesis that the conserved E¹²¹ residue of S1P receptors is key in
controlling ligand selectivity. This was achieved by introduction of the
equivalent mutation in the S1P₄ receptor and evaluation of its consequences on
activation of[^35S]GTPγS binding by S1P and LPA after expression of the mutant
receptor in mammalian cells.
Chapter 2 – Materials and Methods

2.1 General chemicals and materials

General laboratory chemicals were purchased from Sigma Aldrich Company Ltd., (Gillingham, Dorset, U.K.) or BDH Ltd., (Poole, Dorset, U.K.) unless stated otherwise. Ethanol and Biphenol/Tris were from Hayman Ltd., (Witham, Essex, U.K.) and Camlab Ltd., (Cambridge, U.K.), respectively. Bacto-tryptone, bacto-agar and bacto-yeast extract were from Difco Ltd., (Detroit, MI., U.S.A.). Restriction endonucleases, dNTPs, DNA molecular weight markers, T4 DNA ligase, calf intestinal phosphatase and amplification grade deoxyribonuclease I were purchased from New England Biolabs Ltd., (Hitchin, Herts., U.K.) or Roche Diagnostics Ltd., (Lewes, East Sussex, U.K.). DNA sequences were cloned into pCR4-TOPO, pIRESpuro or pIND expression vectors, Invitrogen Ltd., (Paisley, Scotland, U.K.). QIAGEN Miniprep, Maxiprep, QIAquick Gel Extraction, PCR purification, DNeasy genomic DNA and RNeasy Mini RNA purification kits were from Qiagen Ltd., (Crawley, West Sussex, U.K.). Apparatus for horizontal gel electrophoresis was supplied by Invitrogen Ltd., (Paisley, Scotland, U.K.). Big Dye Terminator Cycle Sequencing Ready Reaction DNA sequence kit and Amplitaq DNA polymerase were obtained from Applied Biosystems, (Warrington, Cheshire, U.K.). Sequencing reactions were carried out using a Biometra Trio-Thermoblock (Anachem Ltd., Luton, Herts., U.K.) or Primus 96 Plus (MWG-Biotech Ltd., Milton Keynes, Bucks., U.K.). All other PCR reactions were performed using Biometra Personal Cycler (Anachem Ltd., Luton, Herts., U.K.) or Primus 96 Plus (MWG-Biotech Ltd., Milton Keynes,

Media and supplements for tissue culture and transfection reagents were supplied by Invitrogen Ltd., (Paisley, Scotland, U.K.); non-enzymatic cell dissociation solution was from Sigma Aldrich Company Ltd., (Gillingham, Dorset, U.K.); foetal bovine serum was from Helena Biosciences Ltd., (Sunderland, U.K.) or PAA Labs GmbH., (Linz, Austria) and tissue culture plastics were from BD Falcon (Erembodegem, Belgium). CHO-K1 and HEK-293 cells were from ECACC (CAMR, Porton Down, Wilts., U.K.) and CHO-EcR cells were supplied by Invitrogen Ltd., (Paisley, Scotland, U.K.).

Sphingosine-1-phosphate was obtained from Biomol Research Laboratories Inc., (Plymouth Meeting, PA., U.S.A.) and UK14304 was from Tocris Cookson Ltd., (Bristol, U.K.). Pertussis toxin was purchased from CN Biosciences Ltd., (Nottingham, U.K.). Lysophosphatidic acid (18:1, 16:0 and 14:0) was from Avanti Polar Lipids Inc., (Alabaster, AL., U.S.A.). Other chemicals (FTY720 and AAL) were synthesised by the Medicinal Chemistry department of Celltech R&D Ltd.

The radiochemical guanosine 5'-[\gamma^{35}\text{S}]triphosphate, triethylammonium salt ([\gamma^{35}\text{S}]GTP\gamma S) had a specific activity of > 37 TBq/mmol and was purchased from Amersham Biosciences (Amersham, Bucks., U.K.).
Tris-Glycine SDS Page gels and SeeBlue prestained protein markers were supplied by Invitrogen Ltd., (Paisley, Scotland, U.K.). Immobilon-P PVDF membrane was from Millipore Ltd., (Watford, Herts., U.K.).

Small scale centrifugation was performed using Eppendorf 5415D (Anachem Ltd., Luton, Herts., U.K.) or Hettich refrigerated EBA12R (Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany) benchtop microcentrifuges; Sorvall RC3B or RC5B refrigerated centrifuges were used for large scale centrifugation.

2.1.1 Media for bacterial growth

<table>
<thead>
<tr>
<th>Media</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB Agar</td>
<td>1 % (w/v) Bacto-tryptone, 0.5 % Bacto-yeast extract, 171 mM NaCl, 1.5 % (w/v) Bacto-agar, pH 7.0</td>
</tr>
<tr>
<td>SOC</td>
<td>2 % (w/v) Bacto-tryptone, 0.5 % Bacto-yeast extract, 8.6 mM NaCl, 25 mM KCl, 10 mM MgCl₂, 20 mM glucose, pH 7.0</td>
</tr>
<tr>
<td>2x TY Broth</td>
<td>2 % (w/v) Bacto-tryptone, 1 % Bacto-yeast extract, 171 mM NaCl, 1.5 % (w/v) Bacto-agar, pH 7.0</td>
</tr>
</tbody>
</table>

2.1.2 Buffer formulations

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>137 mM NaCl, 2.68 mM KCl, 10.1 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4</td>
</tr>
<tr>
<td>TAE</td>
<td>40 mM Tris-acetate pH 8.0, 2.5 mM EDTA</td>
</tr>
</tbody>
</table>
**Chapter 2**

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TE</strong></td>
<td>10 mM Tris-HCl pH 7.4, 1 mM EDTA</td>
</tr>
<tr>
<td><strong>Hi TNE</strong></td>
<td>65 mM Tris-HCl pH 8, 15 mM EDTA, 4 M NaCl</td>
</tr>
</tbody>
</table>

**5x DNA loading buffer**

0.25 % (w/v) bromophenol blue, 30 % (w/v) glycerol

**2x protein sample buffer**

125 mM Tris-HCl pH 6.8, 0.715 M 2-mercaptoethanol, 20 % (w/v) glycerol, 4 % (w/v) SDS, 0.1 % (w/v) bromophenol blue

**5x ligation buffer**

250 mM Tris-HCl pH 7.4, 5 mM ATP, 100 mM DTT, 50 mM MgCl₂

**Clontech Advantage PCR and RT buffers**

- **10x Advantage 2 PCR buffer**
  - 400 mM Tricine-KOH, pH 9.2 at 25 °C
  - 150 mM potassium acetate
  - 35 mM magnesium acetate
  - 37.5 µg/mL BSA
  - 0.05 % (v/v) Nonidet P-40
  - 0.05 % Tween-20

- **5x Advantage-GC 2 PCR buffer**
  - 200 mM Tricine-KOH, pH 9.2 at 25 °C
  - 75 mM potassium acetate
  - 17.5 mM magnesium acetate
  - 25 % (v/v) DMSO
  - 18.75 µg/mL BSA
  - 0.025 % (v/v) Nonidet P-40
  - 0.025 % Tween-20

- **5x Advantage RT-for-PCR buffer**
  - 200 mM Tris-HCl, pH 8.3
  - 375 mM KCl
  - 15 mM MgCl₂
### QIAGEN kit purification buffers

<table>
<thead>
<tr>
<th>Buffer Name</th>
<th>Composition Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer AB (elution buffer)</td>
<td>10 mM Tris-HCl, pH 8.5</td>
</tr>
<tr>
<td>Buffer AL (lysis buffer)</td>
<td>Contains chaotropic salts *</td>
</tr>
<tr>
<td>Buffer AW1 (wash buffer 1)</td>
<td></td>
</tr>
<tr>
<td>Buffer AW2 (wash buffer 2)</td>
<td></td>
</tr>
<tr>
<td>Buffer EB (elution buffer)</td>
<td></td>
</tr>
<tr>
<td>Buffer N3 (neutralisation buffer)</td>
<td>50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/mL RNase A</td>
</tr>
<tr>
<td>Buffer PI (resuspension buffer)</td>
<td>200 mM NaOH, 1% (w/v) SDS</td>
</tr>
<tr>
<td>Buffer P2 (lysis buffer)</td>
<td></td>
</tr>
<tr>
<td>Buffer P3 (neutralisation buffer)</td>
<td>3.0 M potassium acetate, pH 5.5</td>
</tr>
<tr>
<td>Buffer PB (binding buffer)</td>
<td>Contains chaotropic salts *</td>
</tr>
<tr>
<td>Buffer PE (wash buffer)</td>
<td></td>
</tr>
<tr>
<td>Buffer QBT (equilibration buffer)</td>
<td>1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, 15% (v/v) isopropanol</td>
</tr>
<tr>
<td>Buffer QG (solubilisation buffer)</td>
<td></td>
</tr>
<tr>
<td>Buffer RLT (lysis buffer)</td>
<td></td>
</tr>
<tr>
<td>Buffer RW1 (wash buffer 1)</td>
<td></td>
</tr>
<tr>
<td>Buffer RPE (wash buffer 2)</td>
<td></td>
</tr>
</tbody>
</table>

* The composition of these QIAGEN buffers is withheld by the manufacturer.
2.1.3 Sources of cDNA

The human peripheral blood mononuclear (PBMC) cDNA library had been previously prepared at Celltech R&D Ltd. Go(1)(C^{35}I) and α_2AAR-GO(1)(C^{35}I) constructs were kindly provided by Professor Graeme Milligan (University of Glasgow, Scotland, U.K.).

2.1.4 Bacterial strains

Routine propagation of recombinant plasmids was performed using Novablue chemically competent E. Coli cells (endA1 hsdR17(rK12 mK12^+ ) supE44 thi-1 recA1 gyrA96 relA1 lacF',proA',B',lacP',Z M15::Tn10 (Tc^)), supplied by Novagen Inc., (Madison, WI, U.S.A.). TOPO cloning reactions were transformed into TOP10 chemically competent E. Coli cells (F mcrA Δ(mrr-hsdRMS-merBC) φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str^) endA1 mupG), supplied by Invitrogen Ltd., (Paisley, Scotland, U.K.).

2.1.5 Antibodies

High affinity rat anti-HA monoclonal antibodies (unconjugated and Fluorescein-conjugated), which recognises the HA epitope tag (YPYDVPDYA) were from Roche Diagnostics Ltd. Rabbit polyclonal anti-GO(1) (sc-391) and anti GO(1/2/3) (sc-262) antibodies were supplied by Autogen Bioclear Ltd., (Calne, Wilts., U.K.). The rabbit polyclonal SGI antibody was raised against the decapeptide KENLKDCGLF [Grant et al., 1997] and cross-reacts with GO(1/2). Peroxidase conjugated anti-rat and anti-rabbit IgG antibodies were purchased from Jackson ImmunoResearch Laboratories Inc., (West Grove, PA., U.S.A.). Isotype matched negative control antibody for use with the rat anti-HA-Fluorescein
antibody was FITC conjugated rat IgG1,κ and was supplied by BD Pharmingen (Erembodegem, Belgium). The anti-SIP₄ antibody was a rabbit polyclonal antibody that was raised against a peptide corresponding to amino acids 226-243 of the human SIP₄ sequence (Lifespan Biosciences, Inc., Seattle, WA., U.S.A.).

2.1.6 Oligonucleotides

Oligonucleotides were designed using sequences in the Genbank nucleotide sequence database. Shown below are sequences for oligonucleotides that were used for PCR cloning, PCR mutagenesis and RT-PCR; vector specific and sequencing oligonucleotides are not shown.

**Human SIP₄ (Edg6) – Genbank accession number AJ000479**

**F7240 (forward)**
5'-GAGAGAGCGGCGGCAACATGAACGACCCCGGGACC-3'  
*NotI*  
Start

**F7241 (reverse)**
5'-GAGAGAGAATTCTTTATCACTGCTCCGACGCTGGAG-3'  
*EcoRI*  
Stop  
Stop

**F12582 (forward)**
5'-GAGAGAGCGGCGGCACCATTGTATCCATATGATGTTCCAGATTAT  
*NotI*  
Start  
HA-tag  
GCTAACGCACCGGGAGCCCGGGTG-3'

**F12581 (reverse)**
5'-GAGAGAGACCATGGTGGCAAAGCGCTCC-3'  
*NcoI*

**F27384 (forward)**
5'-GAGAGAGGATCCTGGCCTGGCCGTC-3'  
*BamHI*

**F36663 (reverse)**
5'-GAGAGAGAATTCGCGATGCTCCGACGCTGGAGATG-3'  
*EcoRI*  
A(aa)
Oligonucleotides were designed to allow PCR amplification of G\(\alpha_{II}\), which had previously been modified to contain the C\(^{351}\)I mutation, which confers insensitivity to pertussis toxin [Bahia et al., 1998]; the presence of this mutation affected design of the reverse primer and its location in this primer is shown.

Cloning of partial sequences of Edg receptor genes from Chinese hamster genomic DNA was achieved using the following oligonucleotide pairs, which had been designed using alignments of nucleotide and protein sequences from available species. The suffix B indicates oligonucleotides which were used for
cloning fragments of Chinese hamster Edg receptor genes; those designated with
the suffix C were used for RT-PCR.

**Chinese hamster S1P₁ (Edg1)**

1170B (forward)
5'-GCTGGGTCATCTCCCTCATCCTGG-3'

1171B (reverse)
5'-GGTCAGAGTGTAGATGATGGGTTG-3'

1364C (forward)
5'-CCATCAGGGCTGGAACCTGCATC-3'

1365C (reverse)
5'-ACCTGAGTTTCAGCACAGCCAGAA-3'

**Chinese hamster S1P₂ (Edg5)**

1176B (forward)
5'-AACAGCAAGTTCCACTCACGCAATGTA-3'

1177B (reverse)
5'-GCAGCCAGCAGYATGATGCAGCAG-3'

1372C (forward)
5'-CTGTTTCCTCGCAACCTGGCAG-3'

1373C (reverse)
5'-CAGTACGATGGTGACTGTCTTGAG-3'

**Chinese hamster S1P₃ (Edg3)**

1215B (forward)
5'-CTGATGTTTTTGATTGCCATCTCGA-3'

1217B (reverse)
5'-CCAGCGTGTAGATGACNGGGTTCA-3'

1368C (forward)
5'-AACAATAAAATTTCAACAACCAGCATGTAC-3'
1369C (reverse)
5'-GATGAACACACTCACTACTACATCACG-3'

Chinese hamster S1P₄ (Edg6)

53B (forward)
5'-GAGAGTGAGGGCCACCAAGACCCAGCCG-3'

54B (reverse)
5'-CCTCACGGCTGCGGAAGGAGTAGATGA-3'

1374C (forward)
5'-GTGTATGTTGCTGCGGCTGTG-3'

1375C (reverse)
5'-GGGTGATGGCAGGTTGAGTACG-3'

Chinese hamster S1P₅ (Edg8)

1182B (forward)
5'-GGTTTGCGCCGNGARGGRGGYTCTG-3'

58B (reverse)
5'-GTCCGGCAGGTCKCGTGGTGTA-3'

1378C (forward)
5'-GTGGCAGTGGCCGCGATCGGTG-3'

1379C (reverse)
5'-GAACGATGATGATGGGATTCAG-3'

Chinese hamster LPA₁ (Edg2)

45B (forward)
5'-CACAGCCCCAGTTACACAGCCCATG-3'

46B (reverse)
5'-GTTGAAATGGCCAGAAGACTAAG-3'

1366C (forward)
5'-CGAACCCCATGCTTCTACAACG-3'
1367C (reverse)
5'-GTAGGAGTCACTATAGAGGGGTG-3'

Chinese hamster LPA$_2$ (Edg4)

49B (forward)
5'-GGCCAGTGCTACTACAACGAGACCA-3'

1174B (reverse)
5'-AGACAACGGAGCTSGAYAGRGCCCA-3'

1370C (forward)
5'-ATTGGCTTTTTCTACAATAACAGTGG-3'

1371C (reverse)
5'-CACAGCCAGGTATGAGCGGCTG-3'

Chinese hamster LPA$_3$ (Edg7)

1178B (forward)
5'-GAGTGTCATATGACAAGCNCATGG-3'

1180B (reverse)
5'-CAGTCATCACYGTCTTCATTAGCTTCA-3'

1376C (forward)
5'-GACTTTTTTCAACAGGAGCAACTC-3'

1377C (reverse)
5'-AGCCCTCCCGCGCTGATGGA-3'

Chinese hamster GAPDH – Genbank accession number X52123

59C (forward)
5'-GGTCGGCGTGAACGGATTTGGCCG-3'

60C (reverse)
5'-CGCATTGCTGACAATCTTGAGGGAG-3'
2.1.7 Mammalian cell lines

CHO-K1 cells (85051005) and HEK-293 cells (85120602) were from ECACC (CAMR, Porton Down, Wilts., U.K.) and CHO-EcR cells were supplied by Invitrogen Ltd., (Paisley, Scotland, U.K.).

2.2 DNA methods

2.2.1 PCR amplification of DNA fragments

Due to its high GC content, amplification of S1P₄ sequences was performed using Advantage-GC 2 PCR kit. Typically, PCR reactions were carried out in a total volume of 20 μL and contained approximately 100 ng cDNA, genomic DNA or plasmid DNA template, 10 pmol each oligonucleotide, 4 μL Advantage-GC 2 PCR buffer, 0.5 M GC Melt, 0.2 mM dNTP mix (dATP, dCTP, dGTP, dTTP), 1 μL Advantage 2 Polymerase and sterile dH₂O.

Other PCR reactions were performed using the Advantage 2 PCR kit. Reactions were typically set up in a final volume of 20 μL and comprised approximately 100 ng cDNA or plasmid DNA template, 10 pmol each oligonucleotide, 2 μL Advantage 2 PCR buffer, 0.2 mM dNTP mix (dATP, dCTP, dGTP, dTTP), 1 μL Advantage 2 Polymerase and sterile dH₂O.

When PCR reactions were performed to generate DNA for use in cloning, reactions were scaled up to 50 μL, with the components in the same proportions as described above for 20 μL reactions.

Oligonucleotides were normally designed to have a melting temperature (Tₘ) of at least 68 °C.
Reactions were cycled under the following conditions:

“2 step program”

95 °C  1 minute
95 °C  30 seconds
68 °C  3 minutes
68 °C  3 minutes
4 °C  constant

x35 cycles

The two step PCR program was used for general PCR amplification and cloning activities. Amplification of partial Edg receptor sequences from Chinese hamster genomic DNA, and from first strand cDNA prepared from Chinese hamster RNA, used either this two step program, or the three step program shown below; details of which program was used for amplification of which partial sequence are shown in Table 2.1.

“3 step program”

94 °C  1 minute
94 °C  15 seconds
60 °C  30 seconds
68 °C  1 minute
68 °C  5 minutes
4 °C  constant

x30 cycles

Post-reaction, samples were analysed using agarose gel electrophoresis.
Table 2.1
Oligonucleotides and PCR programs used to amplify S1P and LPA receptor gene fragments from Chinese hamster genomic DNA or first strand cDNA. The suffix B denotes oligonucleotides used for cloning gene fragments from Chinese hamster genomic DNA; the suffix C denotes use in RT-PCR with first strand cDNA. Sequences of these oligonucleotides are shown in this chapter, section 1.6.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Forward oligonucleotide</th>
<th>Reverse oligonucleotide</th>
<th>PCR program</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1P_1</td>
<td>1170B</td>
<td>1171B</td>
<td>2 step</td>
</tr>
<tr>
<td></td>
<td>1364C</td>
<td>1365C</td>
<td>2 step</td>
</tr>
<tr>
<td>S1P_2</td>
<td>1176B</td>
<td>1177B</td>
<td>3 step</td>
</tr>
<tr>
<td></td>
<td>1372C</td>
<td>1373C</td>
<td>2 step</td>
</tr>
<tr>
<td>S1P_3</td>
<td>1172B</td>
<td>1173B</td>
<td>3 step</td>
</tr>
<tr>
<td></td>
<td>1368C</td>
<td>1369C</td>
<td>3 step</td>
</tr>
<tr>
<td>S1P_4</td>
<td>53B</td>
<td>54B</td>
<td>2 step</td>
</tr>
<tr>
<td></td>
<td>1374C</td>
<td>1375C</td>
<td>2 step</td>
</tr>
<tr>
<td>S1P_5</td>
<td>1182B</td>
<td>58B</td>
<td>2 step</td>
</tr>
<tr>
<td></td>
<td>1378C</td>
<td>1379C</td>
<td>3 step</td>
</tr>
<tr>
<td>LPA_1</td>
<td>45B</td>
<td>46B</td>
<td>2 step</td>
</tr>
<tr>
<td></td>
<td>1366C</td>
<td>1367C</td>
<td>2 step</td>
</tr>
<tr>
<td>LPA_2</td>
<td>49B</td>
<td>1174B</td>
<td>3 step</td>
</tr>
<tr>
<td></td>
<td>1370C</td>
<td>1371C</td>
<td>3 step</td>
</tr>
<tr>
<td>LPA_3</td>
<td>1178B</td>
<td>1180B</td>
<td>3 step</td>
</tr>
<tr>
<td></td>
<td>1376C</td>
<td>1377C</td>
<td>3 step</td>
</tr>
</tbody>
</table>

2.2.2 Analysis of DNA fragments by gel electrophoresis

DNA samples were analysed by horizontal electrophoresis, typically using 1 % (w/v) agarose gels in TAE buffer, which were run at 100 V/cm. Appropriate size markers were included on each gel. Ethidium bromide was added to both gel and running buffer, to a final concentration of 30 µg/mL. DNA bands were visualised using uv transillumination at 254 nm. Agarose gel electrophoresis was also used for the purification of DNA fragments for cloning; this was performed in the same manner as analytical gel electrophoresis.
2.2.3 Spin column purification of PCR products

Purification of PCR products which were to be ligated into expression vectors (after digestion with appropriate restriction enzymes) was performed using QIAGEN QIAquick PCR Purification Kit, in accordance with the manufacturer’s protocol; all centrifugation steps were performed using a benchtop centrifuge at maximum speed (16,100 x g). Briefly, 5 volumes buffer PB were added to 1 volume of PCR reaction and mixed; the sample was then applied to a QIAquick spin column and centrifuged for 1 minute; the flow-through was discarded. Column-bound DNA was washed with 0.75 mL buffer PE and centrifuged for 1 minute, the flow-through discarded and the column centrifuged for a further minute. 30 μL buffer EB was applied to the centre of the column, which was allowed to stand for 1 minute at room temperature prior to centrifugation for 1 minute; this eluted the DNA solution.

2.2.4 Purification of DNA fragments from agarose gels

DNA fragments were purified from agarose gels using QIAGEN QIAquick Gel Extraction Kit, in accordance with the manufacturer’s instructions; all centrifugation steps were performed using a benchtop centrifuge at maximum speed (16,100 x g). Briefly, the desired band was excised from the agarose gel and weighed; 3 volumes buffer QG were added per gel volume (and the assumption was made that 100 mg gel was approximately equal to 100 μL volume). The sample was incubated at 50 °C for 10 minutes in order to melt the agarose and mixed well; 1 volume isopropanol was mixed with the sample before it was applied to a QIAquick spin column and centrifuged for 1 minute. The flow-through was discarded and the column washed with 0.5 mL buffer QG
and centrifuged for 1 minute. Column-bound DNA was washed with 0.75 mL buffer PE and centrifuged for 1 minute, the flow-through discarded and the column centrifuged for a further minute. 30 µL buffer EB was applied to the centre of the column and allowed to stand for 1 minute at room temperature prior to centrifugation for 1 minute; this eluted the DNA solution.

2.2.5 Analysis of DNA fragments by restriction enzyme digest

For general restriction enzyme analysis, 1-2 µg DNA was incubated in an appropriate enzyme buffer with 5 units of each restriction enzyme for 2-4 hours at 37 °C. One unit is defined as the amount of enzyme required to completely digest 1 µg of substrate DNA in 1 hour. When the DNA was to be used in subsequent sub-cloning procedures, the PCR product (which had been purified according to this chapter, sections 2.3-2.4), or approximately 10 µg plasmid DNA was incubated with 20 units of each restriction enzyme, overnight at 37 °C; it was then subject to phenol-chloroform extraction prior to purification by gel extraction (this chapter, sections 2.4 and 2.7).

2.2.6 Alkaline phosphatase treatment of digested vector

Prior to ligation with DNA fragments, vector that had been digested with appropriate restriction enzymes was treated with alkaline phosphatase to prevent religation of partially digested vector. The reaction comprised approximately 10 µg DNA, an appropriate volume of 10x phosphatase buffer and alkaline phosphatase (100 units). One unit is defined as the enzyme activity which hydrolyses 1 µmol of 4-nitrophenyl phosphate in 1 minute at 37 °C. After incubation at 37 °C for 30 minutes, a further 100 units alkaline phosphatase were
added and the reaction incubated for another 30 minutes at 37 °C. The treated DNA was then purified by phenol-chloroform extraction (this chapter, section 2.7).

2.2.7 Phenol-chloroform extraction of DNA

After digestion with restriction enzymes or alkaline phosphatase treatment, DNA was subject to extraction with phenol/chloroform/isoamyl alcohol. Briefly, to a 100 μL reaction, 20 μL Hi TNE buffer, 80 μL TE buffer and 700 μL phenol/chloroform/isoamyl alcohol (25:24:1) were added and mixed well. Centrifugation (1 minute at 16,100 x g) was used to separate the aqueous and organic layers. The aqueous layer was carefully removed into a clean tube and the phenol/chloroform/isoamyl alcohol re-extracted after the addition of 20 μL Hi TNE buffer and 80 μL TE buffer. The aqueous layer was combined with the first aqueous fraction and extracted with chloroform. DNA in the resultant aqueous layer was then precipitated by the addition of 2.5 volumes of 100 % ethanol and incubation at -20 °C for 1 hour. The DNA was collected by centrifugation at 16,100 x g for 10 minutes, washed once with cold 70 % ethanol and resuspended in an appropriate volume of TE buffer.

2.2.8 Ligation of DNA fragments

Ligation of DNA fragments was performed in a total reaction volume of 20 μL, comprising 4 μL 5x ligation buffer, 400 units T4 DNA ligase, approximately 50 ng vector DNA and a molar equivalent of insert fragment(s). Control reactions, containing no insert DNA, were routinely performed to allow assessment of the background of uncut vector as well as the efficiency of
transformation. Ligation reactions were incubated overnight at 16 °C before transformation of a proportion of the reaction into competent \textit{E. Coli} cells.

\textbf{2.2.9 Transformation of DNA into competent \textit{E. Coli} cells}

Novablue competent \textit{E. Coli} cells were transformed in accordance with manufacturer's recommendations. Briefly, 50 \textmu L Novablue cells were gently thawed on ice and 1-2 ng DNA was added. The cells were incubated on ice for 5 minutes, heat-shocked at 42 °C for 30 seconds then incubated on ice for a further 2 minutes. 800 \textmu L SOC medium was added and cells were incubated on a shaker platform at 37 °C for 30 minutes. An appropriate volume of sample was then plated out onto LB Agar plates supplemented with 100 \mu g/mL ampicillin, which were then grown overnight at 37 °C.

\textbf{2.2.10 TOPO cloning of PCR products}

PCR products which were amplified with the specific purpose of sequencing (i.e. partial sequences of SIP- and LPA-receptor coding sequences which were amplified from Chinese hamster genomic DNA) were cloned into pCR4-TOPO vector using the TOPO TA Cloning Kit for Sequencing, in accordance with the manufacturer's recommendations. Briefly, PCR products were purified from agarose as described in this chapter, section 2.4 and cloned into the TOPO TA vector in a 5 minute reaction. 2 \textmu L of each reaction was then transformed into TOP10 One Shot chemically competent cells, which were incubated on ice for 5 minutes, heat shocked at 42 °C for 30 seconds and returned to ice. 250 \textmu L SOC medium was added and the tube incubated on a shaker platform for 1 hour at 37 °C after which time appropriate volumes were plated out onto LB Agar.
plates supplemented with 100 \( \mu \text{g/mL} \) ampicillin; these plates were then grown overnight at 37°C.

### 2.2.11 Isolation of plasmid DNA using QIAGEN Miniprep columns

Miniprep columns were used to prepare recombinant plasmid DNA for subsequent analysis via restriction enzyme digest or DNA sequencing. The method used was broadly in accordance with the manufacturer’s recommendations and centrifugation steps were performed using a benchtop centrifuge at maximum speed (16,100 \( \times \) g), unless stated otherwise. Briefly, a single colony of transformed \textit{E. Coli} was picked from a selective LB Agar plate and used to inoculate a 10 mL 2x TY culture (containing 100 \( \mu \text{g/mL} \) ampicillin). The culture was grown overnight at 37°C and bacteria collected by centrifugation (1,500 \( \times \) g for 15 minutes). The cell pellet was resuspended in 250 \( \mu \text{L} \) buffer P1, then 250 \( \mu \text{L} \) lysis buffer P2 was added and mixed by gentle inversion. The lysis reaction was neutralised by the addition of 350 \( \mu \text{L} \) buffer N3 and was immediately mixed by gentle inversion. Centrifugation (10 minutes) was used to clear the cell lysate and the supernatant fraction was applied to a QIAprep spin column. The column was placed in a 2 mL collection tube and centrifuged for 1 minute. Bound DNA was washed with 0.5 mL buffer PB then with 0.75 mL buffer PE; centrifugation for 1 minute followed each wash step. After an additional 1 minute centrifugation to remove any remaining PE buffer, DNA was eluted from the column in 180 \( \mu \text{L} \) buffer EB, which was collected by centrifugation, into a clean tube. The DNA was then precipitated by the addition of 20 \( \mu \text{L} \) Hi TNE buffer and 600 \( \mu \text{L} \) 100 % ethanol with incubation at -20°C for
Chapter 2

1 hour. Precipitated DNA was collected by centrifugation for 10 minutes, washed once with 70 % ethanol and resuspended in 30 μL buffer EB.

2.2.12 Isolation of plasmid DNA using QIAGEN Maxiprep kit

Larger quantities of plasmid DNA were prepared from overnight cultures (200 mL 2x TY medium containing 100 μg/mL ampicillin) using QIAGEN-tip 500, following the manufacturer’s guidelines; centrifugation steps were performed using Sorvall RC5B refrigerated centrifuge. In brief, cells were collected by centrifugation (4,600 x g rpm for 15 minutes) and the cell pellet resuspended in 10 mL buffer P1. 10 mL buffer P2 was added and mixed gently to lyse the cells. After an incubation of 5 minutes at room temperature, 10 ml buffer P3 (which had been chilled to 4 °C) was added and the lysate was immediately poured into a QIAfilter cartridge and incubated for 10 minutes at room temperature. The cell lysate was then filtered through the QIAfilter cartridge and applied to a QIAGEN-tip 500 (which had been previously equilibrated using 10 mL buffer QBT) and allowed to flow under gravity pressure. Bound DNA was washed twice with 30 mL buffer QC then eluted with 15 mL buffer QF. 10.5 mL isopropanol was added to the eluted DNA and mixed well then centrifuged (17,000 x g, at 4 °C for 40 minutes). Precipitated DNA was washed once with 70 % ethanol, air-dried and resuspended in an appropriate volume of TE buffer.

2.2.13 Optical density measurement of DNA

The concentration of DNA was determined via measurement of the optical density at 260 nm, using a quartz cuvette. A Genova uv/vis spectrophotometer
(Jenway Ltd., Dunmow, Essex, U.K.) was used and an optical density reading of 1.0 was assumed to be equivalent to a concentration of 50 μg/mL.

2.2.14 DNA sequencing

DNA sequencing reactions were performed in 0.2 mL thin-walled tubes (Anachem Ltd.) for analysis using either 373A or 3100 automated DNA sequencer (Applied Biosystems, Warrington, Cheshire, U.K.)

For analysis using 373A sequencer, each reaction comprised 5 pmol sequencing primer, 1.0-1.5 μg plasmid DNA, 1.5 μL sequencing buffer and 1.5 μL Big Dye Terminator Reaction Ready sequencing premix (Applied Biosystems). Distilled water was added to bring the volume to 7.5 μL and the reaction was cycled under the following conditions:

\[
\begin{align*}
96 \, ^\circ C & \quad 30 \text{ seconds} \\
96 \, ^\circ C & \quad 10 \text{ seconds} \\
50 \, ^\circ C & \quad 5 \text{ seconds} \\
60 \, ^\circ C & \quad 4 \text{ minutes} \\
4 \, ^\circ C & \quad \text{constant}
\end{align*}
\]

The DNA was then precipitated by the addition of 2 μL 3 M sodium acetate (pH 4.6) and 50 μL 95 % ethanol. Samples were incubated on ice for 10 minutes and centrifuged at maximum speed (16,100 x g) for 15 minutes. The supernatant was removed and the pellet was air-dried, then resuspended in sample loading buffer.

For analysis using 3100 sequencer, reactions were prepared in the same manner as described above, except that 250 ng DNA was used per reaction. After cycling, samples were batch precipitated by the addition of 0.76 μL 3 M sodium acetate (pH 4.6) and 19.23 μL 95 % ethanol to each tube. Samples were
incubated at room temperature for 15 minutes and centrifuged at 1,500 x g for 45 minutes at 4°C. The resultant pellets were washed with 70% ethanol, air dried and resuspended in 10 μL formamide.

2.2.15 Isolation of genomic DNA from mammalian cells

Genomic DNA was prepared from CHO-K1 cells using QIAGEN DNeasy kit, in accordance with the manufacturer’s recommendations; centrifugation steps were performed using a benchtop centrifuge at maximum speed (16,100 x g). In brief, approximately 5 x 10^6 cells were resuspended in 200 μL PBS then 20 μL proteinase K and 200 μL buffer AL added to the sample which was mixed by vortexing before incubating for 10 minutes at 70 °C. 200 μL absolute ethanol was added and mixed by vortexing and the sample applied to a DNeasy spin column, which was placed in a collection tube and centrifuged for 1 minute. Bound DNA was washed first with 500 μL AW1 then with 500 μL AW2 buffers; before each wash step the spin column was placed into a fresh collection tube and centrifuged for 1 minute (AW1 wash) or 3 minutes (AW2 wash). DNA was eluted from the column by the application of 200 μL buffer AE and was collected by centrifugation after an incubation of 1 minute. The elution step was repeated and the eluates combined. Genomic DNA was stored at 4 °C.

2.2.16 Isolation of total RNA from mammalian cells

Total RNA was prepared from CHO-K1 cells using QIAGEN RNeasy Mini kit, in accordance with the manufacturer’s recommendations; centrifugation steps were performed using a benchtop centrifuge at maximum speed (16,100 x g). Briefly, approximately 4 x 10^6 cells, which had been washed with PBS, were lysed in 350 μL buffer RLT (which had been supplemented with 143 mM...
2-mercaptoethanol) and mixed by vortexing. The lysate was homogenised by application to a QIAshredder column, which was placed in a 2 mL collection tube and centrifuged for 2 minutes. 350 µL 70 % ethanol was added to the homogenised lysate, mixed by pipetting and then applied to an RNeasy Mini column, which was placed in a 2 mL collection tube. The sample was bound to the column by centrifugation for 15 seconds and then washed with 700 µL buffer RW1 followed by 2 x 500 µL washes with buffer RPE; centrifugation followed each wash step. Bound RNA was eluted by the application of 50 µL RNase-free water and collected by centrifugation for 1 minute; the elution was repeated and the eluates were pooled.

Purified total RNA was treated with amplification grade deoxyribonuclease I (DNase I) to eliminate any contaminating genomic DNA. Approximately 5 µg RNA was treated in a 50 µL reaction, which also contained 5 µL 10x DNase I reaction buffer, 5 units DNase I and an appropriate volume of DEPC-treated water. After incubation at room temperature for 15 minutes, the reaction was terminated by the addition of EDTA to a final concentration of 2.2 mM, followed by heat inactivation of the enzyme at 65 °C for 10 minutes.

2.2.17 Synthesis of first strand cDNA by RT-PCR

First strand cDNA was prepared from CHO-K1 total RNA (preparation of which is described in this chapter, section 2.16) using Advantage RT-for-PCR kit, following the manufacturer’s protocol. In essence, approximately 1 µg total RNA was mixed with oligo dT primer and heated for 2 minutes at 70 °C then immediately cooled on ice. 5x reaction buffer, dNTPs, recombinant RNase inhibitor and MMLV reverse transcriptase were added to give a 20 µL reaction
which was incubated at 42 °C for 1 hour then heated at 94 °C to terminate the reaction, denature RNA-cDNA hybrids and destroy DNase activity. The resultant cDNA was stored at -80 °C until use and 2 µL used as template in 20 µL gene specific amplifications, which were carried out as described in this chapter, section 2.1.

2.3 Cell culture and cell biology methods

2.3.1 General cell culture

HEK-293 and CHO-K1 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 2 mM L-glutamine, 10 % (v/v) foetal calf serum and 1x non essential amino acids (“complete DMEM”). The CHO-EcR cell line was maintained in Ham’s F-12 Nutrient Mix, supplemented with 10 % (v/v) foetal calf serum, 2 mM L-glutamine and 250 µg/mL Zeocin (“Complete F12”). Stable cell lines were cultured in the appropriate medium, to which 7.5 µg/mL Puromycin (CHO-K1) or 500 µg/mL Geneticin (CHO-EcR) had been added.

CHO and HEK cells adhere to tissue culture plastics and were normally cultured in T175 flasks. Routine passage of cells was performed using trypsin-EDTA to dislodge the adherent cell monolayer; medium was removed from the flask and the adherent cells were washed once with 10 mL Dulbecco’s PBS before 3 mL trypsin-EDTA in Hank’s Balanced Salt Solution was added. Cells were incubated at 37 °C for approximately 5 minutes until cells could be dislodged by tapping the flask. 7 mL “complete DMEM” was added and an appropriate volume of cell suspension transferred to a fresh flask together with 40 mL medium which was incubated at 37 °C with 5 % CO₂. Cells were subcultured at
a ratio which achieved confluence after approximately 4 days: CHO-K1 cells were split 1 in 100; CHO-EcR were split 1 in 50; HEK-293 were split 1 in 10.

Cell lines were cryopreserved for long term storage. Cell monolayers were removed by trypsinisation, centrifuged, the cell pellet was resuspended in freezing medium (90 % (v/v) foetal calf serum + 10 % (v/v) DMSO) and dispensed into cryovials. Aliquots of cells were packed in expanded polystyrene and chilled to -70 °C before transfer of the aliquots to liquid nitrogen.

2.3.2 Transient transfection of HEK-293 cells

HEK-293 cells were transiently transfected to express α2AR-Gα1 fusion protein, which was to be used as a positive control in the [35S]GTPγS binding assay. Cells were seeded into poly-D-lysine coated T175 flasks (2 x 10^7 cells/flask) on the day prior to transfection. The resulting sub-confluent cells were transfected using Lipofectamine reagent (Invitrogen Ltd.) in accordance with the manufacturer’s protocol. Briefly, 36 μg plasmid DNA was mixed with 1.8 mL Opti-Mem Reduced Serum Medium to yield Solution A; 144 μL Lipofectamine was mixed with 1.8 mL Opti-Mem to produce solution B. Solutions A and B were combined and incubated at room temperature for 30 minutes to allow DNA-liposome complexes to form. 14.4 mL Opti-Mem was added to the complexes and mixed gently. Diluted complexes were overlaid onto the cells (which had been washed once with Opti-Mem) and incubated for 6 hours at 37 °C with 5 % CO₂ after which time they were removed and 50 mL fresh “complete DMEM” added to the cells. Cells were grown for 2-3 days at 37 °C with 5 % CO₂ before being harvested.
2.3.3 Generation of stable cell lines

CHO-K1 cells were stably transfected to express HA-S1P₄, HA-S1P₄-Gα₁₁(C³⁵¹I) or HA-S1P₄(E¹²²Q)-Gα₁₁(C³⁵¹I) proteins. Cells were transfected essentially as described in this chapter, section 3.2 with the following modifications: 0.67 x 10⁶ cells were seeded into a T25 flask on the day prior to transfection, such that they were 50-80 % confluent on the day of transfection. Solution A was prepared by mixing 5.3 µg plasmid DNA with 267 µL Opti-Mem Reduced Serum Medium; 21.3 µL Lipofectamine reagent was mixed with 267 µL Opti-Mem to yield Solution B. After the combination of solutions A and B and incubation to allow formation of complexes, 6.4 mL Opti-Mem was added and the diluted complexes laid over cells which had been washed once with Opti-Mem. Cells were incubated with the complexes for 6 hours at 37 °C with 5 % CO₂ then the complexes were removed and replaced with 5 mL “complete DMEM”. 2-3 days post transfection, cells were detached using trypsin, counted, resuspended in “complete DMEM” supplemented with 7.5 µg/mL Puromycin and seeded into a number of 96-well plates (200 µL/well); seeding densities of 10, 100 and 1000 cells/well were used. The plates were incubated at 37 °C with 5 % CO₂ for 2-3 weeks before inspection of wells to identify those containing a single colony of Puromycin-resistant cells. Such colonies were detached into 200 µL trypsin and expanded into 24-well plates (1 mL/well); when confluent, cells were seeded into 2 copies of a 6-well plate (one copy was then used to test for expression of the recombinant protein of interest). Positive clones (identified using FACS analysis, described in this chapter, section 3.5) were then expanded into T175 flasks (via T25 flasks) and frozen stocks prepared. A mock transfection was set up in parallel to each transfection; this was to ensure that Puromycin-resistant...
colonies were the result of transfection with plasmid containing Puromycin-resistance gene.

The CHO-EcR cell line was stably transfected to express HA-SIP₄ using Lipofectamine 2000 (Invitrogen Ltd.) as the transfection reagent and following the manufacturer's protocol. Essentially, CHO-EcR cells were seeded in T75 flasks such that they were 90-95 % confluent on the day of transfection. 114 μL Lipofectamine 2000 was mixed with 1.9 mL Opti-Mem Reduced Serum Medium and incubated for 5 minutes at room temperature; this was Solution A. 38 μg plasmid DNA was mixed with 1.9 mL Opti-Mem to form Solution B. Solutions A and B were mixed and incubated at room temperature for 20 minutes to allow formation of DNA-liposome complexes. Cells were washed once with Opti-Mem medium and 8 mL fresh Opti-Mem was added to the cells before the complexes were laid onto cells. The cells were incubated with complexes for 6 hours at 37 °C with 5 % CO₂ after which time complexes were removed and 15 mL “complete F12” medium added to each flask. 2-3 days post-transfection, cells were trypsinised, counted and seeded into 96 well plates in “complete F12” medium supplemented with 500 μg/mL Geneticin and single colonies isolated and expanded as described previously in this chapter. A mock transfection was set up in parallel.

2.3.4 Induction of HA-SIP₄ expression in ecdysone-inducible CHO-EcR cell line

Small scale experiments used to profile this expression system were set up in 24-well tissue culture plates using CHO-EcR cells which had been stably transfected to express HA-SIP₄ under the control of the ecdysone receptor. Cells were normally seeded on the day prior to an experiment with 1.6 x 10⁵ cells per well,
in “complete F12” medium. Plates were placed on an orbital shaker for 30 minutes to aid even settling of the cells and then transferred to 37 °C with 5 % CO₂ for 3 hours to allow attachment to the tissue culture plastic. Medium was then removed and the attached cells washed once with PBS before serum-free F12 medium containing the appropriate concentration of induction agent was applied to the cells. The induction agents, ponasterone A and muristerone A, were prepared as 10 mM DMSO stocks. Cells were then assayed to determine cell-surface receptor expression (this chapter, section 3.5) 24 hours after application of the induction agent. For expression timecourse experiments, cells were seeded in 24 well plates at 0.6 x 10⁵ cells per well three days prior to assay and medium replaced with serum-free F12 medium containing induction agent at appropriate timepoints. Cells were assayed to determine cell-surface receptor expression as normal.

2.3.5 Detection of cell-surface proteins (FACS analysis)

Cells (>1 x 10⁵) were detached from tissue culture plastics using cell dissociation solution (Sigma) and mixed with FACS buffer (PBS containing 3 % (v/v) FCS, 0.1 % (w/v) NaNO₃) in 5 mL polypropylene Falcon tubes (BD Biosciences, Cowley, Oxon., U.K.). After collection by centrifugation (4 minutes at 400 x g), cells were resuspended in 100 µL FACS buffer in the presence of approximately 0.5 µg/mL rat anti-HA-Fluorescein antibody or an isotype matched control antibody, and incubated for 1 hour at 4 °C, in the dark. Cells were washed with 3 mL chilled FACS buffer and resuspended in 200 µL of the same. Staining data was measured using FACScan or FACSCalibur flow cytometers (BD
Chapter 2

Biosciences); staining with the specific antibody was compared to that obtained with the isotype matched control antibody.

2.3.6 Fluorescence microscopy

Cells were non-enzymatically detached from tissue culture plastics using cell dissociation solution and seeded into 8 chamber tissue culture treated glass slides (2 x 10^4 cells/chamber) in the appropriate complete medium. Where appropriate, the induction agent ponasterone A was added to medium. 24 hours after seeding, cells were washed well with medium and stained in complete medium containing the rat anti-HA-Fluorescein antibody (0.5 μg/mL) for 30 minutes in the dark. The nuclear stain DAPI (4',6'-diamidino-2-phenylindole hydrochloride) was added to the medium for the final 10 minutes (10 μg/ml). Cells were washed with medium and the slides mounted using ProLong Antifade Kit (Molecular Probes, Leiden, Holland). Fluorescent images were observed using a Leica DMRXA fluorescence microscope with oil immersion lenses. Fluorescein was excited at 495 nm (520 nm emission) and excitation of the DAPI stain was at 420 nm (345 nm emission). Images were manipulated using Openlab 2.2.0 deconvolution software (Improvision, Coventry, U. K.).

2.4 Protein methods

2.4.1 Preparation of cell membranes

CHO cell lines for use in S1P-receptor activation assays were grown in T175 flasks or 2 L roller bottles and transferred to serum-free medium approximately 24 hours prior to harvest. Briefly, medium was aspirated and cells washed twice with PBS before replacement with serum-free DMEM (containing 100 μg/mL pertussis toxin, where appropriate). HEK-293 cells, which had been transiently
transfected to express $\alpha_{2A}$AR-G$\alpha_{i1}$(C$^{351}$) fusion protein, were grown in “complete” DMEM. To harvest cells, growth medium was removed and the adherent cells washed with PBS. Cells were dislodged from culture vessels using cell dissociation solution and collected by centrifugation; the resultant cell pellet was washed with PBS. Cells were resuspended in an appropriate volume of membrane preparation buffer (20 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM MgCl$_2$, “complete” protease inhibitors (Roche Molecular Biochemicals)). For small scale membrane preparations, cells were ruptured by 35 strokes using a hand held Wheaton tight fit glass douncer; larger scale preparations were homogenised in a nitrogen cavitation chamber (500 psi for 20 minutes). Unbroken cells and nuclei were removed by centrifugation (500 x g, 10 minutes at 4 °C) and the supernatant fraction was centrifuged at 45,000 x g for 45 minutes at 4 °C. Membrane pellets were resuspended in membrane preparation buffer, titurated through a fine gauge needle, aliquotted and stored at -80 °C until required.

2.4.2 Determination of protein concentration

The protein concentration of membrane preparations was determined using Micro BCA Protein Assay Reagents (Perbio Science U.K. Ltd., Tattenhall, Cheshire, U.K.) in accordance with the manufacturer’s protocol. Membrane preparation buffer (this chapter, section 5.2) was used as the diluent for BSA standards and samples.

2.4.3 Immunological detection of protein (Western blotting)

Samples (typically membrane preparations) were prepared for Western blot analysis by the addition of an equal volume of 2x protein sample buffer and
incubation for 1 hour at room temperature. Approximately 10 μg each sample was loaded onto 4-20 % Tris-Glycine pre-cast polyacrylamide gel alongside pre-stained molecular weight markers. Gels were electrophoresed at 100 V / 35 mA for approximately 90 minutes, until the dye front had reached the bottom of the gel. Proteins were transferred to Immobilon-P PVDF membrane, which had been pre-wetted in methanol, overnight at 80 mA; the use of pre-stained molecular weight markers allowed judgement of the success of protein transfer. The resultant blot was blocked in PBS containing 2.5% (w/v) Marvel for 1 hour at room temperature, with constant agitation. Antibodies were prepared in PBS containing 1 % (w/v) Marvel and 0.1 % (v/v) Tween-20. The membrane was incubated with the primary antibody for 4 hours at room temperature, then washed three times in PBS containing 0.1 % (v/v) Tween-20 (5 minutes per wash) before incubation with the appropriate horseradish peroxidase-conjugated secondary antibody for approximately 2 hours at room temperature. After a further three washes (10 minutes per wash), the blot was developed using Pierce SuperSignal reagents (Perbio Science), in accordance with the manufacturer’s instructions. The signal was detected using x-ray film, which was processed using an X-OMat automatic developer (Kodak Ltd., Hemel Hempstead, Herts., U.K.).

2.5 Receptor activity assays

2.5.1 Handling of receptor agonists

Lysophospholid solutions were handled using siliconised Eppendorfs and pipette tips to minimise losses due to adhesion to plastics. Sphingosine-1-phosphate was dissolved in methanol to give a 1 μM solution, this required heating at 60 °C and
repeated vortexing and sonication to disperse the solid. 100 μL aliquots (equivalent to 0.1 μmoles compound) were dispensed into 2 mL Wheaton brown glass vials (Sigma) and the solvent gently evaporated under a stream of nitrogen. Aliquots were then stored at -80 °C prior to use. To reconstitute an aliquot of S1P, 250 μL “assay buffer” (this chapter, section 5.2) supplemented with 1 % (w/v) fatty acid free BSA was added and incubated at 37 °C for 30 minutes, with repeated vortexing. A further 1 in 4 dilution in assay buffer supplemented with 1 % (w/v) fatty acid free BSA yielded a 100 μM solution of S1P for use in receptor activation assays.

Lysophosphatidic acid (18:1) was prepared as a 2 mM DMSO solution (repeated vortexing and sonication were used to disperse the solid) and aliquotted for storage at -20 °C. 16:0 and 14:0 lysophosphatidic acids were prepared in 1:1 ethanol:water as 2 mM stock solutions, aliquotted and stored at -20 °C until use. Dilution of these stock solutions with “assay buffer” (this chapter, section 5.2) supplemented with 1 % (w/v) fatty acid free BSA gave a solution of LPA, which could be used in assays.

The α2AR agonist, UK14304 was prepared as a 5 mM stock solution in 1:1 DMSO:water and aliquots stored at -80 °C.

Compounds FTY720 and AAL were synthesised by the medicinal chemistry department of Celltech R+D using synthetic schemes described previously [Kiuchi et al., 2000]. Each compound was then prepared as 1mM stock solution in ethanol and stored at -20 °C. In vitro phosphorylation of FTY720 and AAL was achieved via incubation with rabbit blood (essentially as described by
Mandala et al., [2002]). Heparinised venous rabbit blood was mixed with three volumes of RPMI medium (supplemented with 10 % FCS, 2 mM L-glutamine and 1x non essential amino acids). FTY720 or AAL compounds were added to the diluted blood and the phosphorylation reaction incubated for the stated time at 37 °C, without additional CO₂. Post reaction, four volumes of acetonitrile were added and mixed well before centrifugation at approximately 2000 x g for 15 minutes. The supernatant was then analysed by liquid chromatography-mass spectrometry (LC-MS).

2.5.2 \[^{35}\text{S}]\text{GTP}γ\text{S binding assay}\n
\[^{35}\text{S}]\text{GTP}γ\text{S binding experiments were performed in accordance with the method described by Stevens et al., [2001]. Reagents were prepared in “assay buffer” (20 mM HEPES (pH 7.4), 3 mM MgCl₂, 100 mM NaCl). Membranes (20 µg/point) were incubated with or without ligand for an appropriate time at 30 °C in “assay buffer” containing \[^{35}\text{S}]\text{GTP}γ\text{S} (100 nCi/point), and an appropriate concentration of GDP, in a final volume of 100 µL/point. Where indicated, saponin (20 µg/point) was included. Lysophospholipid ligands were prepared in “assay buffer” supplemented with 1 % (w/v) fatty acid free BSA (this chapter, section 5.2), such that the final BSA concentration in the assay was 0.1 % (w/v). UK14304, an α₂AAR agonist, was diluted in “assay buffer” and used at a final concentration of 1 µM.

Following incubation, 500 µL chilled “assay buffer” containing “complete” protease inhibitors was added to each tube and membranes collected by centrifugation (14,000 rpm (16,100 x g) for 15 minutes at 4 °C). Membranes were solubilised for 1 hour at 4 °C, mixing end-over-end, in 50 µL 0.4 % (w/v)
SDS in chilled “solubilisation buffer” (100 mM Tris-HCl (pH 7.4 at 4 °C), 200 mM NaCl, 1 mM EDTA, “complete” protease inhibitors and 1.25 % (v/v) NP-40). This solution was then precleared by the addition of 60 μL/point of a mixture containing normal rabbit serum (diluted in “solubilisation buffer” to give 1 in 100 final concentration) and protein G Sepharose beads (6 μL/point diluted in “bead buffer” (2 % (w/v) BSA, containing 0.1 % (w/v) NaN₃ and “complete” protease inhibitors)) and mixed for 1 hour at 4 °C. Beads were pelleted by centrifugation (14,000 rpm for 5 minutes at 4 °C) and the supernatant was immunoprecipitated with SGI antiserum, which directed against Gα₁/2 G proteins. 50 μL of a mix containing SGI (diluted to give 1 in 200 final concentration in “solubilisation buffer”) and protein G Sepharose beads (5 μL/point diluted in “bead buffer”) was added to each tube and mixed overnight at 4 °C. Beads were collected by centrifugation and washed twice with 500 μL “solubilisation buffer”. 1 mL Ultima Gold XR scintillation fluid (Perkin Elmer, Cambridge, U.K.) was added to each tube and bound radioactivity measured using liquid scintillation spectrometry. Non-specific binding in the presence of 100 μM GTPγS was also determined in each experiment.

2.5.3 Data analysis

Analysis of numerical data was performed using Graphpad Prism, version 3.00. Data were corrected for non-specific binding and where appropriate, fitted using nonlinear (sigmoidal dose-response, fixed slope) regression. This method is reliant on the presence of at least two data points on the top and bottom sections of the curve, and at least one data point on the sigmoidal section of the curve in order to generate a dose-response curve; where this does not occur, interpretation
of the resultant \( \text{EC}_{50} \) value should be cautious. Results were expressed as the mean of triplicate determinations \( \pm \text{SEM} \). Typically, assays were performed at least three times, using membranes prepared from at least two individual cell passages. Statistical tests applied to data were one-way ANOVA, with Dunnett’s multiple comparison post test. Investigations of the statistical relationship between two data sets were performed using Pearson’s correlation.
Chapter 3 – Cloning of Human S1P\textsubscript{4} and Production of Expression Constructs

3.1 Introduction

This chapter describes the cloning and construction of plasmids for expression in mammalian cells and the application of RT-PCR to determine the profile of endogenous S1P and LPA receptor mRNA expression in CHO-K1 cells.

The S1P\textsubscript{4} receptor was cloned from a human PBMC cDNA library and modified to contain an amino-terminal HA epitope-tag. It was also constructed as an in-frame fusion between HA-tagged S1P\textsubscript{4} and a pertussis toxin resistant G\textsubscript{\alpha\textsubscript{i}}(C\textsuperscript{351}) G protein. These constructs were made using the pIRESpuro mammalian expression vector (Figure 3.1). HA-S1P\textsubscript{4} and the HA-S1P\textsubscript{4}-G\textsubscript{\alpha\textsubscript{i}}(C\textsuperscript{351}) fusion protein were stably expressed in CHO-K1 cells and used in functional assays as described in chapters 4 and 6.

![Figure 3.1 Schematic diagram of the pIRESpuro mammalian expression vector.](image)

The vector is approximately 5.2 kb in size and includes the internal ribosome entry site (IRES), which permits the translation of two open reading frames from one mRNA. This expression cassette contains the human cytomegalovirus (CMV) major intermediate early promoter/enhancer which drives expression of cloned genes; a multiple cloning site for insertion of cloned genes; a synthetic intervening sequence (IVS) intron to enhance mRNA stability; the encephalomyocarditis virus (ECMV) IRES followed by the Puromycin resistance marker; bovine growth hormone polyadenylation signal for proper processing of the 3' end of the cloned mRNA. The vector backbone also contains the β-lactamase gene to confer resistance to ampicillin and the pBR322 origin of replication for propagation in *E. Coli.*
HA-tagged S1P4 was also constructed in the pIND expression vector (Figure 3.2) and stably transfected into CHO-EcR cells for the inducible expression studies described in chapter 5.

![Figure 3.2](image)  
**Figure 3.2**  
Schematic diagram of the pIND ecdysone-inducible mammalian expression vector.  
This vector is approximately 5.0 kb in size and contains five ecdysone/glucocorticoid receptor elements (E/GRE), which allow binding of the modified ecdysone-receptor for activation of gene transcription and inducible expression of the gene of interest. Its other essential elements include the minimal heat shock promoter, which drives expression of the gene of interest; a multiple cloning site; bovine growth hormone (BGH) polyadenylation signal to allow efficient transcription termination and polyadenylation of cloned mRNA; fl origin of replication; SV40 early promoter and origin of replication to drive high level expression of the neomycin resistance gene, which confers resistance to the antibiotic Geneticin and SV40 polyadenylation signal. The vector also contains β-lactamase gene and pUC origin for high copy number replication and propagation in *E. Coli*.

Residue 122 (glutamic acid) of S1P4 was mutated to glutamine to allow investigation into the role of this residue in the ligand preference of S1P4. This mutant was constructed with an HA-epitope tag as an in-frame fusion with the pertussis toxin resistant mutant of Gα11(C35I) G protein, using the pIRESpuro expression vector. These proteins were then stably expressed in CHO-K1 cells and their response in functional assays measured as described in chapter 6.
3.2 Results

3.2.1 Cloning of HA-tagged S1P₄

In order to clone S1P₄, the published sequence (Genbank accession number AJ000479) was used to design primers F7240 and F7241 to allow amplification of the coding sequence of the S1P₄ gene. The 5' oligonucleotide, F7240, contained a NotI restriction site to facilitate cloning and a partial Kozak translational initiation sequence for optimal gene expression, upstream of the initiator methionine. F7241, the 3' oligonucleotide, contained the terminal coding sequence of S1P₄, an additional stop codon and an EcoRI restriction site. S1P₄ was amplified from a human peripheral blood mononuclear cell (PBMC) cDNA library using PCR. Since the S1P₄ sequence was GC rich (approximately 67% GC content), it was necessary to use a "GC-rich" PCR kit to amplify this sequence. The resultant PCR product was digested with the appropriate restriction enzymes to yield a fragment of approximately 1152 bp. This fragment was gel-purified and ligated into the mammalian expression vector, pIRESpuro (Figure 3.3 (A)), which had been digested with NotI and EcoRI, then transformed into chemically competent E. Coli. Single colonies were cultured and plasmid DNA purified and the presence of insert verified by restriction digest and analysis by gel electrophoresis. The entire insert was sequenced using a combination of sequencing primers designed from the published S1P₄ sequence, and vector-specific primers. A number of clones were sequenced and were all found to contain PCR errors, which may have been due to the use of modifiers in the PCR mix and were necessary for amplification of this GC rich sequence. These were identified as PCR errors since they were random errors.
Figure 3.3
Construction of S1P$_4$/pIRES.
S1P$_4$ was amplified from a human peripheral blood mononuclear cell (PBMC) cDNA library using the 5’ oligonucleotide F7240, which contained the NotI restriction site and a partial Kozak sequence, and the 3’ oligonucleotide F7241, which contained an EcoRI restriction site. The fragment was digested with NotI and EcoRI and ligated into pIRES (A). Due to the presence of PCR errors in the clones which were sequenced, it was necessary to generate an authentic construct via restriction digest and three-way ligation of two clones, each containing a single random PCR error either side of the unique Ncol site located at position 437, into the pIRES vector (C). Two unique restriction sites located within the S1P$_4$ gene: Ncol and BamHI, are also shown. These sites were used to facilitate construction of this construct, epitope-tagged S1P$_4$, a fusion of S1P$_4$ with the Ga$_{i1}$ G protein and a fusion of S1P$_4$(E$^{122}$Q) with the pertussis toxin-insensitive Ga$_{i1}$(C$^{351}$) G protein.
that were not present in every clone sequenced. Two clones were identified which each contained a single error: clone number 10 had an error at position 348, whilst in clone number 5 there was a single error at position 642. A unique NcoI restriction site at position 437 was utilised to produce a complete error free construct. DNA from each clone was digested with appropriate restriction enzymes (clone 5 with NotI/NcoI; clone 10 with NcoI/EcoRI) to excise the error-free section of the amplified sequence (Figure 3.3 (B)). These 5' and 3' portions were then ligated into pIRESpuro, which had been digested with NotI and EcoRI enzymes. DNA sequencing was used to confirm the absence of errors in the resultant construct and the sequence obtained was identical to that described by Graeler et al., [1998] and this plasmid was named pIRE/S1P4, (Figure 3.3 (C)).

It was necessary to epitope tag the S1P4 sequence since no antibodies to this receptor were available and, being an orphan GPCR at the time this work was undertaken, there was no possibility of detecting expression of protein using a functional assay. Therefore, pIRE/S1P4 was modified by PCR. The 5' oligonucleotide F12582 contained a NotI restriction site for cloning, a partial Kozak sequence and the HA-epitope (YPYDVPDYA) inserted between the first and second codons of the S1P4 coding sequence. The 3' oligonucleotide, F12581, was designed across the unique NcoI site present in the S1P4 sequence such that only the 5' end of the gene would be amplified (Figure 3.4 (A)). The product of this PCR was digested with NotI and NcoI restriction enzymes to yield a 449 bp fragment. The 3' end of the S1P4 coding sequence with which this 5' fragment was to be ligated was produced by digestion of the pIRE/S1P4 plasmid with NcoI and EcoRI restriction enzymes and gel purification (Figure
Figure 3.4  
Construction of HA-SIP4/pIRES. 
SIP4 was HA-epitope tagged by PCR amplification of the 5’ portion of the sequence, using the forward oligonucleotide F12582, which contained the HA epitope sequence (YPYDVPDYA) immediately after the initiator methionine, as well as a partial Kozak sequence and NotI restriction site, and the reverse oligonucleotide F12581 which utilised the unique NcoI site present in the SIP4 sequence. This fragment (A) was joined with fragment (B), produced by NcoI/EcoRI restriction digest of SIP4/pIRES, in a three-way ligation reaction with NotI/EcoRI digested pIRES to yield HA-SIP4/pIRES (C).

3.4 (B)). The complete construct was then generated by three-way ligation reaction of the PCR-generated HA-tagged 5’ fragment, the restriction digest-produced 3’ fragment and NotI/EcoRI-digested pIRES. Plasmid DNA was extracted from transformed E. Coli colonies and digested with restriction
enzymes to check for the presence of insert. The entire insert was sequenced with vector- and insert-specific primers and was found to be error free. This construct was named HA-S1P4/pIRES (Figure 3.4 (C)).

3.2.2 Generation of HA-tagged S1P4-G\(\alpha_{41}(C^{351})\) fusion construct

The construct to express HA-S1P4 fused to G\(\alpha_{41}\), where the G protein contained the C\(^{351}\)I mutation and was therefore resistant to ADP-ribosylation by pertussis toxin, was produced in several stages. Initially, PCR was used to remove the stop codon present at the 3' end of the HA-S1P4 coding sequence in the previously produced HA-S1P4/pIRES construct. The forward oligonucleotide, F27384, which encompassed a unique BamHI restriction site within the S1P4 sequence, was used in conjunction with the reverse oligonucleotide, F36663, which allowed mutation of the stop codon to alanine and also contained the EcoRI restriction site. The resultant PCR product was digested with BamHI and EcoRI enzymes and gel purified to yield a fragment of approximately 290 bp (Figure 3.5 (A)). Digestion of HA-S1P4/pIRES with NotI and BamHI yielded the 910 bp 5' fragment of HA-S1P4 (Figure 3.5 (B)) which was gel purified before three-way ligation with the PCR-generated 3' fragment and pIRES which had been cut with NotI and EcoRI. This ligation reaction was transformed into chemically competent E. Coli and plasmid DNA extracted from cultures of single colonies was analysed by restriction digest and gel electrophoresis to check for the presence of insert. The entire insert of a single clone was sequenced on both strands and found to be error free. This intermediate construct was named HA-S1P4(No Stop)/pIRES and is shown in Figure 3.5 (C).
A construct of HA-S1P₄ which lacked the 3' stop codon was generated by PCR amplification of a 3' fragment of HA-S1P₄ using the forward primer F27384, which encompassed a unique BamHI site within the S1P₄ sequence, and the reverse oligonucleotide F36663, which mutated the stop codon to alanine and contained an EcoRI restriction site to facilitate cloning. Restriction digest and gel purification of the PCR product yielded a fragment of approximately 290 bp (A). This was ligated in a three-way reaction with fragment (B), which had been produced by NotI/BamHI digestion of HA-S1P₄, and NotI/EcoRI-digested pIRES, to produce HA-S1P₄(No Stop)/pIRES (C).

The Gα₁(C₃₅₁) G protein was modified by PCR such that it contained restriction sites which would allow in-frame fusion with the HA-S1P₄(No Stop) sequence. The forward oligonucleotide F36661 introduced an EcoRI restriction site before the initiator methionine, and the reverse oligonucleotide F36662 created a
BamHI site after the stop codon of $\alpha_4(C^{351})$. Digestion of the PCR product with appropriate restriction enzymes and subsequent gel-purification, yielded a fragment of approximately 1083 bp which was ligated into pIRES, which itself had been digested with EcoRI and BamHI. Chemically competent E. Coli were transformed with this ligation reaction and plasmid DNA extracted from cultures grown from single colonies. Analysis of this DNA by restriction digest and gel electrophoresis confirmed the presence of the insert, after which the insert of a single clone was sequenced on both strands, using $\alpha_4$- and vector-specific sequencing primers. This clone was found to be error free and was named $\alpha_4(C^{351})$/pIRES (shown in Figure 3.6).

Figure 3.6
Construction of $\alpha_4(C^{351})$/pIRES.
$\alpha_4(C^{351})$ was modified using PCR such that it contained an EcoRI restriction site at the 5' end and a BamHI restriction site at the 3' end. These sites were present in the forward oligonucleotide, F36661, and the reverse oligonucleotide, F36662, respectively. Digestion of the PCR product with EcoRI and BamHI yielded a fragment of approximately 1083 bp, which was ligated into pIRES, which had also been cut with EcoRI and BamHI. This produced the $\alpha_4(C^{351})$/pIRES construct.

The in-frame fusion between HA-S1P$_4$(No Stop) and $\alpha_4(C^{351})$ was generated via restriction digest and three-way ligation. The 1200 bp HA-S1P$_4$(No Stop) insert was released by NotI/EcoRI digest of the HA-S1P$_4$(No Stop)/pIRES
construct (Figure 3.7 (A)), whilst the G\(\alpha_{11}(C^{351})/pIRES\) plasmid was digested with EcoRI and BamHI to yield the G\(\alpha_{11}(C^{351})\) insert, which was approximately 1083 bp in size (Figure 3.7 (B)). These two fragments were gel-purified and combined with pIRES, which had been cut with NotI and BamHI restriction enzymes, in a three-way ligation reaction, which was transformed into competent E. Coli bacteria. Single colonies were cultured and plasmid DNA extracted. Analysis of this DNA by restriction digest and gel electrophoresis confirmed the presence of the expected insert and one of the clones was designated HA-SIP\(_4\)-G\(\alpha_{11}(C^{351})/pIRES\) (Figure 3.7 (C)). The use of this cloning strategy resulted in the addition of a five amino acid artificial linker between the two elements of the fusion, which is shown in Figure 3.7 (C).

### 3.2.3 Generation of HA-tagged SIP\(_4\) construct for inducible expression

In order to control the level of HA-SIP\(_4\) expression in mammalian cells, a construct was made for use with the ecdysone-inducible mammalian expression system (Invitrogen Ltd.).

The insert of the HA-SIP\(_4\)/pIRES construct was modified by PCR such that the 5' end of the sequence contained an EcoRI restriction site, and a NotI site was present at the 3' end. These restriction sites were present in the forward and reverse oligonucleotides, 110A and 111A, respectively (see Figure 3.8 (A)). The PCR product was digested with the appropriate restriction enzymes and gel-purified before being ligated into the pIND vector, which had also been digested with EcoRI and NotI restriction enzymes. Chemically competent E. Coli were transformed and single colonies cultured before plasmid DNA was purified and...
Chapter 3

Figure 3.7
Construction of HA-S1P4-Gα11(C351I)/pIRES.
The insert of the HA-S1P4(No Stop)/pIRES construct was released by digest using NotI and EcoRI restriction enzymes (A). Fragment (B) was produced by restriction digest of the Gα11(C351I)/pIRES with EcoRI and BamHI enzymes. Ligation of these fragments into pIRES, which had been digested with NotI and BamHI restriction enzymes, produced HA-S1P4-Gα11(C351I)/pIRES (C).

analysed by restriction digest and gel electrophoresis to confirm the presence of the expected insert. The insert of a single clone was sequenced over both strands, using a combination of the previously designed S1P4-specific and
vector-specific sequencing primers. This clone was error free and was designated HA-S1P₄/pIND (shown in Figure 3.8 (B)).

Figure 3. 8
Construction of HA-S1P₄/pIND.
PCR was used to modify the restriction sites at the 5’ and 3’ ends of the HA-S1P₄ insert such that they were compatible with the pIND vector. The forward oligonucleotide, 110A contained a partial Kozak sequence and introduced an EcoRI restriction site prior to the initiator methionine; the reverse oligonucleotide, 111A contained a NotI site after the stop codon of HA-S1P₄. The HA-S1P₄/pRES construct was used as a template and the resultant PCR product (A) was digested with EcoRI and NotI restriction enzymes. Ligation into pIND, which had also been digested with EcoRI and NotI produced the construct named HA-S1P₄/pIND (B).

3.2.4 Generation of HA-tagged S1P₄(E₁²²Q) mutant fused with pertussis toxin-insensitive Gα₃₁(C₃₅₁T)

The E₁²²Q mutation was introduced into HA-S1P₄ using PCR. Two complementary oligonucleotides, 58A and 59A, were designed across residue ₁²². Each oligonucleotide contained the necessary base modifications to mutate glutamic acid ₁²² to glutamine. Parallel PCR reactions were set up, each using
HA-S1P<sub>4</sub>/pIRES as template DNA. The first reaction used oligonucleotides F12582 and 59A to produce a fragment of approximately 405 bp; the second reaction was performed using oligonucleotides 58A and F7241 and yielded a fragment of approximately 795 bp. After gel purification, equimolar amounts of these PCR products were mixed and a further reaction performed, using oligonucleotides F12582 and F7241. This product was gel purified then digested of with NotI and NcoI restriction enzymes, gel purified to yield the 5' fragment of approximately 476 bp, which included the desired mutation (Figure 3.9 (A)). Digestion of the previously produced HA-S1P<sub>4</sub>(No Stop) construct with NcoI and EcoRI restriction enzymes was followed by gel purification and yielded a 3' fragment, of approximately 727 bp (Figure 3.9 (B)). The Gα<sub>11</sub>(C<sup>351</sup>I)/pIRES plasmid (produced in this chapter, section 2.2) was digested with EcoRI and BamHI to yield the Gα<sub>11</sub>(C<sup>351</sup>I) insert, which was approximately 1083 bp (Figure 3.9 (C)). An in-frame fusion between the E<sup>122</sup>Q mutant of HA-S1P<sub>4</sub> and Gα<sub>11</sub>(C<sup>351</sup>I) was generated via four-way ligation of these fragments into NotI/EcoRI-digested pIRES. Single colonies of transformed E. Coli were cultured and restriction digestion followed by gel electrophoresis used to check for the presence of insert in the purified plasmid DNA. The HA-S1P<sub>4</sub> insert of a single clone was sequenced on both strands and was found to contain the desired mutation and to be free from PCR errors. This construct was named HA-S1P<sub>4</sub>(E<sup>122</sup>Q)-Gα<sub>11</sub>(C<sup>351</sup>I)/pIRES and is shown in Figure 3.9 (D). In common with the wild type HA-S1P<sub>4</sub>-Gα<sub>11</sub>(C<sup>351</sup>I) fusion protein, HA-S1P<sub>4</sub>(E<sup>122</sup>Q)-Gα<sub>11</sub>(C<sup>351</sup>I) contained an artificial five amino acid linker present between the two elements of the fusion protein, which was a consequence of the cloning strategy used.
Construction of HA-S1P₄(E¹²²Q)-Gα₆(C³⁵¹I)/pIRES.
Mutation of glutamic acid at position ¹²² of S1P₄ to glutamine was achieved by parallel PCR reactions. The 5' portion of the sequence was amplified using the oligonucleotides F12582 (forwards) and 59A (reverse), whilst the forward oligonucleotide 58A and the reverse oligonucleotide F7241 were used to amplify the 3' portion of the sequence. The PCR products were combined and a further amplification performed using the oligonucleotides F12582 and F7241. This product was digested with NotI and NcoI restriction enzymes (A). Other fragments to produce this construct were generated by restriction digest: (B) 3' NcoI/EcoRI fragment of HA-S1P₄(No Stop) and (C) EcoRI/BamHI insert from Gα₆(C³⁵¹I)/pIRES. These fragments were ligated into NotI/BamHI digested pIRES to yield HA-S1P₄(E¹²²Q)-Gα₆(C³⁵¹I)/pIRES (D).
3.2.5 Determination of expression of endogenous S1P and LPA receptors in CHO-K1 cells by RT-PCR

Untransfected CHO-K1 cells respond to exogenous S1P in a variety of in-vitro assays, which is thought to be due to the expression of endogenous S1P receptors by these cells. Although several mammalian cell lines have been characterised with respect to expression of endogenous S1P and LPA (Edg) receptors (examples in Graeler et al., 1998; Motohashi et al., 2000; Fueller et al., 2003), very little data of this nature has been reported for the CHO-K1 host. This may be due to this cell line being derived from Chinese hamster. Very few gene sequences have been published for this species and of those which had been deposited at the time of the current investigation, none were of S1P or LPA receptors.

Such a lack of sequence data meant that the design of oligonucleotides for use in RT-PCR experiments to determine which of the S1P and LPA receptors were endogenously expressed by CHO-K1 cells was not straightforward. Human and rodent sequences for each of the eight Edg receptors were aligned, both at the nucleotide and protein level, and areas of high similarity between species were identified as the optimal areas over which oligonucleotides could be designed. It was noted that, for each of the Edg receptors, there was a strong degree of conservation between species at the protein level, although this was less obvious in nucleotide sequences since there were often changes at the 3-position between different species, which were silent with respect to the protein sequence. Hence, it was necessary to design primers which contained a degree of redundancy. The LPA receptors were known to contain an intron in TM VI, the location of which is absolutely conserved between human and mouse [Contos and Chun, 1998;
Contos and Chun, 2000; Contos and Chun, 2001]. Oligonucleotides to amplify a fragment of each of the LPA receptor genes were designed such that this intron boundary was not spanned by the expected product. Table 3.1 shows the identity of the forward and reverse primers designed to amplify each of the S1P and LPA receptor genes from Chinese hamster.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Forward oligonucleotide</th>
<th>Reverse oligonucleotide</th>
<th>Expected fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1P1</td>
<td>1170B</td>
<td>1171B</td>
<td>440</td>
</tr>
<tr>
<td>S1P2</td>
<td>1176B</td>
<td>1177B</td>
<td>560</td>
</tr>
<tr>
<td>S1P3</td>
<td>1172B</td>
<td>1173B</td>
<td>610</td>
</tr>
<tr>
<td>S1P4</td>
<td>53B</td>
<td>54B</td>
<td>485</td>
</tr>
<tr>
<td>S1P5</td>
<td>1182B</td>
<td>58B</td>
<td>625</td>
</tr>
<tr>
<td>LPA1</td>
<td>45B</td>
<td>46B</td>
<td>610</td>
</tr>
<tr>
<td>LPA2</td>
<td>49B</td>
<td>1174B</td>
<td>590</td>
</tr>
<tr>
<td>LPA3</td>
<td>1178B</td>
<td>1180B</td>
<td>720</td>
</tr>
</tbody>
</table>

Table 3.1
Oligonucleotides used to clone fragments of S1P and LPA receptor genes from Chinese hamster genomic DNA.

Oligonucleotides were tested using genomic DNA prepared from CHO-K1 cells as template in PCR reactions (Figure 3.10); products of the expected sizes were obtained for each primer pair. PCR products were cloned into the TOPO TA Cloning Kit for Sequencing vector and transformed into TOP10 chemically competent E. Coli. Plasmid DNA was purified from cultures grown from single colonies and digested with EcoRI to confirm the presence of an insert. Vector-specific primers were used to sequence the entire insert of four clones of each receptor construct and the consensus sequence obtained for each Chinese hamster gene fragment aligned with known human and rodent sequences for that gene, at both the nucleotide and protein levels. Alignments of translated partial
Chinese hamster gene fragment sequences with translated coding sequences of human, mouse and, where available, rat genes are shown in Figure 3.11 (A-H).

**Figure 3.10**
PCR amplification of partial S1P and LPA receptor sequences from Chinese hamster genomic DNA.
Oligonucleotides to amplify a fragment of each Chinese hamster S1P and LPA receptor were designed using sequences of human, mouse and, where available, rat S1P and LPA receptors. Genomic DNA was prepared from CHO-K1 cells and used as template in PCR reactions with these oligonucleotides pairs and the PCR products analysed using gel electrophoresis. This Figure is representative of three independent experiments.

<table>
<thead>
<tr>
<th>Lane</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S1P₁</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S1P₂</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S1P₃</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S1P₄</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S1P₅</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LPA₁</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LPA₂</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LPA₃</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LPA₃ (no DNA)</td>
</tr>
</tbody>
</table>
Figure 3.11
Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.
Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

<table>
<thead>
<tr>
<th>Section 1</th>
<th>Section 2</th>
<th>Section 3</th>
<th>Section 4</th>
<th>Section 5</th>
<th>Section 6</th>
<th>Section 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1P1 (mouse)</td>
<td>(1)</td>
<td>MVSTSPIUKALRSVSVDYQNYDIIVPHNYTGTKLNGADBHDGKLTSVFPILICC</td>
<td>FIEFLIFVULLTITKFMHPHYFICGNMLSDLALLACVAYTNLLSAGATTYKLTPA</td>
<td>QWFLERGSMFLVSLAIAEYITMLKMKHNCSSRSFILLISACWVSLI</td>
<td>SRLTFPKNISKASSSKLALLKTIVLTVSFIACMAPFLILLLLVDGKAKTDI</td>
<td>MEFSFSKDNSHSHPQDDCGNPEITMSCGVNSSS</td>
</tr>
<tr>
<td>S1P1 (hamster)</td>
<td>(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1P1 (human)</td>
<td>(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1P1 (rat)</td>
<td>(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A S1P1 (Edg1)
Figure 3.11 (continued)

Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.

Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

B  S1P2 (Edg5)
Figure 3.11 (continued)
Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.
Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

SIP3 (Edg3)
Figure 3.11 (continued)
Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.
Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

D  S1P4 (Edg6)
Alignment of human, mouse and rat SIP and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.

Amplification of a portion of the coding sequence of each of the SIP and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat SIP and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent SIP and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

S1P\(_5\) (Edg8)
<table>
<thead>
<tr>
<th>Protein</th>
<th>Alignment</th>
<th>Translation</th>
</tr>
</thead>
</table>
| **LPA1 (mouse)** | MAA...CFYN... | RQRTHRHSRHSSGPRRNRDTHHSLLKTVVIVL
| **LPA1 (hamster)** | VCFI... | QVFHNLNL...CFYN... |
| **LPA1 (human)** | HAA...CFY... | AIVMCA... |
| **LPA1 (rat)** | MAA...CFY... | AIVMCA... |

Figure 3.11 (continued)

Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.

Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

**F** LPA₁ (Edg2)
### Figure 3.11 (continued)
Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.

Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

G  \( \text{LPA}_2 \) (Edg4)

<table>
<thead>
<tr>
<th>LPA2 (mouse)</th>
<th>LPA2 (hamster)</th>
<th>LPA2 (human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>59</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LMP2 (mouse)</th>
<th>LMP2 (hamster)</th>
<th>LMP2 (human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(117) 117</td>
<td>(117) 117</td>
<td>(117) 117</td>
</tr>
<tr>
<td>TM II 130</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td>140</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>160</td>
<td>160</td>
<td>160</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LMP2 (mouse)</th>
<th>LMP2 (hamster)</th>
<th>LMP2 (human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(175) 175</td>
<td>(175) 175</td>
<td>(175) 175</td>
</tr>
<tr>
<td>TM III 190</td>
<td>190</td>
<td>190</td>
</tr>
<tr>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>210</td>
<td>210</td>
<td>210</td>
</tr>
<tr>
<td>220</td>
<td>220</td>
<td>220</td>
</tr>
<tr>
<td>232</td>
<td>232</td>
<td>232</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LMP2 (mouse)</th>
<th>LMP2 (hamster)</th>
<th>LMP2 (human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(233) 233</td>
<td>(233) 233</td>
<td>(233) 233</td>
</tr>
<tr>
<td>TM IV 250</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>260</td>
<td>260</td>
<td>260</td>
</tr>
<tr>
<td>270</td>
<td>270</td>
<td>270</td>
</tr>
<tr>
<td>280</td>
<td>280</td>
<td>280</td>
</tr>
<tr>
<td>292</td>
<td>292</td>
<td>292</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LMP2 (mouse)</th>
<th>LMP2 (hamster)</th>
<th>LMP2 (human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(351) 351</td>
<td>(351) 351</td>
<td>(351) 351</td>
</tr>
<tr>
<td>TM V 300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>310</td>
<td>310</td>
<td>310</td>
</tr>
<tr>
<td>320</td>
<td>320</td>
<td>320</td>
</tr>
<tr>
<td>330</td>
<td>330</td>
<td>330</td>
</tr>
<tr>
<td>348</td>
<td>348</td>
<td>348</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LMP2 (mouse)</th>
<th>LMP2 (hamster)</th>
<th>LMP2 (human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(340) 340</td>
<td>(340) 340</td>
<td>(340) 340</td>
</tr>
<tr>
<td>STL</td>
<td>STL</td>
<td>STL</td>
</tr>
</tbody>
</table>
Alignment of human, mouse and rat S1P and LPA receptor protein sequences with translated partial hamster sequences obtained from PCR reactions using Chinese hamster genomic DNA.

Amplification of a portion of the coding sequence of each of the S1P and LPA receptors present in the genome of the Chinese hamster was achieved via PCR using oligonucleotide pairs which had been designed using alignments of human, mouse and, where available, rat S1P and LPA receptor sequences. The products of these PCR reactions were cloned and sequenced. The translated sequence was aligned with protein sequences of human and rodent S1P and LPA receptors and the alignment annotated to show the location of each transmembrane (TM) domain.

Figure 3. 11 (continued)

H LPA3 (Edg7)
Both nucleotide and protein sequences of the partial Chinese hamster sequences were found to be very similar to those from human, rat and mouse. Percent identities were calculated for novel Chinese hamster sequences compared to human, mouse or rat orthologues using pairwise comparisons of sequences at the nucleotide (Table 3.2 (A)) and translated protein level (Table 3.2 (B)).

### A

<table>
<thead>
<tr>
<th></th>
<th>% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human</td>
</tr>
<tr>
<td>S1P1</td>
<td>88.7</td>
</tr>
<tr>
<td>S1P2</td>
<td>82.6</td>
</tr>
<tr>
<td>S1P3</td>
<td>89.5</td>
</tr>
<tr>
<td>S1P4</td>
<td>83.3</td>
</tr>
<tr>
<td>S1P5</td>
<td>81.9</td>
</tr>
<tr>
<td>LPA1</td>
<td>90.9</td>
</tr>
<tr>
<td>LPA2</td>
<td>86.1</td>
</tr>
<tr>
<td>LPA3</td>
<td>87.3</td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th></th>
<th>% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human</td>
</tr>
<tr>
<td>S1P1</td>
<td>94.6</td>
</tr>
<tr>
<td>S1P2</td>
<td>90.6</td>
</tr>
<tr>
<td>S1P3</td>
<td>95.2</td>
</tr>
<tr>
<td>S1P4</td>
<td>81.8</td>
</tr>
<tr>
<td>S1P5</td>
<td>88.7</td>
</tr>
<tr>
<td>LPA1</td>
<td>97.9</td>
</tr>
<tr>
<td>LPA2</td>
<td>95.0</td>
</tr>
<tr>
<td>LPA3</td>
<td>93.3</td>
</tr>
</tbody>
</table>

Table 3.2
Summary of similarities between cloned partial Chinese hamster S1P and LPA receptor sequences and those from mouse, rat and human.

Pairwise comparisons of the cloned Chinese hamster sequence of each S1P and LPA receptor gene fragment with the mouse, rat or human orthologues were performed and the percentage identity determined for the range of the partial Chinese hamster sequence.

* - sequence not available from National Centre for Biotechnology Information (NCBI) Entrez database.

A  Summary of nucleotide sequence identities
B  Summary of protein sequence identities
The cloned Chinese hamster gene fragments shared a high degree of identity with their mouse, rat and human orthologues, showing between 81.9 and 95.0% identity at the nucleotide level and between 81.8 and 99.5% at the protein level. In general, the level of identity was higher for the protein sequence than the nucleotide sequence for a given receptor gene, although this was not the case for the comparison between human and hamster S1P4 (where the level of identity at the nucleotide level was slightly higher than at the protein level). It was observed that many differences in the nucleotide sequences of a given S1P or LPA receptor gene from each of the species examined, occurred at the third position of a codon and hence were not observed as a difference in the protein sequence. Chinese hamster S1P and LPA receptor gene fragments always shared greater sequence identity with mouse and rat orthologues than with human.

Each Chinese hamster gene consensus sequence was then used to facilitate design of non-redundant oligonucleotides for use in the RT-PCR experiment. The identity of these oligonucleotides is shown in Table 3.3.

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Forward oligonucleotide</th>
<th>Reverse oligonucleotide</th>
<th>Expected fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1P1</td>
<td>1364C</td>
<td>1365C</td>
<td>380</td>
</tr>
<tr>
<td>S1P2</td>
<td>1372C</td>
<td>1373C</td>
<td>510</td>
</tr>
<tr>
<td>S1P3</td>
<td>1368C</td>
<td>1369C</td>
<td>560</td>
</tr>
<tr>
<td>S1P4</td>
<td>1374C</td>
<td>1375C</td>
<td>425</td>
</tr>
<tr>
<td>S1P5</td>
<td>1378C</td>
<td>1379C</td>
<td>580</td>
</tr>
<tr>
<td>LPA1</td>
<td>1366C</td>
<td>1367C</td>
<td>560</td>
</tr>
<tr>
<td>LPA2</td>
<td>1370C</td>
<td>1371C</td>
<td>540</td>
</tr>
<tr>
<td>LPA3</td>
<td>1376C</td>
<td>1377C</td>
<td>670</td>
</tr>
</tbody>
</table>

Table 3.3
Oligonucleotides used for RT-PCR analysis of CHO-K1 mRNA to identify endogenously expressed S1P and LPA receptor genes.
Total RNA was prepared from CHO-K1 cells and treated to remove contaminating genomic DNA (described in chapter 2, section 2.16). Reverse transcription using an oligo dT(18) primer yielded first strand cDNA which was then used as template in PCR reactions with the oligonucleotide pairs shown in Table 3.3 to determine which S1P and LPA receptor mRNA's were expressed in CHO-K1 cells (Figure 3.13 (A)). The ability of each oligonucleotide pair to amplify a product of the expected size was demonstrated using CHO-K1 genomic DNA (Figure 3.13 (B)) and the integrity of the first strand cDNA preparation was verified using control GAPDH oligonucleotides. The absence of DNA contamination in the RNA preparation was confirmed by the inability of the primer pairs to amplify products when the reverse transcription step was omitted (not shown) and the identity of each amplified S1P and LPA receptor fragment was confirmed by size and restriction digest of that product.

Fragments of the expected sizes were amplified using oligonucleotide pairs specific for the S1P1, S1P2, S1P4 and LPA1 receptors; the control GAPDH primers also amplified a product of the expected size. The intensity of the fragments amplified in this qualitative experiment varied; the S1P2 fragment was the most intense, fragments of the S1P1 and LPA1 receptors, as well as GAPDH had lower intensity but were still clearly visible, whilst the S1P4 fragment was very faint. Although this experiment was not quantitative, this result may suggest that the transcript for S1P4 was less abundant than those of the S1P1, S1P2 and LPA1 receptor genes.
Figure 3.12
Expression of S1P and LPA receptor mRNA in CHO-K1 cells by RT-PCR.
RT-PCR was performed using total RNA that had been extracted from CHO-K1 cells, with pairs of oligonucleotides designed to amplify specific regions of hamster S1P and LPA receptor coding sequences (A). The ability of these primer pairs to amplify products of the expected size was confirmed using CHO-K1 genomic DNA as template in a parallel set of PCR reactions (B). 5 µL of a 20 µL reaction was loaded in each lane. This Figure is representative of two independent experiments.

Lane 1  S1P₁
Lane 2  S1P₂
Lane 3  S1P₃
Lane 4  S1P₄
Lane 5  S1P₅
Lane 6  LPA₁
Lane 7  LPA₂
Lane 8  LPA₃
Lane 9  GAPDH
Chapter 3

3.3 Discussion

At the time this work was started, S1P₄ had recently been cloned as the orphan receptor Edg6 [Graeler et al., 1998]. This novel sequence was discovered in an effort to clone new chemokine receptors and was assigned to the then named Edg receptor family on the basis of sequence homology with other Edg receptors. The study of an orphan receptor presents a number of challenges, one of the most obvious being the inability to use a functional method for screening antibiotic-resistant clones which result from a stable transfection of the orphan into a suitable cell line. Similarly, commercial antibodies to orphan receptors are frequently unavailable, so the use of such an antibody in a technique such as Western blotting is also prohibited. For these reasons, S1P₄ expression plasmids were engineered to contain the HA epitope (YPYDVPDYA) immediately after the initiator methionine. This allowed detection of cell-surface receptor via FACS analysis, and of full length protein via Western blotting, using an anti-HA antibody. The addition of such a tag to the amino-terminus of a GPCR has been used previously and was shown to have no effect on the ability of ligand to bind the receptor [Koller et al., 1997]. In the case of the S1P₄ receptor, the ligand was expected to be S1P or a related lysophospholipid, and as such would be most likely to bind to the transmembrane helices of the receptor so the inclusion of a nine amino acid epitope at the amino-terminus was not expected to interfere with ligand binding.

The use of fusion proteins constructed between a GPCR and a G protein is a popular approach and has a number of advantages over systems where non-fused receptors are studied (reviewed by Seifert et al., 1999; Milligan, 2000; Wurch and Pauwels, 2001). The key benefit of constructing such fusions include a
defined 1:1 stoichiometry between receptor and G protein, and a physical proximity between the proteins. Additionally, construction of a GPCR-G\(\alpha\) fusion may be achieved using a G\(\alpha\) G protein which has been mutated such that it is rendered insensitive to ADP-ribosylation by pertussis toxin [Milligan, 1988; Bahia et al., 1998], therefore allowing isolation of the response from the fused receptor. This was of particular relevance when applied to a receptor such as S1P\(\lambda\), since it is one of a sub-family of receptors that are activated by S1P and are almost ubiquitously expressed in mammalian cells. This has meant that functional responses by untransfected cells are commonplace and can interfere with interpretation of results generated using transfected cells. The lack of S1P receptor-selective agonists combined with widespread expression of the receptors in mammalian cells has complicated the characterisation of this GPCR family. Generation of the pertussis toxin-insensitive HA-S1P\(\lambda\)-G\(\alpha_{11}(C^{351})\) fusion protein in this chapter enabled the use of pertussis toxin to eliminate interfering signals arising from the presence of endogenous S1P receptors in the host CHO-K1 cells when measuring activation of G\(\alpha_{i}\) G proteins by S1P. Indeed, a similar approach was adopted in an early study which examined activation of the related LPA\(1\) (Edg2) receptor in HEK-293 cells [McAllister et al., 2000].

The cloning strategy adopted to produce fusions between S1P\(\lambda\) and G\(\alpha_{11}\) resulted in the introduction of a five amino acid artificial linker sequence between the two elements of the fusion. The presence of this short linker is unlikely to impact on the ability of the receptor to signal via the tethered G protein and a published study showed that the presence of the large green fluorescent protein (GFP) in a
fusion protein between the A1 adenosine receptor, GFP and Goαi1 had little effect on ligand binding or effector regulation by this GPCR [Bevan et al., 1999].

The final section of work undertaken in this chapter was to profile the expression of endogenous S1P and LPA receptors in CHO-K1 cells. Whilst it was known that most cell types respond to S1P and LPA, and presumably express at least one receptor from each of these families, there had been no concerted study to determine the repertoire of expression of these receptors in CHO-K1 cells. Some interesting observations had been made using Northern blotting techniques, however the probes used in these studies were not hamster-gene specific, and therefore interpretation of the results needs caution [Okamoto et al., 1998; Sato et al., 1999].

The approach taken in this thesis was to perform RT-PCR using Chinese hamster mRNA with oligonucleotides specific to each of the eight Chinese hamster S1P and LPA gene coding sequences. Searches of the NCBI Entrez database revealed that no S1P or LPA gene sequences from Chinese hamster had been deposited. Therefore, semi-redundant oligonucleotides were designed using nucleotide and amino acid sequence alignments for each of the eight genes from human, mouse and rat. These were used to amplify a portion of each of the eight S1P and LPA genes from Chinese hamster genomic DNA. Sequencing of the resultant PCR products generated novel sequence data for these Chinese hamster genes and comparison of partial sequences with human, mouse and rat orthologues demonstrated high levels of identity at both nucleotide and protein levels. The amino acid residue present in TM III, which has been proposed to control S1P and LPA receptor ligand selectivity [Wang et al., 2001], was found
to be present in all sequences analysed (Chinese hamster gene fragments for the S1P$_4$ and S1P$_5$ receptors did not cover this region, however it is probable that this residue is also conserved within these sequences). It is therefore unlikely that there would be significant species differences with regard to ligand selectivity among the orthologues for the S1P and LPA receptors. Such differences are sometimes observed and can complicate drug discovery programs due to species-selectivity of ligands and small molecule therapeutics (e.g. chemokine receptors).

Each of the partial hamster receptor sequences analysed shared greatest identity with rodent orthologues and slightly lower identity with the equivalent human sequence. The relative order of identity of Chinese hamster sequences compared to mouse, rat and human orthologues was not consistent with respect to the order of identity with mouse or rat sequences for the eight S1P and LPA receptors, but the rank order for a given receptor was the same at nucleotide and protein levels.

Species-specific oligonucleotides were designed using sequences of the Chinese hamster gene fragments for use in the RT-PCR analysis of Chinese hamster total RNA to indicate which S1P and LPA receptor genes were endogenously expressed in this cell line. This study showed the presence of mRNA for S1P$_1$, S1P$_2$, S1P$_4$ and LPA$_1$ receptors in CHO-K1 cells, although the result suggested that the S1P$_4$ transcript was not very abundant since it only yielded a faint product in the RT-PCR experiment. It would have been interesting to continue this study to profile which of these genes were expressed at the protein level, but it was not clear whether the available antibodies to human S1P and LPA receptors would cross-react with hamster protein and generation of hamster-
specific antibodies for these four GPCRs was beyond the scope of this investigation.

The information from this RT-PCR study may be compared with published reports describing expression profiles of the eight S1P and LPA genes in ovarian type tissues. This information is summarised in Table 3.4.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Previous reports of expression in ovary or related cell lines (method used)</th>
<th>Result from CHO-K1 RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1P1</td>
<td>Not detected in CHO-K1 cells using rat S1P1 TM domains as probe (Northern blot)</td>
<td>++</td>
</tr>
<tr>
<td>S1P2</td>
<td>Expresed in rat ovary (EST expression profiling)</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Detected in CHO-K1 cells using rat S1P2 5' fragment as probe (Northern blot)</td>
<td>-</td>
</tr>
<tr>
<td>S1P3</td>
<td>Not detected in CHO-K1 cells using human S1P3 coding sequence as probe (Northern blot)</td>
<td>-</td>
</tr>
<tr>
<td>S1P4</td>
<td>Not detected in human ovary (Northern blot)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Not detected in CHO-K1 cells using human S1P4 3'-coding sequence fragment as probe (Northern blot)</td>
<td>-</td>
</tr>
<tr>
<td>S1P5</td>
<td>No data published</td>
<td>-</td>
</tr>
<tr>
<td>LPA1</td>
<td>Expressed in human ovary (Northern blot)</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Not detected in CHO-K1 cells using human LPA1 3'-UTR as probe (Northern blot)</td>
<td>-</td>
</tr>
<tr>
<td>LPA2</td>
<td>Not detected in human ovary (Northern blot)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Not detected in CHO-K1 cells using human LPA2 3'-UTR as probe (Northern blot)</td>
<td>-</td>
</tr>
<tr>
<td>LPA3</td>
<td>Expressed in human ovary – faint (Northern blot)</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.4
Summary of S1P and LPA expression data in ovarian tissues and CHO-K1 cells.
Data were taken from published reports and RT-PCR analysis of expression in CHO-K1 cells described in this chapter.
++ strongly positive in RT-PCR; + faintly positive in RT-PCR; - negative in RT-PCR.

References
1 Okamoto et al., 1998
2 Sato et al., 1999
3 Glickman et al., 1999
4 Graeler et al., 1998
5 Yamazaki et al., 2000
6 An et al., 1998
7 Bandoh et al., 1999
The detection of mRNA for S1P and LPA receptors in CHO-K1 cells did not always correlate with detection of these transcripts in ovarian tissues and probably reflects the heterogeneity of cell types present within ovarian tissues. This investigation confirmed the previous observations, that CHO-K1 cells express S1P₂ but not S1P₃ or LPA₂ [Okamoto et al., 1998], although detection of transcripts for S1P₁ and LPA₁ contradicted this earlier study. This may suggest that the use of species-specific reagents is important in such an investigation, however it should also be noted that the current study used RT-PCR as the analytical tool, which is a more sensitive technique than Northern blotting.

The following chapter describes expression of the HA-S1P₄/pIRES and HA-S1P₄-Gα₁₁/pIRES constructs in CHO-K1 cells and analysis of the functional responses of the resultant recombinant proteins.
Chapter 4 – Expression and Functional Responses of S1P₄

4.1 Introduction

The lysophospholipid ligand, S1P, is known to elicit myriad cellular responses, including stimulation of calcium flux, alterations in cell morphology and enhanced cell survival (reviewed by Pyne and Pyne, 2000a; Pyne and Pyne, 2000b; Maceyka et al., 2002; Pyne and Pyne, 2002; Spiegel et al., 2002; Hla, 2003; Spiegel and Milstien, 2003). The sensitivity of some of these responses to pertussis toxin was an indicator of the involvement of GPCRs in these processes and the subsequent cloning of high affinity receptors for S1P confirmed a role for this bioactive lipid as a GPCR agonist. Of the six Edg receptors which had been identified when the work described in this chapter was started, three were known to be activated by S1P whilst the two remaining Edg receptors to which ligands had been assigned were activated by LPA [Graeler et al., 1998]. The orphan receptor S1P₄ (then named Edg6) showed greater sequence similarity with the S1P-activated Edg receptors than with those activated by LPA and it therefore seemed possible that S1P might be an agonist for the S1P₄ receptor. Early characterisations of the known S1P receptors showed that all three coupled to Go₄ G-proteins and the S1P₂ and S1P₃ receptors could activate multiple G-proteins [Zhang et al., 1999; Kon et al., 1999; Windh et al., 1999] in response to S1P stimulation. Given the common ability of these known S1P receptors to couple to Go₄ G-proteins, it was postulated that the S1P₄ receptor would also activate this class of G-proteins.

Activation of a cell-surface GPCR results in the downstream activation of many signalling pathways, which may be conveniently monitored and interpreted with...
respect to receptor activation. These include modulation of adenyl cyclase activity, flux of intracellular calcium and activation of MAPK enzymes. There is a potential advantage, however, in measuring the consequence of receptor activation at the G protein level. Intracellular signalling cascades often involve signal amplification and may be subject to positive and negative influences, which may complicate interpretation of data. The immediate function of GPCR activation is to promote exchange of the guanine nucleotides, GDP and GTP, on the Gα G protein and this may be represented as the four stage cycle shown in Figure 4.1.

Figure 4.1
Schematic diagram showing guanine nucleotide exchange cycle on Gαβγ G proteins.
Exchange of guanine nucleotides on Gαβγ G proteins is stimulated by the binding of an agonist (A) to a GPCR located on the cell membrane.
During this cycle, occupation of a GPCR by an agonist causes it to become activated. Guanine nucleotide exchange is initiated by the binding of the activated GPCR to the membrane-associated heterotrimeric G protein. This event stimulates the release of bound GDP from the Gα G protein and allows binding of endogenous GTP, which in turn promotes dissociation of the GPCR from the Gα-GTP and Gβγ G protein subunits. These Gα-GTP and Gβγ G protein subunits then activate effector molecules, such as adenylyl cyclase, phospholipase C and ion channels. The intrinsic GTPase activity of the Gα G protein inactivates the Gα G protein entity via hydrolysis of the bound GTP back to GDP, with concomitant release of inorganic phosphate. The Gα-GDP subunit is then able to reassociate with the Gβγ G protein subunit to reform the GDP-associated heterotrimer, which is ready for another round of activation, thus completing the cycle. The existence of this cycle may be exploited in two ways to measure receptor activation: one possibility is to measure the rate of hydrolysis of [γ^32P]GTP and this is employed in the GTPase assay. Alternatively the binding of a radiolabelled non-hydrolysable analogue of GTP, [^35S]GTPγS may be determined [Windh and Manning, 2002; Milligan, 2003]. These assays are typically performed using crude membranes prepared from cells expressing the receptor of interest.

The focus of the work reported in this chapter was to try to detect direct activation of a G protein in response to stimulation of S1P₄ by S1P using an adapted[^35S]GTPγS binding assay where specific antisera were used to immunoprecipitate Gα₄ G proteins from detergent extracts prepared from membranes after incubation with S1P, guanine nucleotides and[^35S]GTPγS.
This type of GTPγS/immunoprecipitation (GTPγS/IP) assay has been successfully used to examine the differential coupling of S1P1/2/3 receptors to different G proteins [Windh et al., 1999].
4.2 Results

4.2.1 Transient expression of \( \alpha_{2A}\)AR-G\( \alpha_{i1}(C^{351}I) \) fusion in HEK-293 cells

HEK-293 cells were transiently transfected to express the \( \alpha_{2A}\)AR-G\( \alpha_{i1}(C^{351}I) \) fusion protein (detailed in chapter 2, section 3.2), to provide a positive control for use in \( ^{35}S \)GTP\( \gamma \)S binding assays. Approximately 48 hours after transfection, cells were harvested non-enzymatically and membranes prepared, as described in chapter 2, section 4.1. Analysis of the resultant membranes by Western blotting using an anti-G\( \alpha_{i1} \) antibody (Figure 4.2) showed the presence of a polypeptide with apparent mass of approximately 40 kDa in both parental and transiently transfected cells and is due to expression of endogenous G\( \alpha_{i1} \) G protein. Membranes from transfected cells also contained a polypeptide with apparent Mr of 120 kDa, which reflects expression of the \( \alpha_{2A}\)AR-G\( \alpha_{i1}(C^{351}I) \) fusion protein. This mass was greater than that predicted on the basis of amino acid sequence and may reflect post-translational modification of the receptor.

4.2.2 Comparison of immunoprecipitation efficiency of different anti-G protein antisera in \( ^{35}S \)GTP\( \gamma \)S binding assay

In order to optimise conditions before work started with S1P\(_4\)-transfected membranes, a comparison was made between two antibodies (sc-262 and SGI) for their ability to immunoprecipitate G\( \alpha_{i} \) subunits from HEK-293 membranes transiently expressing \( \alpha_{2A}\)AR-G\( \alpha_{i1}(C^{351}I) \). A \( ^{35}S \)GTP\( \gamma \)S binding assay was performed, as detailed in chapter 2, section 5.2, using these \( \alpha_{2A}\)AR-G\( \alpha_{i1}(C^{351}I) \)-expressing membranes, which were incubated with or without the agonist UK14304 (used at a single concentration of 1 \( \mu \)M).
Expression of $\alpha_{2A}$AR-$G_{\alpha 1}(C^{351})$ in HEK-293 cells.
Membranes from HEK-293 cells which had been transiently transfected to express the $\alpha_{2A}$AR-$G_{\alpha 1}(C^{351})$ fusion protein (lane 1) and untransfected HEK-293 cells (lane 2) were analysed by Western blotting using an anti-$G_{\alpha 1}$ antibody. Visualisation of immunoreactive proteins was achieved via chemiluminescence after incubation of the blot with an appropriate HRP-conjugated secondary antibody. The position of the $\alpha_{2A}$AR-$G_{\alpha 1}(C^{351})$ fusion is indicated by an arrow.

Both the sc-262 antibody, which cross-reacts with $G_{\alpha 1/2/3}$, and the SG1 antiserum, which recognises $G_{\alpha 1/2}$ were used at their previously determined maximally effective dilutions of 1 in 100 and 1 in 200, respectively. Whilst an agonist-dependent increase in $[^{35}\text{S}]{\text{GTP}}_\gamma{\text{S}}$ binding was observed using each antibody, the window of stimulation was significantly greater using the SG1 antiserum (>8-fold compared to 2.5-fold stimulation with sc-262) (Figure 4.3) and may have reflected the ability of SG1 to immunoprecipitate a greater proportion of the $G_{\alpha 1/2}$ G protein from the detergent extract. On the basis of these results, the SG1 antiserum was used for the immunoprecipitation step in all subsequent $[^{35}\text{S}]{\text{GTP}}_\gamma{\text{S}}$ assays.
**Chapter 4**

4.2.3 **Stable expression of HA-S1P<sub>4</sub> in CHO-K1 cells**

The plasmid HA-S1P<sub>4</sub>-pIRES (described in chapter 3, section 2.1) was stably transfected into CHO-K1 cells, as described in chapter 2, section 3.3. Transfection with the HA-S1P<sub>4</sub> construct resulted in the presence of a polypeptide with apparent Mr of 65 kDa, which cross-reacted with anti-HA and anti-S1P<sub>4</sub> antibodies (shown in Figure 4.4 (A)) and was consistent with expression of full length HA-tagged S1P<sub>4</sub> protein. CHO-K1 membranes (both parental and transfected) expressed a protein of approximately 40 kDa, which was identified using the anti-Gα<sub>4</sub> antibody and reflected expression of endogenous Gα<sub>4</sub> G proteins in these cells (not shown). Cell-surface expression of HA-tagged S1P<sub>4</sub> was confirmed by FACS analysis using an anti-HA antibody (shown in Figure 4.4 (B)).
Figure 4.4

Expression of HA-S1P₄ in CHO-K1 cells.

A Membranes from CHO-K1 cells stably expressing HA-S1P₄ (lane 1) and untransfected CHO-K1 cells (lane 2) were analysed by Western blotting using anti-S1P₄ (panel I) or anti-HA (panel II) antibodies. Visualisation of immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with appropriate HRP-conjugated secondary antibodies. The position of HA-S1P₄ is indicated by an arrow.

B Cell-surface expressed HA-S1P₄ was detected by FACS analysis using a Fluorescein conjugate of the anti-HA antibody, represented by the blue trace. Cells were also stained with an isotype matched control antibody, denoted by the red trace. Panel I shows the staining obtained using HA-S1P₄-transfected CHO-K1 cells whilst panel II illustrates staining of untransfected CHO-K1 cells. Data are presented as overlay histograms and are representative of at least five independent experiments.
4.2.4 Optimisation of HA-S1P₄ response in [³⁵S]GTPγS binding assay

The concentration of GDP can markedly alter the capacity to monitor specific binding of [³⁵S]GTPγS. To determine the optimal GDP concentration for use in [³⁵S]GTPγS assays with HA-S1P₄, membranes were incubated with or without 10 μM S1P in the presence or absence of varying concentrations of GDP before immunoprecipitation of detergent extracts with the SG1 antiserum (Figure 4.5).

![Figure 4.5](image)

**Figure 4.5**
Determination of optimal GDP concentration using HA-S1P₄ in [³⁵S]GTPγS binding assay.
Membranes from CHO-K1 cells stably expressing HA-S1P₄ were incubated for 10 minutes at 30 °C with [³⁵S]GTPγS in the absence (basal) or presence (stimulated) of 10 μM S1P and varying concentrations of GDP. The membranes were then solubilised and SG1 antiserum used to immunoprecipitate Gα_i subunits after preclearance with non-immune serum. Data are presented as means of triplicate determinations ± SEM.

An agonist-dependent increase in [³⁵S]GTPγS binding was observed and was found to be greatest in the presence of 0.1 μM GDP. The level of S1P-mediated stimulation observed at the optimal concentration of GDP was relatively small, so attempts were made to optimise the assay conditions. Time course experiments were performed to determine how the length of incubation could
influence the window of stimulation. Figure 4.6 (A) shows the variation of response with incubation time; the “standard” incubation length of 10 minutes appeared to be preferred to longer incubations, although the general trend of basal and stimulated counts increasing with time suggested that a shorter incubation time could give an improved window of response. The relationship between basal or S1P-stimulated [\(^{35}\)S]GTP\(\gamma\)S binding with time was not linear and appeared to have reached a plateau after incubation for 20 minutes. This may suggest that [\(^{35}\)S]GTP\(\gamma\)S binding was rapid and the linear portion of binding occurred within the first few minutes.

The effect of the permeabilising agent saponin was also examined. Saponin has been reported to increase signal-to-noise ratio without altering pharmacological characteristics of the receptor preparation [Lazareno, 1997] and its effect on the window of stimulation achieved using HA-S1P\(_4\)-expressing membranes treated with or without 10 \(\mu\)M S1P is shown in Figure 4.6 (B). Addition of saponin to assay tubes incubated for 10 minutes was found to increase the level of stimulation over basal from approximately 30 \% to greater than 90 \% and was therefore chosen to be included in subsequent assays. After a 10 minute incubation, the level of counts in stimulated samples was greater than 10 \% of total radioactivity added and so incubation for 5 minutes was deemed preferable, to avoid substrate-depletion effects. This length of incubation gave approximately 2-fold stimulation over basal (shown in Figure 4.6 (B)), with an incorporation of less than 10 \% of the radionucleide and was therefore used as the incubation time for subsequent assays of HA-S1P\(_4\).
Membranes from CHO-K1 cells stably expressing HA-SIP4 were assayed for $[^{35}S]$GTPγS binding in the absence (basal) or presence (stimulated) of 10 μM SIP. The assay was incubated at 30 °C for the times shown (A) and where indicated, saponin was included in the assay mix (B). The membranes were then solubilised and SG1 antiserum used to immunoprecipitate Gαi subunits after preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment and are representative of two independent experiments.
4.2.5 Constitutive activity of HA-SIP<sub>4</sub> in [³⁵S]GTPγS binding assay

In comparison with membranes expressing α<sub>2A</sub>AR-Gα<sub>z1</sub>, the level of basal [³⁵S]GTPγS binding observed using HA-SIP<sub>4</sub>-expressing membranes during the initial optimisation experiments was noted as being markedly higher. Although this could have been due to the difference in host system (α<sub>2A</sub>AR-Gα<sub>z1</sub> was expressed in HEK-293 cells whilst HA-SIP<sub>4</sub> was expressed CHO-K1 cells), this observation coupled with the relatively small effects of S1P suggested that HA-SIP<sub>4</sub> might be constitutively active. This was examined further: [³⁵S]GTPγS binding was measured using HA-SIP<sub>4</sub>-transfected, or untransfected, CHO-K1 membranes treated with or without 10 μM S1P, or with a vehicle (0.1% fatty acid free BSA) control. Expression of HA-SIP<sub>4</sub> in CHO-K1 membranes resulted in a significant increase in the level of basal [³⁵S]GTPγS binding (Figure 4.7). This agonist-independent activity represented constitutive activity of the SIP<sub>4</sub> receptor and was also observed with a second clone expressing the same construct (data not shown). Treatment with S1P further increased the extent of [³⁵S]GTPγS binding in HA-SIP<sub>4</sub>-expressing membranes and was shown to be S1P<sub>4</sub>-dependent since no such increase was observed using untransfected membranes.

4.2.6 Pertussis toxin-sensitivity of HA-SIP<sub>4</sub> dose-response to S1P in [³⁵S]GTPγS binding assay

The previous observations that suggested HA-SIP<sub>4</sub> was constitutively active were examined in detail during the course of EC<sub>50</sub> determinations for the ligand S1P. S1P-mediated increases in [³⁵S]GTPγS binding were concentration-
**Figure 4.7**

**Constitutive activity of HA-S1P4 after expression in CHO-K1 cells.** Membranes from parental or HA-S1P4-transfected CHO-K1 cells were incubated for 5 minutes at 30 °C with [35S]GTPγS in the absence (basal) or presence (stimulated) of 10 μM S1P, or 0.1 % fatty acid free BSA vehicle control (vehicle). G protein α subunits were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment, and are representative of three such experiments performed. Statistical significance from the basal response of parental cells is denoted by ** (P < 0.01).

...dependent (Figure 4.8). The mean EC₅₀ obtained for activation of HA-S1P₄ by S1P was 565 ± 245 nM (n=4). Both S1P-induced and agonist-independent [35S]GTPγS binding were shown to be sensitive to ADP-ribosylation by pertussis toxin. Pre-treatment of cells with 100 ng/mL pertussis toxin for 24 hours prior to harvesting resulted in [35S]GTPγS binding levels that were unaffected by the presence of S1P and were similar to those seen using parental CHO-K1 membranes (Figure 4.7). A second CHO-K1 clone expressing HA-S1P₄ gave similar results (data not shown), with an EC₅₀ for activation by S1P of 320 nM (n=1), significant constitutive activity and complete sensitivity of basal and agonist-induced [35S]GTPγS binding to ADP-ribosylation by pertussis toxin.
Figure 4.8
Concentration dependent stimulation of HA-S1P<sub>4</sub>-mediated $[^{35}S]GTP\gamma S$ binding by S1P.
Membranes from CHO-K1 cells transfected with HA-S1P<sub>4</sub> which had been cultured in the absence (- PTx) or presence (+ PTx) of 100 ng/mL pertussis toxin for 24 hours prior to harvest were stimulated with varying concentrations of S1P for 5 minutes at 30 °C in the $[^{35}S]GTP\gamma S/IP$ binding assay. Go, G proteins were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment, and are representative of four such experiments performed.

4.2.7 Stable expression of HA-S1P<sub>4</sub>-G<sub>ai1</sub>(C<sup>351</sup>I) fusion in CHO-K1 cells
CHO-K1 cells were stably transfected with the plasmid HA-S1P<sub>4</sub>-G<sub>ai1</sub>(C<sup>351</sup>I) (described in chapter 3, section 2.2). Membranes from cells expressing HA-S1P<sub>4</sub>-G<sub>ai1</sub>(C<sup>351</sup>I) contained a polypeptide with an apparent Mr of 110 kDa which reacted with the anti-HA, anti-S1P<sub>4</sub> and anti-G<sub>ai1</sub> antibodies, (Figure 4.9 (A)). This was indicative of expression of the HA-tagged S1P<sub>4</sub>-G<sub>ai1</sub>(C<sup>351</sup>I) fusion protein. As had been observed with the unfused HA-S1P<sub>4</sub>-transfected membranes, the apparent mass of the HA-S1P<sub>4</sub>-G<sub>ai1</sub>(C<sup>351</sup>I) was greater than that predicted on the basis of amino acid sequence and suggested the presence of post-translational modifications. FACS analysis using the anti-HA antibody was used to confirm the presence of cell-surface fusion protein (Figure 4.9 (B)).
Figure 4.9

Expression of HA-S1P$_4$-G$\alpha_{i1}$(C$^{351}$I) in CHO-K1 cells.

A Membranes from CHO-K1 cells stably expressing HA-S1P$_4$-G$\alpha_{i1}$(C$^{351}$I) (lane 1) and untransfected CHO-K1 cells (lane 2) were analysed by Western blotting using anti-S1P$_4$ (panel I), anti-HA (panel II) or anti-G$\alpha_{i1}$ (panel III) antibodies. Visualisation of immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with appropriate HRP-conjugated secondary antibodies. The position of HA-S1P$_4$-G$\alpha_{i1}$(C$^{351}$I) is indicated by an arrow.

B Cell-surface expressed HA-S1P$_4$-G$\alpha_{i1}$(C$^{351}$I) was detected by FACS analysis using a Fluorescein conjugate of the anti-HA antibody, represented by the blue trace. Cells were also stained with an isotype matched control antibody, denoted by the red trace. Panel I shows the staining obtained using HA-S1P$_4$-G$\alpha_{i1}$(C$^{351}$I)-transfected CHO-K1 cells whilst panel II illustrates staining of untransfected CHO-K1 cells. Data are presented as overlay histograms and are representative of at least five independent experiments.
4.2.8 Optimisation of HA-S1P$_4$-G$\alpha_{i1}(C^{351}I)$ response in [$^{35}$S]GTP$\gamma$S binding assay

A similar approach to that used with the HA-S1P$_4$-expressing membranes (detailed in this chapter, section 2.4) was applied to optimise the response of HA-S1P$_4$-G$\alpha_{i1}(C^{351}I)$ in the [$^{35}$S]GTP$\gamma$S binding assay. Figure 4.10 shows the effect of GDP concentration on the ability to detect agonist-mediated increases in [$^{35}$S]GTP$\gamma$S binding. Similar to the result obtained with the unfused HA-S1P$_4$ construct, the optimal concentration of GDP was approximately 0.1 $\mu$M. Time course experiments were performed to determine the optimal incubation time for this receptor construct. Saponin was included in these incubations as it had successfully increased the window of stimulation observed with the unfused HA-S1P$_4$ receptor (described in this chapter, section 2.4).

![Figure 4.10](image_url)

**Figure 4.10**

**Determination of optimal GDP concentration using HA-S1P$_4$-G$\alpha_{i1}(C^{351}I)$ in [$^{35}$S]GTP$\gamma$S binding assay.**

Membranes from CHO-K1 cells stably expressing HA-S1P$_4$-G$\alpha_{i1}(C^{351}I)$ were incubated for 10 minutes at 30 °C with [$^{35}$S]GTP$\gamma$S in the absence (basal) or presence (stimulated) of 10 $\mu$M S1P and varying concentrations of GDP. The membranes were then solubilised and SG1 antiserum used to immunoprecipitate G$\alpha_i$ subunits after preclearance with non-immune serum. Data are presented as means of triplicate determinations ± SEM.
Figure 4.11 shows the effect of incubation time on the stimulation window. In contrast to the unfused HA-S1P\(_4\) construct, which gave an optimal level of stimulation above basal after 5-10 minutes, the optimal incubation time for the HA-S1P\(_4\)-G\(\alpha_{41}(C^{351})\) fusion was 30 minutes and was used in subsequent experiments. Similar to observations with the unfused HA-S1P\(_4\) receptor, basal and agonist-stimulated \(^{35}\text{S}\)GTP\(\gamma\text{S}\) binding did not show a linear relationship with time which suggested that the linear portion of \(^{35}\text{S}\)GTP\(\gamma\text{S}\) binding occurred in the first few minutes.

![Graph showing [\(^{35}\text{S}\)GTP\(\gamma\text{S}\) binding](image)]

**Figure 4.11**

Optimisation of response of HA-S1P\(_4\)-G\(\alpha_{41}(C^{351})\) in \(^{35}\text{S}\)GTP\(\gamma\text{S}\) binding assay. Membranes from CHO-K1 cells stably expressing HA-S1P\(_4\)G\(\alpha_{41}(C^{351})\) were assayed for \(^{35}\text{S}\)GTP\(\gamma\text{S}\) binding in the absence (basal) or presence (stimulated) of 10 \(\mu\text{M}\) S1P and incubated at 30 °C for varying lengths of time. Post assay, membranes were solubilised and G\(\alpha_{4}\) subunits immunoprecipitated using SG1 antiserum after preclearance with non-immune serum. Data shown are the mean of three determinations ± SEM from a single experiment and are representative of two independent experiments.
4.2.9 Constitutive activity of HA-SIP₄-Gα₄₁(C³⁵¹I) in [³⁵S]GTPγS binding assay

Measurement of [³⁵S]GTPγS binding using HA-SIP₄-Gα₄₁(C³⁵¹I)-transfected or untransfected CHO-K1 membranes was made after treatment with or without 10 µM S1P, or with a vehicle (0.1 % fatty acid free BSA) control. Expression of HA-SIP₄-Gα₄₁(C³⁵¹I) in CHO-K1 cells resulted in higher levels of basal [³⁵S]GTPγS binding than that observed in untransfected CHO-K1 cells and treatment with S1P further increased [³⁵S]GTPγS binding in HA-SIP₄-Gα₄₁(C³⁵¹I)-expressing membranes in a receptor-dependent manner (Figure 4.12). This was similar to observations made with the unfused HA-SIP₄ receptor (this chapter, section 2.5).

![Bar chart showing constitutive activity of HA-SIP₄-Gα₄₁(C³⁵¹I) expressed in CHO-K1 cells.](image)

**Figure 4.12**
Constitutive activity of HA-SIP₄-Gα₄₁(C³⁵¹I) expressed in CHO-K1 cells. Membranes from parental or HA-SIP₄-Gα₄₁(C³⁵¹I)-transfected CHO-K1 cells were incubated with [³⁵S]GTPγS in the absence (basal) or presence (stimulated) of 10 µM S1P, or 0.1 % fatty acid free BSA vehicle control (vehicle) for 30 minutes at 30 °C. G protein α subunits were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment, and are representative of three such experiments performed. Statistical significance from the basal response of parental cells is denoted by **(P < 0.01).
4.2.10 Pertussis toxin-sensitivity of HA-S1P$_4$-G$\alpha$$_i$(C$^{35}$I) dose-response to S1P in [$^{35}$S]GTP$\gamma$S binding assay

The potency of the ligand S1P as an agonist of the HA-S1P$_4$-G$\alpha$$_i$(C$^{35}$I) fusion construct was determined in the [$^{35}$S]GTP$\gamma$S binding assay (Figure 4.13). The EC$_{50}$ for S1P activation was 355 nM ± 155 nM (n=3). In contrast to the pertussis toxin-sensitivity seen with HA-S1P$_4$ membranes, HA-S1P$_4$-G$\alpha$$_i$(C$^{35}$I) membranes treated with pertussis toxin for 24 hours prior to harvest were able to exhibit increased [$^{35}$S]GTP$\gamma$S binding after S1P stimulation, with an EC$_{50}$ of 805 nM ± 190 nM (n=3) which reflected activation of the fused, pertussis toxin-insensitive G$\alpha$$_i$ G protein. This construct also appeared to be constitutively active because basal counts obtained, even after pertussis toxin treatment, were appreciably higher than those obtained with untransfected CHO-K1 membranes incubated for the same length of time under the same assay conditions (shown in Figure 4.12). There was a marked reduction in basal [$^{35}$S]GTP$\gamma$S binding, however, following pertussis toxin treatment. This may reflect the ability of HA-S1P$_4$-G$\alpha$$_i$(C$^{35}$I) to interact constitutively with endogenously expressed G$_i$ G proteins. Pretreatment with pertussis toxin also produced a small decrease in the extent of the signal produced by S1P. This also suggested a degree of coupling of the HA-S1P$_4$-G$\alpha$$_i$(C$^{35}$I) fusion to endogenous pertussis toxin-sensitive G proteins. However, the majority of the signal from the fusion protein was clearly mediated via the fused, pertussis toxin-insensitive G protein. A second clone expressing the HA-S1P$_4$-G$\alpha$$_i$(C$^{35}$I) protein was also tested (data not shown). This clone expressed very low levels of HA-S1P$_4$-G$\alpha$$_i$(C$^{35}$I) and whilst EC$_{50}$ values for activation by S1P were somewhat higher than for the original clone tested, the overall profile of response was similar: constitutive activity was
evident even after pertussis toxin treatment and S1P was able to stimulate activation of the receptor both before and after pretreatment of cells with pertussis toxin.

Figure 4.13
Concentration dependent stimulation of HA-S1P₄-Gα₁₁(C₃₅₁)-mediated [³⁵S]GTPγS binding by S1P.
Membranes from CHO-K1 cells transfected with HA-S1P₄-Gα₁₁(C₃₅₁) which had been cultured in the absence (- PTx) or presence (+ PTx) of 100 ng/mL pertussis toxin for 24 hours prior to harvest were stimulated with varying concentrations of S1P for 30 minutes at 30 °C in the [³⁵S]GTPγS/IP binding assay. Gα₁₁ G proteins were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment, and are representative of three such experiments performed.
4.3 Discussion

SIP4 was cloned as the orphan GCPR named Edg6 [Graeler et al., 1998]. Sequence similarity with other Edg receptors suggested the lysophospholipid SIP might be an agonist of this receptor, and that it may couple to Goi G proteins. This hypothesis was tested in this chapter.

Initially, HA-SIP4 was expressed in RH7777 cells, which have been reported to be unresponsive to SIP and LPA [Zhang et al., 1999]. Preliminary attempts had been made to measure SIP-induced activation of the SIP4 receptor expressed in this cell line using conventional [35S]GTPγS binding, inhibition of cAMP accumulation and calcium flux assays. Unfortunately, it was not possible to detect activation of this receptor in these assays. The receptor was then expressed in CHO-K1 cells and the modified [35S]GTPγS/IP binding assay used to try to measure activation of SIP4 at the level of receptor-G protein interaction.

The concentration of GDP used in this assay was optimised since GDP affects the magnitude of agonist stimulated [35S]GTPγS binding [Windh and Manning, 2002]. High GDP concentrations suppress basal activity whilst at low GDP concentrations, basal activity is unrestricted so the ability of an agonist to further stimulate [35S]GTPγS binding is limited. The optimal concentration of GDP for the detection of SIP-stimulated [35S]GTPγS binding for the SIP4 receptor was 0.1 μM. The time of incubation was optimised such that the window of stimulation was maximised. The non-linear profile of the timecourses suggested that [35S]GTPγS binding plateaued rapidly and was not unexpected, since a similar profile was reported for the 5-HT1A receptor [Windh and Manning, 2002]. Further optimisation of the signal was obtained via the addition of the
permeabilising agent saponin. In this study, saponin was used at a 1:1 ratio relative to the amount of membranes, however it may have been beneficial to test other ratios. A study of the SLC-1 receptor (somatostatin-like receptor 1) by Audinot and et al., [2001] investigated the effect of different saponin ratios on ligand-induced \[^{[35]S}\]GTP\(\gamma\)S binding for this receptor. Saponin was shown to produce a bell-shaped curve and the peak stimulation corresponded to a ratio of approximately 1:2.5 (saponin:SLC-1 membranes).

Whilst this work was in progress, two independent groups reported the ability of S1P to bind to S1P\(_4\) and stimulate ERK phosphorylation [Van Brocklyn et al., 2000] and calcium flux [Yamazaki et al., 2000]. However, this receptor was reported to be non-functional in a study using a conventional GTP\(\gamma\)S assay to compare the activation of S1P\(_{1/2/3}\) with S1P\(_5\), expressed in HEK-293 cells, by various analogues of S1P [Im et al., 2001a]. Therefore the results described in this chapter were the first demonstration of S1P\(_4\) activation measured at the G protein level. Subsequently, two groups have shown activation of S1P\(_4\) using a conventional \[^{[35]S}\]GTP\(\gamma\)S assay [Brinkmann et al., 2002; Mandala et al., 2002] and the data presented in this chapter concurs with these reports.

In this chapter, it has been shown that 1) S1P\(_4\) couples to Goq G proteins; 2) S1P\(_4\) is strongly constitutively active when expressed in CHO-K1 cells and 3) S1P\(_4\) can be further activated by treatment with S1P. These results support published observations that S1P\(_4\) is activated by S1P [Van Brocklyn et al., 2000; Yamazaki et al., 2000] but in addition are the first to show the extent of constitutive activity displayed by S1P\(_4\). It may be that previous difficulties in observing S1P activation of S1P\(_4\) reflect this high level of constitutive activity. The increase in
[\textsuperscript{35}S]GTP\gamma S binding in S1P\textsubscript{4}-transfected CHO-K1 membranes when compared to untransfected membranes is strongly indicative of a high level of constitutive activity of the receptor and warrants further investigation. Additionally, the S1P\textsubscript{4} receptor was seen to exhibit significant levels of basal activity in a high affinity GTPase assay (data not shown). The ability of S1P\textsubscript{4} to couple to G\alpha\textsubscript{i} G proteins is a feature shared by all of the S1P-activated Edg receptors. In common with S1P\textsubscript{2/3/5}, S1P\textsubscript{4} couples to multiple G proteins and has been reported to activate G\alpha\textsubscript{i} and G\alpha\textsubscript{12/13} [Van Brocklyn \textit{et al.}, 2000; Yamazaki \textit{et al.}, 2000; Graeler \textit{et al.}, 2003].

The use of fusion proteins constructed between a GPCR and the \alpha subunit of a G protein has become widespread and offers several advantages over systems where non-fused receptors are studied (reviewed by Seifert \textit{et al.}, 1999; Milligan, 2000; Wurch and Pauwels, 2001). Construction of a GPCR-G\alpha fusion using a mutated G\alpha G protein that is insensitive to ADP-ribosylation by pertussis toxin allowed isolation of the response from the fused receptor [Milligan, 2000]. In this study, a pertussis toxin-insensitive HA-S1P\textsubscript{4}-G\alpha\textsubscript{i1}(C\textsuperscript{351}I) fusion protein was used, enabling the elimination of signalling from endogenously expressed S1P receptors by treatment with pertussis toxin. The ability of the tethered G protein to transduce the activation of S1P\textsubscript{4} was demonstrated using membranes prepared from pertussis toxin-treated cells, where S1P was shown to promote a dose-dependent increase in [\textsuperscript{35}S]GTP\gamma S binding. The HA-S1P\textsubscript{4}-G\alpha\textsubscript{i1}(C\textsuperscript{351}I) fusion protein also exhibited constitutive activity.

The lysophospholipid, S1P has been identified as the high affinity ligand for several members of the Edg receptor family and these S1P receptors bind S1P
with dissociation constants in the range 2 nM for S1P_3 to approximately 25 nM for S1P_{2/3} [Lee et al., 1998; Kon et al., 1999; Im et al., 2000a]. In a recent parallel study [Im et al., 2001a] a conventional GTP\gamma S binding assay was used to compare the efficacy of various agonists, including S1P, to activate S1P_{1/2/3} and S1P_5, using membranes prepared from HEK-293 transfected cells. Values for the EC_{50} of S1P-induced GTP\gamma S binding were in the range 0.9 - 2.9 nM for S1P_{1/2/3} and 44 nM for S1P_5; these data may be compared with EC_{50} values obtained for activation of the S1P_4 constructs described here: 565 nM for HA-S1P_4 and 355 - 805 nM for the HA-S1P_4-G\alpha_11(C^{351}I) fusion protein. Statistical analysis indicated that these EC_{50} values were not significantly different. This probably reflects the standard errors associated with these values and is a result of each S1P dose-response experiment having been set up using membranes prepared from separate cell passages (which expressed different levels of cell-surface S1P_4). S1P has previously been shown to bind to the S1P_4 receptor, albeit with a lower affinity than that with which it binds other S1P receptors. Dissociation constants for S1P binding to S1P_4 expressed in mammalian cells fall in the range 20 nM - 95 nM [Van Brocklyn et al., 2000; Yamazaki et al., 2000; Mandala et al., 2002]. Therefore, whilst S1P does bind to, and activate S1P_4, it does so with lower affinity and potency than it does at the other S1P receptors, which may suggest that it is not the endogenous ligand for the S1P_4 receptor. Phylogenetic analysis of the Edg receptors (shown in chapter 1, Figure 1.6) shows that whilst the other S1P receptors form a discrete cluster, S1P_4 is on the edge of this cluster and could represent the first member of a new subset of Edg receptors, which are activated by a lipid that is structurally distinct from S1P and LPA. A recent survey of GPCRs in the human genome [Vassilatis et al.,
2003] predicted that the Edg receptor family will not extend past the eight members already cloned. It should be noted however, that a related family of orphan GPCRs (GPR3, 6 and 12) were recently identified as S1P receptors, indicating that there are certainly other GPCRs which respond to S1P and related lipids [Uhlenbrock et al., 2002].

In the following chapter, further investigations into the constitutive activity displayed by S1P₄ are described.
Chapter 5 – Use of an Inducible Expression System to Examine the Constitutive Activity of S1P₄

5.1 Introduction

In chapter 4 it was shown that the S1P₄ receptor is constitutively active and whilst this may have been a consequence of the high levels of recombinant receptor present in membranes used for these investigations, it could also reflect an inherent property of S1P₄. This chapter describes work carried out to further characterise the ability of S1P₄ to signal in the absence of ligand stimulation.

Constitutive activity is defined as ligand-independent activity and results in G protein activation and second messenger production in the absence of stimulation by an agonist. The phenomenon of GPCR constitutive activity has been widely reviewed [Lefkowitz et al., 1993; Leurs et al., 1998; de Ligt et al., 2000] although it is not yet clear whether this property is physiologically relevant. Ligand-independent activation may be observed for almost any GPCR, although the introduction of a constitutively activating mutation (CAM) is often required to induce significant levels of agonist-independent activity (reviewed by Parnot et al., [2002]).

The finding that certain receptors exhibit constitutive activity has led to a modification of traditional receptor theory [Samama et al., 1993]. It is now believed that receptors can exist in at least two conformations, an inactive (R) and an active (R*) state and the equilibrium between these two conformations strongly favours the inactive receptor state. In some native receptors, and in those which have been modified by the introduction of a constitutively activating mutation, this equilibrium appears to be shifted so that there is a sufficient
number of receptors in the active state to initiate signalling, in the absence of agonist stimulation.

Constitutive activity is sensitive to the actions of inverse agonists. These ligands were previously defined as antagonists, since they were known to bind to, but not activate GPCRs. The discovery that many of these as "antagonists" were able to decrease agonist-independent activity of constitutively active receptors, and therefore possessed negative efficacy, led to their reclassification as inverse agonists. Inverse agonists have now been characterised for many receptor families and are valuable pharmacological tools that allow definitive demonstration of GPCR constitutive activity. At the time of these investigations however, no such compounds had been identified for the S1P receptor family.

Studies into the effect of expression level on receptor functionality may be undertaken as a way of characterising constitutive activity in the absence of such pharmacological tools. One of the more simple methods is to isolate a number of clonal cell lines which express a range of receptor densities and use these cell line in subsequent functional assays. This method utilises the positive correlation which exists between receptor density and agonist-independent activity. A potential drawback with such an approach is that it may not be possible to isolate clones which express significantly different levels of receptor, and by definition, comparisons would be made between different cell lines.

An attractive alternative is to employ an expression system that permits control over the level of protein expression via incubation of the cells with an agent which drives transcription of the gene of interest (reviewed by Clackson [1997]). This technique has the advantage of permitting examination into the effect of receptor expression level using the same clonal population of cells. The key
prerequisites for such a system are low levels of uninduced (background) expression and a specific induction agent which induces a large range of expression levels, in a dose-dependent manner. This is generally achieved using a heterologous transcription factor which binds a DNA sequence that is not recognised by endogenous transcription factors, and a small molecule which can modulate the activity of the transcription factor. There are two such commercially available systems: the tetracycline- and ecdysone-inducible mammalian expression systems; the latter of these was chosen for use in studies with the S1P₄ receptor.

The ecdysone-inducible expression system was first used to regulate expression of genes in HEK-293 cells [Christopherson et al., 1992] and is classified as an "allosteric on-switch" system [Clackson, 1997] i.e. transcription is stimulated by exposure to the induction agent. It is based on the Drosophila moulting induction system, which has been modified for use in mammalian systems and uses analogues of the ecdysone steroid hormone to potently activate expression of the gene of interest via a heterodimeric nuclear receptor composed of a modified form of the ecdysone receptor and the retinoid X receptor. Figure 5.1 shows a schematic diagram of this system. The ecdysone-inducible system has been used for the inducible expression of numerous recombinant proteins, including the somatostatin SST₂ and dopamine D₂long GPCRs [Choi et al., 2000; Cole et al., 2001].
Figure 5.1
Schematic diagram describing the ecdysone-inducible mammalian expression system.
The retinoid X receptor (RXR) and a modified form of the ecdysone receptor (VgEcR), which contains the VP16 transactivation domain, are expressed in mammalian cells under the control of the rous sarcoma virus (RSV) and cytomegalovirus immediate early (CMV) promoters, respectively. In the presence of a synthetic analogue of ecdysone, such as ponasterone A (PonA) or muristerone A (MuA), the receptors form a heterodimer, the DNA binding domains (DBD) of which bind the hybrid ecdysone response elements (E/GRE). Transcription of the gene of interest (HA-S1P₄ in this instance) is activated from the minimal heat shock promoter (P₃₄hsp) via the VP16 transactivation domain present in the receptor heterodimer.

Synthetic ecdysone receptor hormone analogues commonly used to induce protein expression with this system are muristerone A and ponasterone A, the structures of which are shown in Figure 5.2. These compounds are steroids and possess the characteristic four-ring backbone of this class of molecule. The structure of muristerone A is identical to that of ponasterone A other than the substituents present at the 5- and 11-positions of the ring system: hydroxyl groups in muristerone A and hydrogen atoms in ponasterone A.
In this chapter, the ecdysone-inducible expression system was used to investigate the relationship between S1P₄ expression and agonist-independent activity. These results would indicate whether the S1P₄ constitutive activity observed in chapter 4 represented an inherent property of this receptor, or was a result of the high levels of S1P₄ expression in the cell line used for those studies.
5.2 Results

5.2.1 Generation of HA-S1P₄ inducible expression stable cell line

The plasmid HA-S1P₄/pIND, which was produced in chapter 3, section 2.3 was stably transfected into CHO-EcR cells as described in chapter 2, section 3.3. This commercially available cell line had been transfected to stably express functional ecdysone receptor under selection with Zeocin. Stable transfection of these cells with the pIND plasmid containing the gene of interest followed by dual selection with Zeocin and Geneticin allowed isolation of a stable cell line in which expression of the heterodimeric ecdysone receptor was constitutive and expression of the gene of interest was under the control of the ecdysone receptor hormone analogues, ponasterone A or muristerone A.

5.2.2 Characterisation of the effect of the ecdysone receptor hormone analogues, ponasterone A and muristerone A, on cell-surface expression of HA-S1P₄

HA-S1P₄ receptor expression was measured in the stably transfected CHO-EcR cell line, which had been treated with varying concentrations of ponasterone A and muristerone A or DMSO vehicle control, for 24 hours at 37 °C. Cell-surface expression was determined via FACS analysis using an anti-HA antibody and the resultant data were expressed as fluorescence dot plots, such that staining of cell-surface HA-S1P₄ with the Fluorescein conjugated anti-HA antibody was observed as a shift to the right of the dot plot. Parental CHO-EcR cells cultured in the absence or presence of each ecdysone receptor hormone analogue, or DMSO vehicle control were analysed in this way and shown not to shift to the right under any condition (shown in Figure 5.3).
Figure 5.3

Dot plot analysis of parental CHO-EcR cells.

Parental CHO-EcR cells were treated with the conditions used to induce expression in HA-S1P₄-transfected cells i.e. with DMSO vehicle, 10⁻⁴.5 M ponasterone A (PonA) or Muristerone (MuA), or were left untreated (uninduced) and subject to FACS analysis using a Fluorescein conjugate of the anti-HA antibody. The location of the quadrant was defined using these cells and was such that the population was present in the lower left quadrant. Data presented are from duplicate determinations of a single experiment and are representative of three independent experiments.

Quadrant analysis of the dot plots was used to quantify the percentage of cells expressing HA-S1P₄. The location of the quadrant was set using parental CHO-EcR cells, which had been stained with the anti-HA antibody (Figure 5.3) and the same quadrant location used for the analysis of HA-S1P₄ expression in the transfected CHO-EcR cell line. Comparison of dot plots from parental and HA-S1P₄-transfected cells in this manner was performed for cells treated in the same way and harvested in parallel.

Ponasterone A was titrated over the range 10⁻⁴.5 to 10⁻⁷ M and expression of cell-surface HA-S1P₄ determined. The dot plots (Figure 5.4 (A)) showed a small population of cells in the lower right quadrant in the uninduced and vehicle controls, which indicated that there was some constitutive expression of the receptor. The proportion of cells in this location increased with the concentration of ponasterone A and the level of expression of HA-S1P₄ was dependent on the concentration of ponasterone A used.
Figure 5.4
Concentration dependent induction of cell-surface receptor expression by ponasterone A treatment of ecdysone-inducible HA-S1P$_4$-transfected CHO-EcR cells.
Cells were incubated for 24 hours in serum-free medium supplemented with varying concentrations of ponasterone A (PonA). Levels of cell-surface receptor expression were quantified by FACS analysis using a Fluorescein conjugate of the anti-HA antibody. Quadrant analysis of dot plots was used to determine the percentage of cells expressing HA-S1P$_4$ (A); the location of the quadrant was defined using CHO-EcR cells and those cells present in the lower right quadrant were classified as expressing HA-S1P$_4$. The resultant data are presented graphically (B). Data presented are the mean of duplicate determinations $\pm$ SEM from a single experiment, and are representative of three such experiments performed.
When plotted as a dose-response curve (Figure 5.4 (B)), an EC$_{50}$ value was obtained, although interpretation of this value needed caution, since a plateau of maximal receptor expression was not reached. It was not possible to use ponasterone A at concentrations in excess of $10^{-4.5}$ M since vehicle effects were observed with a vehicle control of the equivalent concentration of DMSO (not shown). At the maximal concentration of ponasterone A used ($10^{-4.5}$ M), 80 % ± 6 % (n=3) cells were determined to be expressing HA-S1P$_4$.

A similar experiment was set up using muristerone A and the profile of HA-S1P$_4$ expression induced by this agent was found to be very similar to that observed with ponasterone A. Analysis of the dot plots (Figure 5.5 (A)) showed that a proportion of cells expressed HA-S1P$_4$ prior to stimulation with muristerone A, and that the number of cells present in the lower right quadrant increased with muristerone A concentration, in a dose-dependent manner (Figure 5.5 (B)). As was seen when using ponasterone A, it was not possible to use sufficiently high concentrations of muristerone A to observe a maximal plateau with this dose-response curve. At the maximal concentration of muristerone A used ($10^{-4.5}$ M), 76 % ± 7 % (n=3) cells expressed HA-S1P$_4$, as determined by quadrant analysis of the FACS dot plots.
Concentration dependent induction of cell-surface receptor expression by muristerone A treatment of ecdysone-inducible HA-S1P4-transfected CHO-EcR cells.

Cells were incubated for 24 hours in serum-free medium supplemented with varying concentrations of muristerone A (MuA). Levels of cell-surface receptor expression were quantified by FACS analysis using a Fluorescein conjugate of the anti-HA antibody. Quadrant analysis of dot plots was used to determine the percentage of cells expressing HA-S1P4 (A); the location of the quadrant was defined using CHO-EcR cells and those cells present in the lower right quadrant were classified as expressing HA-S1P4. The resultant data are presented graphically (B). Data presented are the mean of duplicate determinations ± SEM from a single experiment, and are representative of three such experiments performed.
The data obtained with each induction agent are summarised in Table 5.1.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mean EC₅₀ ± SEM (µM)</th>
<th>Maximal % cells expressing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ponasterone A</td>
<td>5.44 ± 0.71</td>
<td>68 ± 4</td>
</tr>
<tr>
<td>Muristerone A</td>
<td>5.35 ± 1.29</td>
<td>64 ± 4</td>
</tr>
</tbody>
</table>

Table 5.1
Summary of ecdysone analogue potency for induction of HA-SIP₄ expression in HA-SIP₄-transfected ecdysone-inducible CHO-EcR cells. EC₅₀ values were calculated from dose-response experiments using the ecdysone analogues ponasterone A and muristerone A to induce cell-surface expression of the HA-SIP₄ receptor. The proportion of cells expressing the receptor was assessed using FACS analysis with a Fluorescein conjugated anti-HA antibody. The values shown above are the means of three independent determinations.

Fluorescence microscopy was used as an alternative method of showing induction of HA-SIP₄ expression as a dose-dependent response to the induction agent. Cells were cultured on microscope slides, in the presence of varying concentrations of ponasterone A, or vehicle control. Staining was performed with the nuclear stain DAPI, and a Fluorescein conjugate of the anti-HA antibody. Images of green fluorescence (due to anti-HA-Fluorescein) and blue fluorescence (due to DAPI) were viewed using a fluorescence microscope and the two images obtained for each sample were merged. These results (Figure 5.6) correlated well with the previously generated FACS data, although the fluorescence microscope was not as sensitive as the FACS method and this technique was less suitable for quantitation of the proportion of cells expressing HA-SIP₄ at a given concentration of ponasterone A. No staining with anti-HA-Fluorescein was observed when cells were cultured in vehicle control (Figure 5.6 (A)), however at the lowest concentration of ponasterone A used (10⁻⁶.⁵ M, Figure 5.6 (B)), a very small proportion of the cells had been stained with the antibody. As the concentration of ponasterone A was increased (Figure 5.6 (C)
Induction of cell-surface receptor expression by ponasterone A treatment of ecdysone-inducible HA-S1P₄-transfected CHO-EcR cells detected by fluorescence microscopy.

Cells were cultured in the presence of ponasterone A or DMSO vehicle control and analysed by fluorescence microscopy (x16 magnification) using a Fluorescein conjugate of the anti-HA antibody; cell nuclei were counterstained with DAPI. Data are representative of two such experiments performed.
and (D)), the percentage of cells fluorescing green increased and some cells were seen to fluoresce more brightly than other cells. As was observed using FACS analysis, less than 100% of cells were positive for HA-S1P₄ expression at the maximal concentration of ponasterone A used. This was in contrast to the constitutively expressing HA-S1P₄-transfected cell line described in chapter 4, where 100% of cells stained with anti-HA-Fluorescein (data not shown). No staining was seen when untransfected cells were used (Figure 5.6 (E)), or when a Fluorescein conjugate of an isotype control antibody was used (data not shown). Staining of cell nuclei with DAPI allowed visualisation of cells when there was no green fluorescence due to binding of the anti-HA-Fluorescein antibody. Since the cells were unfixed and unpermeabilised, staining with the anti-HA antibody reflected expression of cell-surface HA-S1P₄.

The time dependency of receptor expression in the HA-S1P₄-transfected cell line was examined using a single concentration of ponasterone A or muristerone A. Quantitation of the proportion of cells expressing HA-S1P₄ was performed in the same way as described for the dose-response experiments. Exposure of cells to 10⁻⁵.⁵ M each agent over a range of incubation times from 3 hours to 48 hours resulted in a time-dependent increase of HA-S1P₄ expression (Figure 5.7). The profiles of time-dependent induction obtained with ponasterone A and muristerone A were broadly similar. Exposure to the agent for less than 3 hours did not increase cell-surface expression of HA-S1P₄, as compared to the uninduced control whilst maximal expression levels were achieved after 34-48
Figure 5.7
Time-dependent induction of cell-surface receptor expression by ponasterone A and muristerone A treatment of ecdysone-inducible HA-S1P₄-transfected CHO cells.
Cells were incubated for varying times in serum-free medium supplemented with 10⁻⁵.₅ M ponasterone A (A), 10⁻⁵.₅ M muristerone A (B) or DMSO vehicle control (veh). Cell-surface receptor expression was quantified by FACS analysis using a Fluorescein conjugated anti-HA antibody. Quadrant analysis of dot plots was used to determine the percentage of cells expressing HA-S1P₄. The resultant data presented graphically, are the mean of duplicate determinations ± SEM from a single experiment and are representative of two independent experiments.

hours. As was observed when the concentration of induction agent was titrated, small populations of HA-S1P₄-expressing cells were detected in the uninduced
and vehicle controls. After 24 hours, approximately 25% of cells expressed HA-S1P<sub>4</sub> when induced with either ponasterone A or muristerone A. This was slightly lower than the proportion of cells assessed to be expressing HA-S1P<sub>4</sub> at $10^{-5.5}$ M of either agent in the dose-response experiments and may have been due to differences in cell density at the time of induction.

Since there was no significant difference with respect to the concentration- and time-dependency of HA-S1P<sub>4</sub> expression induced by each induction agent, subsequent experiments were performed using ponasterone A.

Immunoblots of membranes from cells stably transfected to inducibly express HA-S1P<sub>4</sub> contained a polypeptide with an apparent $M_r$ of 65 kDa, which reacted with anti-HA and anti-S1P<sub>4</sub> antibodies (Figure 5.8). It was possible to detect HA-S1P<sub>4</sub> protein in membranes prepared from cells treated with $10^{-4.5}$ M or $10^{-5.0}$ M ponasterone A, but not when lower concentrations of this induction agent were used. This presumably reflected the lower sensitivity of this technique, compared to analysis of expression by whole cells using FACS. The apparent mass of HA-S1P<sub>4</sub> expressed using the ecdysone-inducible system was slightly lower than that observed using the constitutively expressing HA-S1P<sub>4</sub> membranes (chapter 4, section 2.3). However, the apparent mass of HA-S1P<sub>4</sub> expressed using the ecdysone-inducible system was still greater than that predicted on the basis of amino acid sequence (43 kDa), suggesting that the small mass differences observed between expression systems were more likely due to post-translational variations, rather than proteolysis. Cross-reactivity with the anti-HA antibody confirmed the amino-terminus of the protein was intact. The
apparent molecular mass of HA-S1P₄ expressed using this system was found to be consistent over a number of clones tested (data not shown).

![Image of Western blot analysis](image)

**Figure 5.8**
Analysis of receptor expression in ecdysone-inducible HA-S1P₄-transfected CHO-EcR cells using Western blotting.

Membranes from untransfected CHO-EcR cells (Lane 1) and CHO-EcR cells transfected to inducibly express HA-S1P₄, and cultured in the presence of 10⁻⁵.⁵ M (Lane 2), 10⁻⁵ M (Lane 3), or 10⁻⁴.⁵ M ponasterone A (Lane 4) were analysed by Western blotting using anti-HA (panel I) or anti-S1P₄ (panel II) antibodies. Visualisation of immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with appropriate HRP-conjugated secondary antibodies. The position of HA-S1P₄ is indicated by arrow.

This characterisation work enabled conditions under which to culture cells for subsequent preparation of membranes and evaluation of receptor functionality in the [³⁵S]GTPγS binding assay to be chosen.

**5.2.3 The effect of cell-surface receptor expression level on HA-S1P₄ basal activity in [³⁵S]GTPγS binding assay**

In an initial experiment, transfected and parental cells were seeded in complete medium and cultured for 24 hours to allow cells to attach to the tissue culture plastic. The medium was removed, cells were washed and then cultured for 24 hours in serum-free medium supplemented with a range of concentrations of ponasterone A to induce expression of HA-S1P₄. For each concentration of
ponasterone A, two flasks of cells were prepared and cultured with or without 100 ng/mL pertussis toxin. Expression of cell-surface HA-S1P₄ was confirmed using FACS analysis before membranes were prepared. Basal and S1P-stimulated signalling was measured using the [³⁵S]GTPγS binding assay. The results from this single experiment (not shown) indicated that at no concentration of ponasterone A was constitutive activity of HA-S1P₄ observed, however the addition of 10 µM S1P could stimulate [³⁵S]GTPγS binding at the highest concentration of ponasterone A used (10⁻⁴.⁵ M). The ability of S1P to activate the receptor clearly confirmed its functionality but the lack of constitutive activity was unexpected. The induction of HA-S1P₄ expression in serum-free conditions was a consequence of the experimental design and resulted in very artificial circumstances under which the receptor was expressed. A further preliminary experiment was set up where cells were seeded in complete medium supplemented with 10⁻⁴.⁵ µM ponasterone A and cultured for 24 hours before being transferred to serum-free medium containing the same concentration of ponasterone A with and without pertussis toxin. Cells were cultured for 24 hours under these conditions before being harvested. This resulted in expression of HA-S1P₄ for 48 hours, the first 24 of which were in serum-containing conditions. Comparison of the basal and S1P stimulated activities of the 48 hour-induced cells with the original membranes from cells induced with 10⁻⁴.⁵ M ponasterone A for 24 hours in serum-free conditions in the [³⁵S]GTPγS binding assay showed that induction of protein expression in serum-containing medium conferred constitutive activity and increased S1P-induced stimulation of the receptor. The following conditions were therefore chosen for the induction of cells for assay of constitutive activity:
Concentrations of ponasterone A used: $10^{-5.5}$ M, $10^{-5.0}$ M, $10^{-4.5}$ M

Cells seeded in complete medium containing appropriate concentration of ponasterone A and cultured for 24 hours

Medium removed, cells washed and cultured in serum-free medium containing appropriate concentration of ponasterone A, in the presence or absence of 100 ng/mL pertussis toxin for 24 hours. Cells harvested and membranes prepared for use in $[^{35}S]GTP\gamma S$ binding assay.

HA-S1P$_4$-transfected and parental CHO-ECR cells were cultured under these conditions and the resultant membranes used to investigate HA-S1P$_4$ constitutive activity. Increased receptor expression caused by incubation with increased concentrations of ponasterone A resulted in increased basal activity of the membranes (Figure 5.9). Pertussis toxin was used to illustrate the extent of this constitutive activity since the level of $[^{35}S]GTP\gamma S$ binding observed with membranes prepared from pertussis toxin-treated cells was the same regardless of the level of receptor expression.

The level of constitutive activity observed at the highest level of HA-S1P$_4$ expression was less than that typically observed with the constitutively expressing cell lines characterised in chapter 4. This was not entirely unexpected, since the level of receptor expression achieved with the highest concentration of ponasterone A was still lower than that of the constitutively expressing cell line.
Figure 5.9
Constitutive activity of HA-SIP4 expressed in ecdysone-inducible CHO-EcR cells.
Membranes were prepared from parental or HA-SIP4-transfected ecdysone-inducible CHO-EcR cells, which had been cultured in the presence of varying concentrations of ponasterone A (PonA) or vehicle (veh), and in the absence (- PTx) or presence (+ PTx) of 100 ng/mL pertussis toxin for 24 hours prior to harvest. The membranes were incubated for 5 minutes at 30 °C with $[^{35}S]$GTPγS and G protein α subunits immunoprecipitated after solubilisation and preclearance with non-immune serum. Data presented are the mean of three determinations ± SEM from a single experiment and are representative of two such experiments performed. Statistical significance from the response of non pertussis toxin-treated vehicle control HA-SIP4 cells is denoted by ** (P < 0.01).

It was necessary to show that the basal activity of HA-SIP4 was a consequence of the level of expression of this receptor and not due to the effects of residual ponasterone A present in the membrane preparations, although this seemed unlikely since cells were washed thoroughly before being harvested. Membranes from CHO-K1 cells stably expressing HA-SIP4 (described in chapter 4) were tested in the $[^{35}S]$GTPγS binding assay to determine whether the maximal concentration of this agent ($10^{-4.5}$ M) used for induction of HA-SIP4 expression in the ecdysone-inducible cell line stimulated the receptor. Ponasterone A did
not stimulate $^{[35}S]GTP\gamma S$ binding in HA-S1P$_4$-expressing membranes (Figure 5.10). Hence the effect of cell-surface HA-S1P$_4$ expression level on basal $^{[35}S]GTP\gamma S$ binding appeared to reflect genuine constitutive activity of the receptor and was not due to stimulation by residual ponasterone A.

![Figure 5.10](image)

**Constitutive activity of HA-S1P$_4$ is not influenced by the presence of ponasterone A.**

Membranes from HA-S1P$_4$-transfected CHO-K1 cells were incubated for 5 minutes at 30°C with $^{[35}S]GTP\gamma S$ with 0.1% fatty acid free BSA (basal), DMSO control (vehicle), $10^{-4.5}$ M ponasterone A (PonA) or $10^{-5}$ M S1P. G protein $\alpha$ subunits were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data presented are the mean of three determinations ± SEM. Statistical significance from the basal response is denoted by ** ($P < 0.01$).

### 5.2.4 The effect of cell-surface receptor expression level on S1P-stimulated HA-S1P$_4$ activity in $^{[35}S]GTP\gamma S$ binding assay

Membranes, which had been used in section 5.2.3 to characterise the effect of receptor expression on constitutive activity, were then used to investigate the relationship between the expression level of HA-S1P$_4$ and S1P-mediated increases in $^{[35}S]GTP\gamma S$ binding (Figure 5.11).
Figure 5.11
S1P-stimulated activity of HA-SIP₄ expressed in ecdysone-inducible CHO-EcR cells.
Membranes from parental or HA-SIP₄-transfected ecdysone-inducible CHO-EcR cells, which had been cultured in the presence of varying concentrations of ponasterone A (PonA) or vehicle (veh) were incubated with [³⁵S]GTPγS for 5 minutes at 30 °C in the absence (basal) or presence (stimulated) of 10 µM S1P. G protein α subunits were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data presented are the mean of three determinations ± SEM from a single experiment and are representative of two such experiments performed. Statistical significance from the S1P-stimulated response of vehicle control HA-SIP₄ cells is denoted by * (P < 0.05) or ** (P < 0.01).

Treatment of membranes with 10 µM S1P was found to promote increased [³⁵S]GTPγS binding when compared to both the level of basal activity of those membranes, and to membranes prepared from vehicle-treated HA-SIP₄-transfected cells. The extent of [³⁵S]GTPγS binding induced by S1P increased with receptor expression, with maximal activation being obtained at the highest concentration of ponasterone A that was used. S1P was also found to cause a small stimulation of [³⁵S]GTPγS binding in untransfected CHO-EcR membranes, although this increase was not statistically significant in every experiment. Such a stimulation was not observed when parental CHO-K1 membranes were
exposed to 10 μM S1P in experiments described in chapter 4 and may suggest that the pattern, or levels, of expression of endogenous S1P receptors in CHO-EcR cells differed slightly from that of CHO-K1 cells.

5.2.5 Correlation analysis of HA-S1P4 constitutive and S1P-stimulated [35S]GTPγS binding activity with the level of HA-S1P4 cell-surface expression

The relationship between the number of cells expressing HA-S1P4, determined using the criteria described in this chapter, section 2.2, and the activity of the receptor in the [35S]GTPγS binding assay, measured in this chapter sections 2.3 and 2.4, was examined. Data were corrected by subtraction of basal dpm obtained using membranes prepared from cells which had been treated with pertussis toxin such that the resultant value solely represented the activity of HA-S1P4. Figure 5.12 shows these data and the linear regression analysis performed on these data sets.

Both constitutive and S1P-stimulated [35S]GTPγS binding attributed to the presence of HA-S1P4 were found to correlate strongly (Pearson’s $r^2 > 0.96$) with the proportion of cells expressing the receptor; each relationship showed a good approximation to linearity ($r^2 > 0.93$) and the correlation result was statistically significant ($P < 0.001$). A summary of this analysis is shown in Table 5.2.

<table>
<thead>
<tr>
<th></th>
<th>Constitutive activity</th>
<th>S1P-stimulated activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity ($r^2$)</td>
<td>0.9346</td>
<td>0.9537</td>
</tr>
<tr>
<td>Correlation (Pearson’s $r^2$)</td>
<td>0.9667</td>
<td>0.9766</td>
</tr>
<tr>
<td>Significance</td>
<td>$P &lt; 0.0001$</td>
<td>$P &lt; 0.0001$</td>
</tr>
</tbody>
</table>

Table 5.2
Summary of statistical analyses performed on data describing the relationship between the percentage of cells expressing HA-S1P4 and [35S]GTPγS binding attributable to the presence of this receptor.
Figure 5.12
Correlation of HA-SIP₄ constitutive- and S1P-stimulated activity from membranes prepared from ecdysone-inducible CHO-EcR cells with percentage of cells deemed to express HA-SIP₄.
Data previously obtained for basal and S1P-stimulated signalling of membranes prepared from the HA-SIP₄ ecdysone-inducible cell line, which had been cultured in the presence of varying concentrations of ponasterone A or DMSO vehicle, and the % cells expressing HA-SIP₄ determined using FACS analysis with a Fluorescein conjugated anti-HA antibody, were corrected with respect to [³⁵S]GTP₇S bound for samples treated with PTx and plotted. Data plotted are from two independent experiments and are the mean of triplicate determinations ± SEM.
5.3 Discussion

The ecdysone-inducible expression system was used to further investigate the nature of basal and S1P-mediated activity of the HA-S1P₄ receptor. This system exploits the role of steroid receptors as transcription factors, the activity of which is tightly regulated by the binding of the associated steroid ligand, and uses the non-mammalian *Drosophila* ecdysone receptor as a way to achieve a tightly regulated expression system that will not suffer interference from endogenous mammalian steroids.

Since a ligand binding assay was not available for S1P₄, FACS analysis was chosen as the method of quantifying the extent of receptor expression. Flow cytometry is a sensitive technique (reviewed by Nolan *et al.*, [1999]) and the inclusion of the HA tag at the amino-terminus of the S1P₄ sequence facilitated detection of cell-surface expressed S1P₄ (shown previously in chapter 4). Quadrant analysis of FACS dot plots allowed receptor expression level to be determined and is a technique which has been used previously [Cole *et al.*, 2001]. However, this method was limited to a qualitative assessment of the percentage of cells expressing the protein of interest. Although no further information regarding the level of expression could be extracted from these data, it was straightforward to compare receptor expression caused by different concentrations of induction agent. An alternative approach might have been to measure relative receptor levels using an ELISA-based assay on fixed cells [Schoneberg *et al.*, 1995]; however this method was not tested.

Expression of cell-surface HA-S1P₄ was dependent on the concentration and the time of exposure to induction agent (ponasterone A or muristerone A). In the
absence of inducing agent, low levels of cell-surface receptor were observed. The ecdysone-inducible system has been used to express other GPCRs. In one such study low levels of the dopamine D2 receptor were detected using radioligand binding assay in uninduced cells [Choi et al., 2000], whilst Cole et al. [2001] found that expression of mRNA and protein for the somatostatin SST2 receptor in this system was undetectable in the absence of inducing agent.

Ponasterone A and muristerone A were found to have very similar half-maximal concentrations for dose-dependent induction of cell-surface HA-S1P4 expression and the maximal levels of expression achieved using these agents were comparable. It was not possible to induce HA-S1P4 expression in 100 % of cells; this is in agreement with a previous report where less than 50 % of transfected CHO-K1 cells were found to express the SST2 receptor using a maximally effective concentration of muristerone A [Cole et al., 2001]. In this study, a proportion of the SST2-positive cells were collected using FACS and cultured before re-induction of expression. The fraction of cells expressing this receptor decreased from 100 % to approximately 65 % over a four week period. The reason for this instability is not known, but was suggested by the authors to be unrelated to cell cycle events.

Expression of HA-S1P4 was found to reach a maximum after 48 hours exposure to ponasterone A, or after 34 hours using muristerone A. A published study with the SST2 receptor showed maximal expression after 24 hours incubation with muristerone A [Cole et al., 2001] which declined after 40 hours. Invitrogen report that the half-life of ponasterone A in culture is not known, however they observe a 30 % decrease in expression 48 hours after induction, and a 70 %
decrease after 72 hours. Muristerone A is reported to have a half-life of 24 hours. The results obtained in this chapter differ from Invitrogen’s observations since HA-S1P4 expression in response to ponasterone A was maximal after 48 hours and may suggest that temporal dependency of protein expression is related to the half-life of that protein, as well as the half-life of the induction agent in culture.

Treatment of HA-S1P4 transfected cells with ponasterone A or muristerone A induced expression of cell-surface HA-S1P4 in a population of those cells. At a given concentration of induction agent, FACS analysis detected a range of HA-S1P4 expression levels. These ranged from low levels, which corresponded to a small shift into the lower right quadrant of the dot plot, to higher levels, which corresponded to a larger shift in the dot plot. Similar heterogeneity has been reported previously for this expression system [Cole et al., 2001].

Fluorescence microscopy was also used to detect cell-surface expression of HA-S1P4 and the results compared well with those generated using FACS analysis. The Fluorescein conjugated anti-HA antibody was found to give the best results with this technique. The anti-S1P4 antibody recognises an intracellular epitope and preliminary experiments using permeabilised cells found this antibody stained less specifically than the anti-HA antibody, and also showed low levels of staining in the nuclear region of untransfected cells (data not shown).

Membranes were prepared from cells treated with different concentrations of ponasterone A, with or without pertussis toxin, then constitutive and S1P-induced activity was measured using the [35S]GTPγS binding assay. In an initial experiment, protein expression was induced for 24 hours in serum-free
conditions. At none of the concentrations of ponasterone A that were used was it possible to detect S1P4 constitutive activity and stimulation of the receptor by S1P was only observed using membranes expressing the highest level of S1P4. It was postulated that this may have been a consequence of the use of serum-free conditions to express the receptor, so S1P4 expression was induced using a variety of ponasterone A concentrations, in serum-containing medium for 24 hours. These cells were then cultured in serum-free medium, containing the appropriate concentration of ponasterone A in the presence and absence of pertussis toxin. Membranes prepared from these cells were shown to be constitutively active at all concentrations of ponasterone A that were used, and this activity could be further stimulated by addition of 10 µM S1P. It is unlikely that the apparent constitutive activity of the receptor resulted from stimulation of S1P4 by residual S1P since cells were washed thoroughly after removal of serum-containing medium prior to addition of serum-free medium for the final 24 hour culture period and were also washed thoroughly prior to harvest. It is unclear why expression using these modified conditions was necessary for the receptor to exhibit constitutive activity (and to respond to S1P when sub-maximal concentrations of ponasterone A were used) and the levels of cell-surface receptor detected using these different culture conditions were essentially identical. A key difference was the time for which protein expression was induced: 48 hours when expression was started in serum-containing medium compared with 24 hours when serum-free conditions were used. Immunoblot analysis detected no differences in apparent mass when HA-S1P4 was expressed in serum-free conditions, compared to when expression was induced in serum-containing medium and then completed in serum-free conditions (data not
shown). A further possibility to consider is that culture of these cells in the presence of serum sustains the expression of an additional factor that is required for the detection of constitutively active S1P4 receptor. Further investigations are necessary to determine the reason for the disparity of receptor activity between expression conditions, however it should be noted that it is more physiologically relevant to express a receptor in serum-containing conditions since S1P4 expressed \textit{in vivo}, for instance on a circulating T cell, would be exposed to serum continuously.

In the $[^{35}S]$GTP\(_\gamma\)S binding assay, basal activity increased from approximately 4000 dpm, obtained with untransfected and vehicle treated HA-S1P4-transfected cells, to greater than 8000 dpm for cells treated with the highest concentration of induction agent. Increasing receptor expression was also shown to correlate with S1P-mediated activity. No HA-S1P4 activity (constitutive- or agonist-stimulated) was observed when membranes prepared from vehicle treated cells were used, even though these cells expressed low levels of S1P4. This may be a reflection of the sensitivity of the GTP\(_\gamma\)S binding assay or be due to a threshold effect whereby S1P4 is not constitutively active until sufficient receptor is expressed to allow dimerisation or clustering; however further investigations would be needed to confirm if this were the case.

The results obtained in this chapter may be compared with those generated using the constitutively expressing HA-S1P4-transfected cell line presented in chapter 4. Treatment of the ecdysone-inducible HA-S1P4-transfected cell line with $10^{-4.5}$ M ponasterone A resulted in high levels of cell-surface expression of HA-S1P4, although less than 100 % of cells expressed the receptor as determined
using FACS analysis. Basal, or constitutive, activity observed when $10^{-4.5}$ M ponasterone A was used to treat cells was approximately 7300 dpm, and that obtained after stimulation with 10 μM SIP was approximately 15500 dpm. Values obtained using the constitutively expressing HA-S1P₄ cell line were approximately 10000 dpm for basal activity and approximately 19500 dpm for activity stimulated by 10 μM SIP. Therefore, the extent of activation exhibited by the ecdysone-inducible HA-S1P₄-transfected cell line treated with the highest concentration of ponasterone A compares favourably to that obtained with the constitutively expressing cell line, despite the fact that less than 100% of cells expressed the receptor in the inducible cell line.

Both basal and SIP-stimulated S1P₄ activity were found to show a linear relationship with the percentage of cells expressing the receptor. The activity attributable to constitutive or agonist-mediated activation of S1P₄ was calculated by subtracting the mean $[^{35}S]GTP\gamma S$ binding measured using membranes prepared from cells treated with pertussis toxin from the total $[^{35}S]GTP\gamma S$ binding obtained using membranes prepared from cells which had not been pertussis toxin treated. The accumulation of $[^{35}S]GTP\gamma S$ in membranes prepared from pertussis toxin treated cells is not unexpected. Although ADP-ribosylation of Gα₄ G proteins with pertussis toxin prevents interaction of GPCR and G protein, it does not alter the intrinsic G protein functions such as guanine nucleotide exchange and GTPase activity [Gierschik, 1992]. Given that cells were categorised as expressing HA-S1P₄ if they were detected in the lower right quadrant of the FACS dot plot, and that there was no measurement of the level of receptor expression, the linearity of this relationship is intriguing. It tends to
suggest that the shape of the distribution of the extent to which cells express HA-S1P₄ is maintained and that application of ponasterone A acts to alter this population such that there is a progressive shift in the peak of this distribution, and in the size of the population in relation to the concentration of the inducer (shown as a model in Figure 5.13).

![Figure 5.13](image)

**Figure 5.13**
*Model to represent the effect of ponasterone A concentration on cell-surface expression of HA-S1P₄ in ecdysone-inducible CHO-EcR cells.*

The population of ecdysone-inducible cells defined as expressing HA-S1P₄ using FACS analysis may be represented by a bell shaped curve and the area under the curve corresponds to the number of cells within this population. As the concentration of the induction agent ponasterone A is increased, expression of cell-surface HA-S1P₄ is increased and so the location of the population is shifted to the right. Concurrently, the area under the curve increases, reflecting increased number of cells within this population.

This investigation has shown that S1P₄ is constitutively active when expressed in CHO cells. The observed relationship between receptor expression level and constitutive activity strongly suggests that the ability of S1P₄ to signal in the absence of agonist stimulation is an inherent property of the receptor rather than merely arising due to receptor clustering or oligomerisation, which may have been a consequence of high levels of expression in a heterologous system. Constitutive activity of the S1P₄ receptor was shown to be related to expression
level and was not a consequence of stimulation of the receptor by residual ponasterone A. Treatment of membranes prepared from CHO-K1 cell transfected to constitutively express HA-SIP4 with ponasterone A did not increase $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding.

Increased levels of receptor expression are expected to lead to increased constitutive activity and indeed this has been shown for the SIP4 receptor. The seminal study of GPCR constitutive activity [Samama et al., 1993] showed the existence of a linear relationship between expression level and basal activity for both the wild type and constitutively active mutant of $\beta_2$-AR, the difference being that this function was much steeper for the mutant receptor compared with the wild type. Other studies which examined the effect of expression level on basal activity of wild type receptors also showed that increased expression resulted in enhanced constitutive activity [Cohen et al., 1997; Casarosa et al., 2001]. It may have been feasible to further investigate the relationship between expression level and basal activity for SIP4 had it been possible to use a radioligand binding assay to determine absolute levels of SIP4 expression in membranes used for the functional assay. However, since it is not known at what levels SIP4 is expressed in a physiological setting (e.g. T cells), it is difficult to suggest whether the constitutive activity observed using this artificial system is relevant in vivo.

Stimulation of SIP4 was shown using a single concentration of SIP (10 μM) and the extent of activation in response to this maximal concentration of agonist was related to expression level. It was not practical to determine whether agonist EC50 values altered with increased receptor expression, however classical
receptor theory predicts that increasing receptor number would initially increase the maximal response to an agonist, without a change in potency; once that maximal response has been reached, further increases in receptor density are predicted to increase agonist potency, without affecting maximal potency. These predictions showed good agreement with experimental studies of the mGlu1α receptor (using an IPTG inducible expression system [Hermans et al., 1999]) and of the 5-HT2B receptor (using sodium butyrate treatment [Jerman et al., 2001]).

Other SIP receptors have been found to be constitutively active. In a landmark study using the baculovirus expression system, constitutive activation of Goi G protein by S1P1/3 and Goi12/13 G proteins by S1P2 was observed [Windh et al., 1999]. A recent publication describing agonist regulated and constitutive activation of specific signalling pathways by S1P5 [Niedernberg et al., 2003a] found this receptor constitutively inhibited adenylyl cyclase and activation of ERK, but that application of exogenous S1P did not further stimulate these responses. In contrast, S1P5 mediated cell rounding via the Go12/13 pathway was found to be an intrinsic ability of the receptor, which was further modulated by S1P. These data were proposed to suggest that when a receptor is constitutively active, and is known to couple to multiple G proteins, that different effector systems may be activated to different extents.

The cannabinoid (CB) receptors are closely related to the SIP receptors and have also been reported to exhibit constitutive activity when expressed in CHO cells [Bouaboula et al., 1997; Bouaboula et al., 1999]. Similarly, the LPA1 receptor was shown to activate Goi G proteins in the absence of ligand stimulation using a baculovirus expression system [Yoshida and Ueda, 1999]. Ishii et al., [2000],
also reported constitutive activity of LPA\textsubscript{1} and LPA\textsubscript{2} receptors. The recently identified second family of SIP receptors, GPR3, 6 and 12 are closely related to the Edg and CB receptor families, are constitutively active, and lack the generally conserved cysteine residue in the first extracellular loop [Uhlenbrock \textit{et al.}, 2002]. The melanocortin (MC) receptor family comprises five members and, like the SIP and CB receptors, also lack the cysteine residue in the first extracellular loop which is generally present in family A GPCRs. This receptor family has also been shown to be constitutively active and interestingly the ligands agouti and agouti-related protein have been identified as endogenous inverse agonists at the MC receptors [Nijenhuis \textit{et al.}, 2001].

Although the precise structural and molecular changes which correspond to receptor constitutive activity are not known, it has been suggested that conformational flexibility may contribute to this phenomenon. A study of the \( \beta_2 \text{AR} \) showed that a constitutively active mutant of this receptor possessed greater structural instability than the wild type receptor [Gether \textit{et al.}, 1997]. There appears to be evolutionary pressure for most GPCRs to reside in the inactive conformation. Two striking examples are the \( \alpha_{\text{IBAR}} \) and rhodopsin. In a landmark study of the \( \alpha_{\text{IBAR}} \), mutation of A\textsuperscript{293} to any other amino acid conferred a constitutively active phenotype to this receptor [Kjelsberg \textit{et al.}, 1992]. The photoreceptor rhodopsin is maintained in an inactive conformation via covalent binding of the ligand retinal within the binding pocket and as a consequence the visual system is not subject to photobleaching that would result from constitutive activity of this receptor.
The basis for the constitutive activity observed using the S1P₄ receptor is likely to be common to the S1P, CB and MC receptor families. The unique property of these family A receptors is the lack of the generally conserved cysteine residue present in the first extracellular loop. This residue forms a disulphide bond with a second cysteine residue present in the second extracellular loop, which probably serves to constrain the transmembrane helix bundle. Since movement of transmembrane helices, particularly TM III and TM VI has been implicated in GPCR activation [Meng and Bourne, 2001], greater mobility of the helices could conceivably result in receptor constitutive activity. This notion is reinforced by observations that culture of constitutively active GPCRs with agonists and inverse agonists increases cell-surface expression of the receptor. This “ligand rescue” of constitutively active mutant receptors presumably reflects stabilisation of the receptor conformation by the binding of the ligand [Milligan et al., 2002]. Conceptually, it would be interesting to examine the effect of the removal of the cysteine residue in the first extracellular loop of a typical family A receptor. In practice however, interpretation of such an experiment may be challenging since removal of this residue by mutagenesis has been reported to prevent transport of the modified receptor to the cell-surface [Ai and Liao, 2002]. An alternative approach might be to investigate whether the inclusion of a cysteine residue within the first extracellular loop attenuated S1P₄ constitutive activity. This could be achieved using a sequence alignment of family A receptors to identify the position of this cysteine residue and mutation of the appropriate residue in the S1P₄ receptor.

Expression of constitutively active mutants of GPCRs has often been reported to be lower than that of their wild type counterparts. In contrast, expression of high
levels of cell-surface S1P₄ was easy to achieve which may represent a fundamental difference between receptors that possess significant constitutive activity in their wild type state as compared to receptors which are constitutively active as a consequence of mutation.

It appears that receptor constitutive activity may be a relevant pharmacological property of certain GPCRs. The viral receptors ORF74 and US28 are constitutively active [Casarosa et al., 2001] and the histamine H₃ receptor has been shown to be constitutively active in situ in rodent brain [Morisset et al., 2000] and is thought to control histaminergic neuron activity in vivo. The previously discussed observation that the three receptor families which lack the cysteine residue in the first extracellular loop (S1P, CB and MC) are constitutively active lends weight to this concept. The discovery of constitutively active wild type GPCRs begs the question of whether physiological systems expressing such receptors also produce endogenous inverse agonists, which would inactivate their signalling. This has already been shown for the MC receptor family. If this was found to be the case for all constitutively active wild type GPCRs it would be an elegant symmetry of the classical system, in which endogenous agonists activate wild type receptors.

S1P₄ constitutive activity has been shown to be related to expression level and was not merely a consequence of the very high levels of expression which are commonly achieved using recombinant systems. It is unclear whether this receptor is constitutively active in its native environment, and if so, whether this property is central to the function of the S1P₄ receptor and is controlled by the actions of an as yet undiscovered inverse agonist.
Chapter 6 – Investigation into S1P₄ Ligand Preference

6.1 Introduction

It was shown in chapter 4 that S1P₄ coupled to Gαᵣ G proteins and could be activated by the lysophospholipid S1P. Although S1P does function an agonist of this receptor, published reports have shown that S1P binds and activates S1P₄ poorly compared to its actions at other S1P receptors [Brinkmann et al., 2002; Mandata et al., 2002]. It has also been suggested that S1P may not be the true endogenous agonist of S1P₄ [Lynch and Im, 1999].

Significant progress has been made in the field since the start of work detailed in this thesis. Recent publications have described the actions of phosphoryl metabolites of the novel immunomodulatory compound FTY720, and its analogue AAL, as potent agonists some of the S1P receptors, including S1P₄ [Brinkmann et al., 2002; Mandala et al., 2002]. Additionally, the compound phytosphingosine-1-phosphate (phS1P) has been identified as a high affinity agonist for the S1P₄ receptor. A published study [Rios Candelore et al., 2002] found that this compound, which is structurally similar to S1P, binds S1P₄ with approximately 50-fold higher affinity than S1P and suggested that phS1P may represent the true endogenous agonist for the S1P₄ receptor.

The first aim of this chapter was to determine the potency of these recently identified putative endogenous and synthetic S1P₄ receptor agonists using the [³⁵S]GTPγS binding assay.

Recently, efforts have been made to understand how the S1P and LPA receptor families discriminate between their structurally similar ligands. Due to their
highly hydrophobic nature, direct structural studies of GPCRs are challenging, so the technique of mutagenesis is often employed in the characterisation of GPCR structure-function relationships. Alignment of the sequences of GPCRs from related families allows identification of residues which are highly conserved within receptor subfamilies, but which differ between different receptor subfamilies. This approach was adopted with the Edg family of GPCRs, where sequences of the LPA and S1P subfamilies were compared [Parrill et al., 2000a; Parrill et al., 2000b; Wang et al., 2001; Bautista et al., 2002]. A single residue in transmembrane domain III was identified as potentially influencing the ligand selectivity exhibited by each receptor subfamily. Computational modelling of S1P1 and LPA1 receptors, performed by Parrill and co-workers showed a probable interaction between this residue and structurally distinct groups present on the two lysophospholipid ligands, S1P and LPA. In the S1P receptor subfamily, this residue, glutamic acid (E121) was proposed to form an ion-pairing interaction with the positively charged ammonium group of S1P; in the LPA receptor subfamily, the glutamine (Q125) residue which is present at this position was believed to hydrogen bond with a hydroxyl group on LPA [Wang et al., 2001].

The second aim of the work described in this chapter was to extend this hypothesis to investigate the role of this position in the S1P4 receptor by mutation of the naturally occurring glutamic acid to glutamine and subsequent assessment of the ability of the ligands S1P and LPA to activate this E122Q S1P4 mutant. Although the published study used RH7777 cells as a host, it was decided that the E122Q S1P4 mutant should be expressed in CHO-K1 cells. This host had been successfully used in earlier studies (described in chapter 4) and
preliminary work undertaken at the start of this thesis had been unsuccessful in detecting S1P₄-mediated increases in [³⁵S]GTPγS binding using S1P₄-transfected RH7777 cells.

It was shown in chapter 3 that CHO-K1 cells endogenously express the LPA₁ receptor, and preliminary experiments (not shown) showed that LPA stimulated [³⁵S]GTPγS binding in parental CHO-K1 cells. A fusion protein between HA-S1P₄(E¹²²Q) and the pertussis toxin-insensitive Gα₁₁(C³⁵¹I) G protein was therefore used for these studies. Expression of this protein in CHO-K1 cells followed by treatment with pertussis toxin prior to harvest allowed elimination of any signal due to stimulation of endogenous LPA receptors. McAllister et al., [2000], adopted a similar approach for studies of the LPA₁ receptor. The ability of wild type S1P₄ to couple to a tethered, pertussis toxin-insensitive Gα₁ G protein was previously shown in chapter 4.

Unlike S1P, which exists as a single species in vivo, the term LPA actually refers to a family of molecules that take the general form 1-o-acyl-2-hydroxy-sn-glyceryl-3-phosphate. Naturally occurring forms of LPA contain acyl chains of differing lengths, with differing degrees of saturation (see Figure 6.1 for examples). Structure-activity relationship studies have examined the potency of some of the various naturally occurring forms of LPA and synthetic LPA analogues as LPA receptor agonists [Bandoh et al., 1999; Bandoh et al., 2000; Im et al., 2000b; Heise et al., 2001; Tigyi, 2001]. The length and saturation of the LPA acyl group has been shown to influence potency at isolated LPA receptors [Bandoh et al., 1999; Im et al., 2000b] with the general trend of decreasing potency with decreasing acyl chain length (18:1 ≥ 16:0 > 14:0).
Chapter 6

Figure 6.1
Structures of three naturally occurring forms of LPA.
A 18:1 LPA (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate)
B 16:0 LPA (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphate)
C 14:0 LPA (1-myristoyl-2-hydroxy-sn-glycero-3-phosphate)

Comparison of space-filling models of the structures of S1P and these analogues of LPA (shown in Figure 6.2) revealed that the acyl chains of 18:1 and 16:0 LPA were longer than that of S1P. 14:0 LPA most closely resembled S1P in terms of apparent length, which suggested that 14:0 LPA might be a more effective agonist of the mutant S1P4 receptor than 18:1 or 16:0 LPA.

The final aim of this chapter was to assess how the length of the hydrocarbon tail present on the LPA molecule affected its ability to stimulate the HA-S1P4(E122Q) receptor.
Chapter 6

Figure 6.2
Models of LPA analogues and S1P.
Space-filling models which represent the minimised extended conformation of each structure were constructed using SYBYL 6.9 software (Tripos Inc., St. Louis, MO., U.S.A.). The distance between phosphorus and terminal carbon atoms was predicted for each structure.

<table>
<thead>
<tr>
<th></th>
<th>Predicted distance between P and terminal C atom</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>18:1 LPA</td>
</tr>
<tr>
<td></td>
<td>27.0 Å</td>
</tr>
<tr>
<td>B</td>
<td>16:0 LPA</td>
</tr>
<tr>
<td></td>
<td>26.7 Å</td>
</tr>
<tr>
<td>C</td>
<td>14:0 LPA</td>
</tr>
<tr>
<td></td>
<td>24.2 Å</td>
</tr>
<tr>
<td>D</td>
<td>S1P</td>
</tr>
<tr>
<td></td>
<td>24.0 Å</td>
</tr>
</tbody>
</table>
6.2 Results

6.2.1 Activation of HA-S1P<sub>4</sub> by phS1P in [<sup>35</sup>S]GTPγS binding assay

Recently, the lysophospholipid phytosphingosine-1-phosphate (phS1P) was described as a high affinity agonist for the S1P<sub>4</sub> receptor [Rios Candelore et al., 2002]. Its potency in the [<sup>35</sup>S]GTPγS binding assay was determined using membranes prepared from HA-S1P<sub>4</sub>-transfected cells (Figure 6.3). Treatment with this agonist promoted a dose-dependent increase in [<sup>35</sup>S]GTPγS binding with an EC<sub>50</sub> of 40 nM ± 8.2 nM (n=3).

![Graph showing concentration dependent stimulation of HA-S1P<sub>4</sub>-mediated [<sup>35</sup>S]GTPγS binding by phS1P.](image)

**Figure 6.3**

**Concentration dependent stimulation of HA-S1P<sub>4</sub>-mediated [<sup>35</sup>S]GTPγS binding by phS1P.**

Membranes from CHO-K1 cells transfected with HA-S1P<sub>4</sub> were incubated with varying concentrations of phS1P for 5 minutes at 30 °C in the [<sup>35</sup>S]GTPγS binding assay. Gα<sub>i</sub>, G proteins were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data presented are the mean of three determinations ± SEM from a single experiment and are representative of three such experiments performed.
6.2.2 **Inactivity of FTY720 and AAL compounds as HA-S1P₄ receptor agonists in [³⁵S]GTPγS binding assay**

The immunomodulatory compounds FTY720 and AAL have been shown to promote lymphopenia *in vivo*. This is believed to be due to the action of their phosphoryl metabolites, which have been identified as potent agonists at four of the five S1P receptors [Brinkmann *et al.*, 2002; Mandala *et al.*, 2002]. In contrast, the parent FTY720 and AAL compounds reportedly do not possess significant activity as S1P receptor agonists. These parent compounds were tested in the [³⁵S]GTPγS binding assay to determine whether they exhibited agonist activity at the S1P₄ receptor (Figure 6.4). Over the range of compound concentrations tested, neither compound stimulated a statistically significant increase in [³⁵S]GTPγS binding when compared to the basal response. Hence these compounds were considered to be inactive at the S1P₄ receptor.

6.2.3 **In vitro phosphorylation of FTY720 and AAL compounds via incubation with rabbit blood**

Phosphoryl metabolites of the compounds FTY720 and AAL (FTY720-P and AAL-P) have been reported to act as potent agonists of certain S1P receptors, including S1P₄ [Brinkmann *et al.*, 2002; Mandala *et al.*, 2002]. *In vitro* phosphorylation of these compounds may be achieved by incubation with purified sphingosine kinase 1 enzyme or with whole blood from species including rabbit, mouse and human. Parent compounds were synthesised by the Medicinal Chemistry department of Celltech R&D Ltd., and attempts made to convert each compound to the phosphate metabolite via incubation with whole rabbit blood, using a method based on that reported previously [Mandala *et al.*, 2002].
FTY720 and AAL compounds are unable to stimulate HA-S1P$_4$-mediated [$^{35}$S]GTP$_{y}$S binding.

Membranes from CHO-K1 cells transfected with HA-S1P$_4$ were stimulated with varying concentrations of FTY720 or AAL compounds for 5 minutes at 30 °C in the [$^{35}$S]GTP$_{y}$S binding assay. G$_{\alpha_i}$ G proteins were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment and are representative of two such experiments performed.

Incubations of varying concentrations of each compound were performed at 37 °C using undiluted whole rabbit blood, or whole rabbit blood that had been diluted 1 in 4 with supplemented RPMI medium. Samples were removed after various timepoints and the percentage conversion to phosphate derivatives determined using liquid chromatography-mass spectrometry (LC-MS). The profile obtained for FTY720 (Figure 6.5 (A)) showed time-dependent phosphorylation of this compound, with a maximal conversion of approximately 70 % achieved after overnight incubation with an initial concentration of 10 µg/ml FTY720 in undiluted blood. In contrast, the same concentration of compound incubated with diluted blood had only undergone approximately 20 % conversion after this time. The related AAL compound showed a rather different
profile of conversion to AAL-P (Figure 6.5 (B)). The maximal level of conversion was achieved using an initial concentration of 1 μg/ml AAL and was approximately 30 % after incubation for 4 hours.

Figure 6.5
Conversion of FTY720 and AAL compounds to phosphate metabolites via *in vitro* incubation with whole rabbit blood.
Compounds were prepared in ethanol and the concentrations shown incubated at 37 °C with whole rabbit blood that had been diluted 1 in 4 with supplemented RPMI medium, or with undiluted blood. Conversion of parent compound to the phosphoryl metabolite was monitored using liquid chromatography-mass spectrometry (LC-MS).
A  FTY720
B  AAL
These results allowed identification of optimal conditions to try to produce sufficient FTY720-P and AAL-P to allow isolation and purification of these phosphate metabolites. These compounds would then be tested in the \[^{35}\text{S}]\text{GTP}_{\gamma}\text{S} binding assay to determine their potency against the HA-SIP4 receptor. Conditions were chosen using the absolute amount of phosphate produced, rather than merely the most efficient conversion in terms of percentage, so as to minimise the amount of rabbit blood required. Therefore, for FTY720, although incubation of 1 \(\mu\text{g/ml}\) compound overnight with diluted blood gave the highest percentage of conversion, optimal conditions for the preparative experiment were 10 \(\mu\text{g/ml}\) in diluted blood, incubated overnight. The same conditions were chosen for the preparative phosphorylation of the AAL compound.

Due to the scale of the planned preparative phosphorylation experiments, an efficient method for extraction of phosphate from the diluted blood was required. It was necessary to process the sample mix to remove proteins and to reduce the volume so that it could be injected onto a high performance liquid chromatography (HPLC) column so that the phosphate metabolite of each compound could be isolated. Attempts were made to optimise extraction of the metabolites into the organic phase of a dichloromethane (DCM) extract of the sample mix. These included testing whether an initial extraction into acetonitrile was beneficial, comparison of different ratios of DCM to sample and the effect of high and low pH on DCM extraction efficiency. Unfortunately conditions which allowed recovery of a high proportion of the phosphate metabolites from the sample mix could not be identified. This meant that only a fraction of the each phosphate metabolite could have been recovered and hence the size of...
incubation required to produce these phosphate derivatives became prohibitive. It was therefore not possible to produce FTY720-P and AAL-P and consequently the potency of these compounds as agonists of the S1P4 receptor could not be determined.

6.2.4 Stable expression of HA-S1P4(E^{122}Q)-Gα_{i1}(C^{351}I) fusion protein in CHO-K1 cells

HA-S1P4 was mutated at position 122 such that the naturally occurring glutamic acid was replaced with glutamine to generate the construct HA-S1P4(E^{122}Q)-Gα_{i1}(C^{351}I)-pIRES (described in chapter 3, section 2.4). The location of this mutation is shown in Figure 6.6.

Figure 6. 6
Snake plot of S1P4.
The sequence of the S1P4 receptor is represented as a snake plot (generated using the Viseur program and reproduced with the permission of Dr. Fabien Campagne from http://www.gpcr.org/7tm/seq/vis/O95977/O95977.html). The location of the E^{122}Q mutation is shown.
The HA-S1P₄(E¹²²Q)-Gα₁₁(C³⁵¹I)-pIRES construct was stably transfected into CHO-K1 cells (as outlined in chapter 2, section 3.3). Western blotting was used to detect expression of this fusion protein. Membranes of HA-S1P₄(E¹²²Q)-Gα₁₁(C³⁵¹I)-transfected cells contained a polypeptide with an apparent molecular mass of approximately 110 kDa, which reacted with anti-HA, anti-S1P₄ and SG1 antibodies (Figure 6.7 (A)). The mass of this construct corresponded to that of the wild type HA-S1P₄-Gα₁₁(C³⁵¹I) fusion protein detected previously (chapter 4, section 2.7). Confirmation of cell-surface expression of this protein was obtained via FACS analysis using the anti-HA antibody (Figure 6.7 (B)).

6.2.5 Investigation of HA-S1P₄(E¹²²Q)-Gα₁₁(C³⁵¹I) ligand preference in [³⁵S]GTPγS binding assay

HA-S1P₄(E¹²²Q)-Gα₁₁(C³⁵¹I)-expressing membranes were prepared from cells which had been treated with 100 ng/mL pertussis toxin for 24 hours prior to harvest. The response of the HA-S1P₄(E¹²²Q)-Gα₁₁(C³⁵¹I) receptor to the ligands S1P and 18:1 LPA was assessed using the [³⁵S]GTPγS assay. These assays were performed under the same conditions used to profile the response of the wild type HA-S1P₄-Gα₁₁(C³⁵¹I) fusion, described in chapter 4, section 2.8. Stimulation of pertussis toxin treated HA-S1P₄(E¹²²Q)-Gα₁₁(C³⁵¹I)-expressing membranes by S1P and 18:1 LPA was small: approximately 14 % above basal with 10 μM 18:1 LPA and approximately 23 % above basal with 10 μM S1P (Figure 6.8 (A)). Although statistically significant increases in [³⁵S]GTPγS binding were observed at the highest concentration of each agonist used (10 μM), it was not possible to calculate an EC₅₀ value for either compound. The
Expression of HA-S1P₄(E₁²²Q)-Gα₁₁(C₃⁵¹I) in CHO-K₁ cells.

A Membranes from CHO-K₁ cells stably expressing HA-S1P₄(E₁²²Q)-Gα₁₁(C₃⁵¹I) (lane 1) and untransfected CHO-K₁ cells (lane 2) were analysed by Western blotting using anti-S1P₄ (panel I), anti-HA (panel II) or anti-Gα₁₁ (Panel III) antibodies. Visualisation of immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with appropriate HRP-conjugated secondary antibodies. The position of HA-S1P₄(E₁²²Q)-Gα₁₁(C₃⁵¹I) is indicated by an arrow.

B Cell-surface expressed HA-S1P₄(E₁²²Q)-Gα₁₁(C₃⁵¹I) was detected by FACS analysis using a Fluorescein conjugate of the anti-HA antibody, represented by the blue trace. Cells were also stained with an isotype matched control antibody, denoted by the red trace. Panel I shows the staining of HA-S1P₄(E₁²²Q)-Gα₁₁(C₃⁵¹I)-transfected CHO-K₁ cells whilst panel II illustrates staining obtained using untransfected CHO-K₁ cells. Data are presented as overlay histograms and are representative of at least five independent experiments.
Figure 6.8
Ligand preference of HA-SIP₄(E₁₂₂Q)-Ga₁I(C³⁵⁵I) in [³⁵S]GTPγS binding assay.
Membranes from CHO-K1 cells transfected to express HA-SIP₄(E₁₂₂Q)-Ga₁I(C³⁵⁵I) and incubated with 100 ng/mL pertussis toxin for 24 hours prior to harvest, were stimulated with various concentrations of SIP or 18:1 LPA for 30 minutes at 30 °C in the [³⁵S]GTPγS/IP binding assay (A). Membranes from parental CHO-K1 cells, or CHO-K1 cells which had been transfected to express HA-SIP₄-Ga₁I(C³⁵⁵I) and cultured in the presence of 100 ng/mL pertussis toxin for 24 hours prior to harvest, were used as controls in this experiment and stimulated with 10 μM SIP or LPA for 30 minutes at 30 °C (B). Gaᵢ G proteins were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data are the mean of three determinations ± SEM from a single experiment and are representative of three such experiments performed. Statistical significance from the basal responses of each set of membranes tested is denoted by * (P < 0.05) or ** (P < 0.01).
controls for this experiment, shown in Figure 6.8 (B) demonstrated that the pertussis toxin treated wild-type HA-S1P4-Gαi(C351I) receptor was unable to respond to LPA, however the signalling integrity of these membranes was confirmed by stimulation with S1P. Treatment of untransfected CHO-K1 cells (which had not been pertussis toxin treated) with 10 μM 18:1 LPA increased [35S]GTPγS binding and demonstrated that LPA was delivered to the assay system in a form able to activate Edg family receptors. It therefore appeared from these results that mutation of residue 122 to glutamine severely affected the ability of S1P4 to respond to S1P, however this mutation did not appear to be sufficient to confer LPA-responsiveness to this receptor.

6.2.6 The effect of LPA hydrocarbon chain length on activation of HA-S1P4(E122Q)-Gαi(C351I) in [35S]GTPγS binding assay

Previous studies have examined the effect of the length of the LPA acyl chain on agonist potency at receptors of the LPA family [Bandoh et al., 1999; Im et al., 2000b]. The ability of three variants of LPA (18:1, 16:0 and 14:0 LPA) to activate HA-S1P4(E122Q)-Gαi(C351I) was tested in the [35S]GTPγS binding assay using membranes prepared from pertussis toxin-treated cells.

The preliminary experiment, shown in Figure 6.9 compared activation of HA-S1P4(E122Q)-Gαi(C351I)-transfected membranes prepared from PTx-treated cells, by a 10 μM concentration of different forms of LPA. Both 18:1 and 16:0 LPA caused a small stimulation of [35S]GTPγS binding (approximately 14 % above basal for 18:1 LPA and approximately 23 % above basal for 16:0 LPA). The level of stimulation induced by 14:0 LPA was larger (approximately 40 % above basal).
Figure 6.9
The effect of LPA acyl chain length on stimulation of HA-S1P4(E122Q)-
Gα11(C351I)-transfected (+PTx).

Membranes from CHO-K1 cells transfected to express HA-S1P4(E122Q)-
Gα11(C351I), which had been cultured in the presence of 100 ng/mL pertussis
toxin for 24 hours prior to harvest, were stimulated for 30 minutes at 30 °C with
10 μM of various LPA forms which contained acyl chains of different lengths in
the [35S]GTPγS/IP binding assay (A). Membranes from parental CHO-K1 cells,
or CHO-K1 cells which had been transfected to express HA-S1P4-Gα11(C351I)
cultured with 100 ng/mL pertussis toxin for 24 hours prior to harvest, were
used as controls (B). Gαi G proteins were immunoprecipitated after
solubilisation and preclearance with non-immune serum. Data are the mean of
three determinations ± SEM from a single experiment and are representative of
two such experiments performed. Statistical significance from the basal
responses of each set of membranes tested is denoted by * (P < 0.05) or ** (P <
0.01); ## denotes statistical significance from the response to 18:1 LPA (P <
0.01).
These increases were statistically significant when compared to the basal response of untreated membranes and the stimulation promoted by 14:0 LPA was statistically different to that produced by 18:1 LPA.

No form of LPA stimulated a statistically significant response in wild type PTx-treated HA-S1P4-Gα4(C351I)-transfected membranes when compared to the basal response of these membranes, which indicated that the activity observed with the E122Q S1P4 mutant was a result of the introduction of this mutation. However, the responses induced by 16:0 and 14:0 LPA using wild type PTx-treated HA-S1P4-Gα4(C351I)-transfected membranes were statistically different compared to the vehicle response of these membranes (P < 0.05). This is not thought to reflect activation of the wild type receptor by these species of LPA since the responses were not significantly different from the basal response of these membranes, but further replicates of this experiment would be needed to confirm this. Interestingly, the profile of activation of endogenous LPA receptors present on parental CHO membranes was different to that seen with HA-S1P4(E122Q)-Gα4(C351I). Untransfected CHO-K1 cells, which have been shown to express mRNA for LPA1 (described in chapter 3) were activated by each form of LPA tested, with the rank order of maximal stimulation of 18:1 ≈ 16:0 > 14:0, which was in agreement with published data [Im et al., 2000b].

These results suggested that introduction of the E122Q mutation in the S1P4 receptor could confer LPA-responsiveness, and that a short form of LPA was a more effective agonist than the intermediate and longer forms, when tested at this single concentration.
6.2.7 Dose-dependent stimulation of HA-S1P₄(E₁²²Q)-Gα₁₁(C³⁵¹I) by 14:0 LPA in [³⁵S]GTPγS binding assay

The 14:0 form of LPA was tested to determine an EC₅₀ for activation of HA-S1P₄(E₁²²Q)-Gα₁₁(C³⁵¹I) (Figure 6.10). Stimulation of [³⁵S]GTPγS binding was observed at higher concentrations of ligand and was statistically significant when compared to the basal response of the membranes. The EC₅₀ value for activation of HA-S1P₄(E₁²²Q)-Gα₁₁(C³⁵¹I) was calculated to be 3.8 ± 1.4 μM. However, since a plateau of maximal stimulation was not achieved in any experiment, interpretation of this EC₅₀ value needed caution. This result clearly showed that 14:0 LPA was a weak agonist of HA-S1P₄(E₁²²Q)-Gα₁₁(C³⁵¹I) and hence confirmed the involvement of residue ¹²² in S1P₄ ligand preference. Similar results were obtained using a second CHO-K1 clone expressing this fusion protein (not shown).
Figure 6.10
Concentration dependent stimulation of HA-S1P$_d$(E$^{122}$Q)-G$\alpha$$_i$(C$^{351}$I)-mediated [$^{35}$S]GTP$\gamma$S binding by 14:0 LPA.
Membranes from CHO-K1 cells transfected with HA-S1P$_d$(E$^{122}$Q)-G$\alpha$$_i$(C$^{351}$I) which had been cultured in the presence of 100 ng/mL pertussis toxin for 24 hours prior to harvest were stimulated with varying concentrations of 14:0 LPA for 30 minutes at 30 °C in the [$^{35}$S]GTP$\gamma$S binding assay. G$\alpha$$_i$ G proteins were immunoprecipitated after solubilisation and preclearance with non-immune serum. Data presented are the mean of three determinations ± SEM from a single experiment and are representative of three such experiments performed. Statistical significance from the basal response of parental cells is denoted by * (P < 0.05) or ** (P < 0.01).
6.3 Discussion

This chapter describes investigations that were undertaken to characterise the ligand preference of the S1P4 receptor.

A recent publication described the ability of phS1P to act as a high affinity S1P4 receptor agonist and suggested that this lysophospholipid may represent the true endogenous agonist of S1P4 [Rios Candelore et al., 2002]. This group reported that phS1P bound to S1P4 with an apparent dissociation constant of approximately 1.6 nM, compared with the 120 nM Kd of S1P in this system. In contrast, phS1P was reported to be less potent than S1P (30.8 nM and 12.4 nM, respectively) in promoting cell medium acidification and was a partial agonist in this assay. In this chapter, the [35S]GTPγS binding assay was used to show that phS1P was approximately 17-fold more potent than S1P as an agonist of S1P4, which differs with the published study. The reason for this discrepancy is likely to be due to the different assay systems used in these determinations. The observed differences in the potency of phS1P to activate S1P4 in these two assay systems may suggest that phS1P activates opposing pathways (whilst, presumably S1P does not), which are detected using the microphysiometer assay. The reported observation that phS1P was a partial agonist of S1P4 was in agreement with results obtained using the [35S]GTPγS binding assay where phS1P was found to have an efficacy of approximately 60 %, relative to S1P.

The potency of S1P as an agonist of S1P4 in the cell medium acidification assay was in marked contrast to its relatively poor potency in the [35S]GTPγS assay. In experiments described in chapter 4, which agreed with published studies [Brinkmann et al., 2002; Mandala et al., 2002], the EC50 for S1P activation of
SIP4 was approximately 355 nM whereas in the cytosensor assay its EC₅₀ was approximately 12 nM [Rios Candelore et al., 2002]. There was little difference, however, in the EC₅₀ value determined for phSIP activation of SIP₄ in the published cytosensor assay (approximately 31 nM) and its potency in the [³⁵S]GTPγS assay described in this chapter (approximately 40 nM). This might suggest that the range of signalling pathways activated by SIP differs from those activated by phSIP. Results obtained using the [³⁵S]GTPγS assay indicate that each agonist is clearly able to stimulate SIP₄-mediated activation of Go₁ G proteins. Acidification of cell medium is a very general response; in contrast, the highly defined [³⁵S]GTPγS assay system offers a convenient way to directly compare the potency of different agonists, such as SIP and phSIP.

The novel immunomodulatory compounds, FTY720 and AAL were tested for their ability to activate SIP₄ and were found to be unable to stimulate [³⁵S]GTPγS binding at the highest concentration used (10 µM). This is in agreement with the reports of Brinkmann et al., [2002] and Mandala et al., [2002], in which the unphosphorylated FTY720 and AAL compounds exhibited no agonist activity at the SIP receptors.

The activity of phosphoryl metabolites of FTY720 and AAL compounds as potent agonists of four of the five SIP receptors is believed to reflect the in vivo mechanism of action of these compounds. When administered in vivo, FTY720 is rapidly metabolised to the phosphate, which is the predominant form of the compound in plasma [Mandala et al., 2002]. In vitro incubation with whole blood results in phosphorylation of these compounds. This reaction is likely to be mediated by an isoform of the sphingosine kinase enzyme. The localisation
of this reaction has not been reported. Since it has been reported that endothelial
cells constitutively export sphingosine kinase 1 [Ancellin et al., 2002], it is
possible that phosphorylation of FTY720 occurs extracellularly. Alternatively,
the reaction could occur within the cytosol and the resultant phosphoryl
metabolites transported out of the cell by the P-glycoprotein lipid transporter
Abcb1 [Honig et al., 2003]. Further work is needed to understand the precise
location and kinetics of this conversion.

In this chapter, incubation of FTY720 and AAL with whole rabbit blood was
used as a method for producing phosphate metabolites of these compounds.
Initially, small scale incubations were performed to allow identification of
optimal conditions for a larger scale incubation, which would yield sufficient
phosphate derivative of each compound to allow isolation and purification. The
profile of phosphorylation obtained after incubation with whole rabbit blood
differed between FTY720 and AAL. FTY720 was phosphorylated relatively
slowly and the maximal conversion achieved was approximately 70 %. In
contrast, AAL was converted more quickly, but reached a plateau after
approximately 30 % conversion. The basis for the difference in the extent to
which these reactions progressed probably reflects stereoselectivity of the AAL
phosphorylation reaction. The AAL compound possesses a chiral centre at the
C2 atom (which is achiral in FTY720) and was produced as a racemate. It has
been reported that the (R) isomer is a substrate for sphingosine kinase and shows
immunomodulatory activity in vivo, whilst the (S) isomer is inactive in vivo and
cannot be phosphorylated by sphingosine kinase [Brinkmann et al., 2002]. The
extent to which phosphorylation of the AAL compound via incubation with
rabbit blood could progress was therefore limited to 50 %.
The efficiency of conversion of each compound using this in vitro method was low, particularly when higher concentrations of compound were used. Although purified sphingosine kinase 1 has been shown to phosphorylate FTY720 in vitro [Brinkmann et al., 2002], the kinetics with which it does so are poor in comparison to the ability of this enzyme to phosphorylate sphingosine. Recent data have shown that the sphingosine kinase 2 isoform phosphorylates FTY720 efficiently. It may therefore have been more appropriate to attempt to generate FTY720-P and AAL-P compounds via incubation of the parent compounds with conditioned medium from endothelial cell cultures.

Large scale incubations of FTY720 and AAL with diluted rabbit blood were required to produce sufficient phosphate derivative of these compounds to allow purification and accurate quantitation of the mass and purity of the resultant products. Significant efforts were made to optimise procedures for extracting FTY720 and AAL phosphate metabolites into an organic phase prior to purification using HPLC. Although a range of conditions and solvents were tested, it was not possible to identify an efficient method. As a consequence of the low recovery of even the most efficient extraction method, the scale of incubation required to produce sufficient FTY720 and AAL phosphoryl metabolites was prohibitive. It was therefore not possible determine the potency of these compounds as agonists of the S1P4 receptor using the [35S]GTPγS binding assay. It has been reported that the phosphate derivatives of FTY720 and AAL were highly potent S1P4 receptor agonists (EC50 values in [35S]GTPγS binding assay of approximately 63 nM and 4.0 nM, respectively) [Brinkmann et al., 2002].
The role of residue 122 in S1P₄ ligand selectivity was also examined. This amino acid, which is located at the top of the third transmembrane domain, is a conserved glutamic acid in the S1P receptor family, whilst the LPA receptors contain a conserved glutamine at this position. Computational modelling of the S1P₁ receptor suggested this residue (E₁²¹ in S1P₁) was key in defining Edg family ligand selectivity due to its apparent interaction with the positively charged ammonium group present on S1P, whilst other important residues such as R¹²⁰ (present in TM III) and R²⁹² (located in TM VII) are thought to interact with structural elements common to both S1P and LPA [Wang et al., 2001]. Experimental analysis of an E¹²¹Q mutant of S1P₁ suggested this model to be accurate, since the presence of this mutation conferred the ability for S1P₁ to bind, and be activated by, LPA [Wang et al., 2001]. The hypothesis that this conserved residue is key in determining ligand selectivity in the S1P receptors was further tested using the S1P₄ receptor.

The HA-S1P₄(E¹²²Q) mutant, which had been constructed using PCR, and is described in chapter 3, was expressed as a fusion protein, coupled to pertussis toxin-insensitive Gα₁₁(C³⁵¹I) G protein. This was necessary since preliminary experiments performed using the unfused mutant (not shown) were difficult to interpret due to stimulation of endogenous receptors present on CHO-K1 cells by LPA. Pertussis toxin treatment of cells expressing this fusion protein prevented activation of endogenous Gα₁ G proteins by endogenous receptors thus isolating the response from the fused receptor. Expression of the HA-S1P₄(E¹²²Q)-Gα₁₁(C³⁵¹I) fusion in stably transfected CHO-K1 cells was confirmed by Western
blotting using three antibodies, which recognised different components of this protein and FACS analysis showed cell-surface expression of the receptor.

Introduction of this mutation severely affected the response to SIP. In dose-response experiments, SIP was only able to cause minimal stimulation of the HA-SIP\textsubscript{4}(E\textsuperscript{122}Q)-G\textalpha\textsubscript{11i}(C\textsuperscript{351}I) fusion and this occurred only at the highest concentration of ligand used. This is in agreement with published observations for activation of the equivalent SIP\textsubscript{1} mutant [Wang et al., 2001]. However, in contrast to this group’s report, the presence of this mutation in SIP\textsubscript{4} did not appear to confer sensitivity to activation by 18:1 LPA. Stimulation by this ligand was only apparent at the maximal concentration tested and it was not possible to calculate an EC\textsubscript{50} value from these data. The current study did not measure the ability of HA-SIP\textsubscript{4}(E\textsuperscript{122}Q)-G\textalpha\textsubscript{11i}(C\textsuperscript{351}I) to bind 18:1 LPA and therefore no conclusions could be drawn as to how well the receptor could bind this ligand. It is notable, however, that SIP binds and activates the SIP\textsubscript{1} receptor with far higher affinity and potency than it does the SIP\textsubscript{4} receptor. The affinity and potency with which 18:1 LPA bound to and activated the SIP\textsubscript{1} mutant were approximately four-fold lower than those values for SIP at the wild type receptor, and suggest that additional interactions affect binding affinity. If the same were true for the SIP\textsubscript{4} mutant, it might be expected that the potency of 18:1 LPA acting at this receptor would be very low.

Interestingly, although introduction of the E\textsuperscript{122}Q mutation affected responses of SIP\textsubscript{4} to SIP, the mutant receptor was still constitutively active. This presumably reflects perturbation of SIP recognition, whilst not affecting the ability of the receptor to spontaneously adopt an active conformation. Similar observations
have been reported for the β2AR, where a mutation in the sixth transmembrane domain abolished agonist activation but not constitutive activity [Hannawacker et al., 2002].

Investigations into the effect of the length and degree of saturation of the acyl chain of LPA have been undertaken for the LPA receptors [Bandoh et al., 1999; Im et al., 2000b], but no such information is available an S1P receptor which has been mutated to allow recognition of LPA. Therefore, a series of LPA forms containing different acyl chains were compared for their ability to activate the S1P4(E122Q) mutant. Based on the distance between the phosphate and terminal carbon atoms calculated using space-filling models of 18:1, 16:0, 14:0 LPA and S1P molecules, it was predicted that 14:0 LPA might be the most potent agonist of S1P4(E122Q) since it most closely resembled S1P. 18:1 and 16:0 LPA were only able to induce a small stimulation of this receptor, whereas activation in response to 14:0 LPA was larger. It had not been possible to calculate an EC50 value for 18:1 LPA, and 16:0 LPA only induced a small stimulation when tested at 10 μM so the potency of this ligand was not determined. 14:0 LPA was able to induce dose-dependent stimulation of HA-S1P4(E122Q)-Gα1(C351I) with an EC50 of approximately 3.8 μM. Whilst the LPA models used are approximate, and in reality the structures will be highly mobile and flexible, it seems possible that the longer forms of LPA tested may simply be too large to fit into the ligand binding pocket. Ligand binding data would be needed to confirm this suggestion. The results of LPA preference obtained with the S1P4 mutant are in contrast to that shown by LPA receptors, which exhibit the general trend of 18:1 ≥ 16:0 > 14:0 for potency and maximal stimulation [Im et al., 2000b]. It would
be interesting to examine the affinity and potency of this series of LPA molecules with the S1P4(E121Q) mutant to see if the same trend is exhibited. The relatively higher potency of 18:1 LPA at this receptor might make interpretation of data easier than it was for the S1P4 mutant.

Stimulation of endogenous receptors on untransfected CHO-K1 cells by these LPA molecules followed a different trend of maximal stimulation: 18:1 = 16:0 > 14:0, which is in agreement to the trend observed for the LPA$_1$ receptor [Im et al., 2000b] and is consistent with the detection of LPA$_1$ mRNA shown in chapter 3.

Mutation of residue 122 in the S1P4 receptor from the naturally occurring glutamic acid to glutamine conferred responsiveness to 14:0 LPA whilst severely affecting responses to S1P. This observation tends to support the hypothesis that this conserved residue in the third transmembrane domain of the S1P receptors is involved in ligand recognition. This is in contrast to a recent paper describing models of several GPCRs, including S1P4, which had been generated using novel first principle methods [Vaidehi et al., 2002]. In this model of S1P4, interactions between S1P and residues T127 (present in TM III) and W291 and E284 (present in TM VII) were observed. Interaction of E284 with the ammonium group of S1P appeared to control ligand selectivity since the other residues appeared to interact with the phosphate group, which is present on both LPA and S1P. It is therefore surprising that none of these residues are conserved throughout the S1P or LPA receptor families. The data presented in this chapter support the assertion that the glutamic acid residue present in the third transmembrane domain of the S1P receptors controls ligand selectivity and tend to suggest that the S1P4 model
described by Vaidehi et al., [2002] is inaccurate. However, it is also possible that residue 122 is not directly involved in interaction with ligand and that the introduction of the mutation has perturbed the structure of this helix such that interaction of S1P with E284 present in TM VII is hindered. This is one of the limitations associated with GPCR mutagenesis (discussed by Colquhoun [1998]). Further analysis, including the measurement of ligand binding affinities, of the (E^{122}Q)S1P4 receptor is needed to further understand the role of this residue in ligand recognition. It would also be fruitful to use the mutagenesis approach to discover whether residue 284 is involved in S1P recognition, as proposed by the recent model of Vaidehi et al., [2002]. An alternative approach that might be to generate a chimæric S1P4 receptor that contained the third TM domain from LPA1 to determine whether introduction of the entire α-helix from an LPA receptor could confer LPA responsiveness to an S1P receptor.
Chapter 7 – Final Discussion

7.1 Introduction

When work in this thesis was started, S1P$_4$ had recently been described as the orphan receptor Edg6 [Graeler et al., 1998]. Phylogenetic analysis suggested that this GPCR would be activated by the lysophospholipid S1P and would couple to G proteins of the Go$_i$ class. The first aim of this thesis was to test this prediction and attempt to measure receptor activation at the G protein level using a modified [$^{35}$S]GTP$_{\gamma}$S binding assay.

S1P$_4$ cDNA was isolated from a PBMC library using PCR and plasmid constructs were produced to allow recombinant expression of the receptor in mammalian cell lines. One of the complications associated with the analysis of S1P receptor signalling is the presence of endogenous S1P and LPA family receptors in most cell lines. For this reason, S1P$_4$ was expressed both as the native receptor and as a G protein fusion. The fusion was constructed with a mutant of Go$_{i1}$ wherein the native C$^{351}$ residue (which is the site of action of pertussis toxin) had been mutated to isoleucine, and hence was insensitive to pertussis toxin. This permitted the use of pertussis toxin to inactivate endogenous Go$_i$ proteins and therefore eliminate any interfering responses emanating from activation of endogenous S1P receptors.

Most cell lines used for recombinant protein expression respond to S1P and LPA, which presumably reflects activation of endogenous Edg family receptors. Therefore, RT-PCR was used to determine the profile of S1P and LPA family receptors expressed in the CHO-K1 cell line that was used in these studies. Semi-redundant oligonucleotides were used to clone fragments of each of the
eight Edg family receptors from Chinese hamster genomic DNA. Sequencing of these gene fragments permitted the design of RT-PCR oligonucleotides which were specific for Chinese hamster Edg family genes. S1P_{1,2,4} and LPA_{1} receptor transcripts were detected in CHO-K1 mRNA. This was the first concerted investigation into the repertoire of S1P and LPA receptors expressed in this commonly used cell line and unlike previous studies [Okamoto et al., 1998; Sato et al., 1999], the analysis described in chapter 3 used species-specific oligonucleotides.

In chapter 4, it was shown that S1P stimulated the S1P_{4} receptor causing activation of G\alpha_{i} G proteins. These observations concurred with the recently published reports of [Van Brocklyn et al., 2000; Yamazaki et al., 2000] which described S1P as an S1P_{4} receptor agonist that promoted stimulation of pertussis toxin-sensitive pathways. In contrast to these published studies, which measured events significantly downstream of receptor activation, the results described in this chapter showed stimulation at the level of G protein activation. This was at a time when other groups reported difficulty in detecting S1P_{4}-mediated G protein stimulation [Im et al., 2001a] and hence the results in chapter 4 were the first demonstration of S1P_{4} activation at the G protein level.

Within the studies undertaken in chapter 4, it was apparent that S1P_{4} exhibited a significant level of constitutive activity. It was unclear whether this was a consequence of the high levels of S1P_{4} expression present in the recombinant cell lines used for these studies; therefore, further investigations into the nature of this constitutive activity were undertaken. In chapter 5, the ecdysone-inducible expression system was used to determine the relationship between
SIP₄ basal activity and receptor expression level. This study revealed a linear correlation between these variables and confirmed that the observed constitutive activity of SIP₄ represented an inherent property of the receptor and was not simply due to high levels of expression.

In chapter 6, the role of residue E¹²² was examined within the context of SIP₄ ligand preference. Each SIP receptor contains a glutamic acid residue at this position whereas a glutamine residue occupies the equivalent position in the LPA₁₃ receptors. This residue had previously been shown to be critical in the control of ligand selectivity for the SIP₁ receptor [Wang et al., 2001] since mutation of the naturally occurring residue to glutamine attenuated SIP binding whilst conferring LPA-responsiveness to the receptor. For the SIP₄ receptor, mutation of this residue to glutamine was found to severely affect the ability of the receptor to respond to SIP. The wild type SIP₄ receptor did not respond to LPA; in contrast, dose-dependent stimulation of the (E¹²²Q) mutant by LPA was observed. A fusion protein between SIP₄(E¹²²Q) and the pertussis toxin-insensitive Go₆(C³⁵¹I) was used in this investigation so that responses due to activation of endogenous LPA receptors could be eliminated by treatment with pertussis toxin. The results obtained suggested that residue ¹²² was important in controlling ligand selectivity for the SIP₄ receptor. Three forms of LPA were tested for their ability to stimulate the SIP₄ mutant (18:1, 16:0 and 14:0 LPA). 14:0 LPA was the most effective agonist of the SIP₄(E¹²²Q) receptor. The effectiveness of different LPA species in this experiment was proposed to have reflected the dimensions of the SIP₄ binding pocket. Space-filling models had been used to calculate the distance between the phosphorus atom of the head group and the terminal carbon atom and revealed that 14:0 LPA most closely
resembled S1P in this parameter. The other forms of LPA tested (16:0 and 18:1) were significantly longer than S1P and binding within the transmembrane helical pocket may have been compromised. This study did not assess the binding affinity of S1P and the various LPA forms to the wild type and E^{122}Q mutant S1P_{4} receptors and the inclusion of this technique would have been beneficial to this investigation since these results would have illustrated whether the effectiveness of the different LPA species as agonists of the mutant S1P_{4} receptor correlated with affinity. An obvious extension of this investigation would be to apply computational modelling of the S1P_{4} receptor in tandem with the results described here alongside further mutational studies to gain insight into the nature of the S1P_{4} binding pocket.

The use of the \[^{35}\text{S}]\text{GTPyS}\) binding assay for the investigations presented in this thesis permitted detection of S1P_{4} stimulation at the level of G protein activation. This assay may be considered to be somewhat artificial since it does not measure a “real” cellular response such as chemotaxis or activation of transcription factors. In addition, the work described here focussed solely on activation of \(\text{Go}_{i}\) G proteins by this receptor and it was recently reported that S1P_{4} was able to activate both \(\text{G}_{i}\) and \(\text{G}_{12/13}\) G proteins [Graeler et al., 2003]. Clearly, the analysis of \(^{35}\text{S}]\text{GTPyS}\) binding on \(\text{Go}_{i}\) G proteins in response to S1P_{4} activation is not sufficient to predict the cellular consequences of activation of this receptor. However, the use of a simple model system such as the \(^{35}\text{S}]\text{GTPyS}\) binding assay provides insight into the first event occurring downstream of S1P_{4} activation. It is appropriate to complement experiments of this type with those measuring cellular responses that may be subject to many levels of amplification, in order to build a clearer picture of the nature of S1P_{4} signalling.
7.2 The *in vivo* role of S1P₄

Whilst significant progress has been made within the S1P receptor field, the *in vivo* function of S1P₄ is still unclear. Its restricted pattern of expression is suggestive of a role in immune function, however as yet no processes have been identified in which the functionality or presence of this receptor is required. The well documented effects of S1P and sphingoid mimetics (such as the phosphoryl metabolite of FTY720) on T cells appear to be S1P₁ dependent (discussed in chapter 1).

Investigations into the effect of deleting the S1P₄ gene in mice are required, although alternative approaches should also be considered given the lack of obvious phenotype observed in mice in which the related S1P₃ receptor had been disrupted [Ishii *et al.*, 2001]. The use of siRNA to silence S1P₄ in a physiologically relevant setting, for instance in a T cell line, may be useful when coupled with relevant cell based assays such as cytokine production, chemotaxis or differentiation. Alternatively, the production and analysis of “knock-in” mice expressing a non-functional form of the S1P₄ receptor might be useful. This approach is similar to the use of dominant negative enzymes and would permit direct examination of the effect of disrupting S1P₄ function (rather than simply deleting the gene) and would be more representative of the effects of an S1P₄ receptor antagonist or inverse agonist. Experiments of this nature will address the need to identify processes in which S1P₄ is key and hence will assist in elucidating the function of this receptor.
7.2.1 S1P4 constitutive activity

The biological significance of S1P4 constitutive activity is currently unclear, however this property is likely to be functionally relevant given the apparent evolutionary pressure on GPCRs to remain in the inactive conformation (discussed in chapter 1, section 2.4.3). S1P4 is one of a subfamily of GPCRs, which are naturally constitutively active (S1P, LPA, CB, MC and GPR3, 6, 12 receptors). The basis for the common ability of these receptors to signal in the absence of ligand stimulation may be related to the absence of the generally conserved cysteine residue within the first extracellular loop. Although the five S1P receptors are believed to have descended from a common ancestral gene [Contos et al., 2002b], there has been no suggestion that all of these constitutively active GPCRs originate from a shared ancestor. This might imply that the constitutive activity of these different receptor sub-families evolved independently and was achieved via the simplest route possible: reduction in the level of constraint of the transmembrane bundle by the loss or mutation of the cysteine residue present within the first extracellular loop. In addition, it has been reported that receptors which are caused to be constitutively active by the artificial mutation of an appropriate residue can be difficult to express [Milligan et al., 2002]. This does not seem to be the case for the S1P receptors and may indicate that the basis for the constitutive activity exhibited by S1P4 is fundamentally different from the artificial constitutive activity caused by mutation.
7.2.2 Regulation of S1P$_4$

The LPA$_{1/2}$ and S1P$_{2/3}$ receptors have been reported to mediate opposing effects [Zheng et al., 2001; Takuwa, 2002], and a similar scenario may exist between S1P$_4$ and another S1P receptor. S1P$_1$ is an obvious candidate since it is expressed in many of the locations where S1P$_4$ is detected and its presence on T cells has been suggested to account for some of the actions of S1P on these cells [Graeler and Goetzl, 2002]. It may be that S1P$_1$ and S1P$_4$ have opposing actions which could result in the fate of a particular cell being dependent on the relative expression levels of these two receptors, as well as the efficiency of coupling to their cognate G proteins.

Another possibility to consider is the formation of heterodimers between S1P$_4$ and another GPCR such as S1P$_1$ or a chemokine receptor. The phenomenon of receptor dimerisation has been extensively studied (reviewed by Angers et al., [2002]). The formation of GPCR heterodimers can be constitutive or ligand-dependent and can impart a novel pharmacology to that dimer. Heterodimer formation between the $\kappa$ and $\delta$ opioid receptor subtypes was reported to produce a novel receptor with ligand binding and pharmacological properties distinct from those of the constituent receptors [Jordan and Devi, 1999]. In support of this proposal, the ability of S1P receptors to form homo- and heterodimers has been reported [Van Brocklyn et al., 2002], although there is no information regarding the effect of dimerisation on S1P receptor pharmacology. Since S1P may not be the true endogenous agonist for the isolated S1P$_4$ receptor, formation of heterodimers with another GPCR may confer an alternative phenotype that may be related to the $in$ $vivo$ functions of this receptor. Investigations of this nature would be best pursued using a functionally relevant means of detecting
receptor dimerisation, as the traditional technique of co-immunoprecipitation may lead to spurious results. Energy transfer techniques such as BRET and FRET (reviewed by Angers et al., 2000; Angers et al., 2002), are less ambiguous since these methods detect the existence of GPCR oligomers on the surface of living cells.

In vivo, the resting activity of S1P₄ may be controlled by the actions of an endogenous inverse agonist countering constitutive activity. Exposure to S1P, present in normal serum and plasma may cause further stimulation of the receptor. Maximal activation could be achieved after release of S1P and hence exposure of the receptor to higher concentrations of this ligand, or by the production of the high affinity S1P₄ agonist, phS1P. It is possible that different S1P₄ agonists may promote activation of different G protein families (agonist trafficking), although there is currently no evidence to support this contention. The potential for activation of this receptor by multiple ligands, which differ in their affinity and potency, may offer increased control over receptor activation and the resultant consequences.

A further consideration is the potential for S1P₄ receptor activity to be subject to modification by accessory proteins. In recent years, it has become apparent that GPCRs may interact with other (non G protein) proteins and the effect of this interaction can alter the localisation or pharmacology of that receptor (reviewed by Brady and Limbird [2002]). This concept is exemplified by the proteins of the RAMP (receptor activity modifying protein) family, which have been shown to control the pharmacology of the calcitonin receptor-like receptor (CRLR) (reviewed by Foord and Marshall [1999]). Interaction with non G protein
proteins may alter S1P₄ signalling so that the receptor responds to different agonists, has a higher affinity for pre-existing agonists, or demonstrates enhanced or diminished constitutive activity. The identification of such proteins might aid in the elucidation of the function of this receptor.

Regulation of S1P₄ receptor expression by cell stimuli offers another level of control of the signalling of this receptor. For instance, it has been shown that expression of both S1P₁ and S1P₄ on T cells is reduced after T cell activation, and this may reflect one of the in vivo functions of these receptors.

7.2.3 Sphingosine-1-phosphate signalling

The enigmatic nature of S1P signalling is not yet fully understood. Whilst there appears to be a role for S1P as a second messenger, the lack of an identified intracellular target means this idea is still controversial. S1P clearly exerts some of its effects via stimulation of the S1P receptors and genetic deletion studies demonstrate a requirement for these receptors, particularly S1P₁. Studies of the effects of FTY720 on the immune system have suggested a role for this molecule (and by inference S1P) as a regulator of lymphocyte trafficking and the immune response.

Like many GPCRs, S1P receptors have been shown to internalise after ligand stimulation [Liu et al., 1999; Watterson et al., 2002; Graeler et al., 2003] and recently the related LPA₁ receptor was found to be located on the nuclear membrane, where stimulation with LPA promoted calcium release and induction of pro-inflammatory gene expression [Gobeil, et al., 2003]. It is therefore possible that S1P receptors, including S1P₄ are capable of being activated when
located intracellularly, either as a consequence of receptor internalisation or due to constitutive intracellular expression.

One thing that is clear about S1P is that it is a most unusual GPCR ligand since significant concentrations of this molecule are present in normal serum and plasma. Receptor agonists are generally tightly regulated in their production and clearance from the site of action in order that the response be transient and well defined. Both circulating and endothelial cells will be continually exposed to S1P and hence the S1P receptors on those cells may be in a state of continual stimulation, desensitisation and recycling. How this is reconciled with the constitutive activity of these receptors is unclear, however this scenario might suggest the existence of an endogenous inverse agonist. It is possible that part of the in vivo function of the S1P receptors is to maintain a tone via an equilibrium between receptor constitutive activity and/or exposure to S1P, and the opposing effects of any endogenous inverse agonists. Where S1P concentrations are elevated, for instance at sites of injury and inflammation, this equilibrium could be overcome and complete activation of the relevant receptors may be achieved. Similar effects might be achieved by spatial and temporal control of inverse agonist production.
7.3 Summary

Many questions have arisen from the work presented in this thesis, not least the uncertainty about the in vivo role of the S1P$_4$ receptor, the identity of its endogenous ligand(s) and functional relevance of S1P$_4$ constitutive activity.

Over the past 10 years, the field of lysophospholipid related research has grown rapidly and there is now abundant information regarding the signalling pathways activated by the Edg family receptors. Some of this information has been produced using artificial in vitro systems and some has arisen from studies of more physiologically relevant studies, including the analysis of S1P and LPA receptor-null mice. Several areas await further investigation; of particular interest will be the analysis of S1P$_4$ receptor function in complex tissues during normal physiological and pathophysiological situations. This is likely to be achieved via the use of genetic manipulations, possibly using conditional knockout technology. The development of blocking or activating anti-S1P$_4$ receptor antibodies will also be interesting and should offer an alternative, highly selective route for discovering the consequences of activation or antagonism of this receptor. The identification of selective agonists and antagonists/inverse agonists which discriminate between different S1P receptor subtypes is a limiting step in the elucidation of the physiological, and possible pathophysiological roles of the S1P receptors in immune system regulation.
References


References


