Regulation of *Dictyostelium* gene expression and chemotaxis by inositol signalling

Melanie Keim-Reder

MRC Laboratory for Molecular Cell Biology and Department of Biology
University College London
Gower Street
London WC1E 6BT

Research thesis submitted for the University of London Ph.D. examination.

May 2006
I, Melanie Keim-Reder, hereby declare that the work presented in this thesis is my own unless otherwise indicated, and that all published work has been acknowledged. Furthermore, I affirm that I have neither fabricated nor falsified the results reported herein.
Abstract

The mood-stabilising drugs lithium and valproic acid (VPA) are used in the treatment of bipolar disorder, but the molecular mechanisms underlying their therapeutic effects are not well understood at present. Both drugs have been suggested to attenuate inositol-based signalling: Lithium by depleting the intracellular pool of inositol via uncompetitive inhibition of Inositol monophosphatase (IMPase) and Inositol polyphosphate-1-phosphatase (IPPPase), and VPA by inhibition of inositol de novo synthesis. The therapeutic time courses for lithium and VPA treatment suggest that the drugs exert their effects through changing gene expression. Therefore, the aim of the present study was to test the hypothesis that lithium sensitivity in the model system *Dictyostelium discoideum* (*D. discoideum*) is caused by changes in gene expression.

Real-time PCR and motility assays were used to investigate whether (1) changes in gene expression can be observed in lithium-resistant mutants, (2) overexpression of IMPase and Ino1 leads to lithium resistance, and (3) lithium and analogues of the bipolar drug VPA affect gene expression.

I found that (1) loss of prolyl oligopeptidase (DpoA) in the lithium resistant LisA mutant or loss of a chromatin-remodelling factor (mutation remains to be confirmed) in the lithium resistant LisG mutant increased Ino1 and IPP1 expression. (2) Overexpression of Ino1 or IMPase led to resistance against lithium, and also VPA and the VPA-analogue VGD. (3) Lithium and VPA-analogues caused distinct changes in gene expression: Lithium treatment increased the expression of enzymes involved in inositol phosphate signalling, with the exception of IMPase; VGD decreased the expression of IMPase, IPP1 and dpoA. In addition, the LisG mutant, which showed increased IPP1 expression, was found to be cross-resistant to VGD.

The present study shows that lithium sensitivity in *D. discoideum* correlates with changes in gene expression and suggests that increase in Ino1 and IPP1 expression may confer lithium resistance in *D. discoideum*. 
Acknowledgements

During the last three years as a Ph.D. student, I have learned a lot about myself, something about life and a bit about molecular biology. I have only been able to achieve this thanks to the tremendous help and support that I received from so many people.

Adrian J Harwood, who has given me a lot of support and enormous freedom to pursue my own interests, while at the same time providing just the right amount of guidance to keep me on track.

Robin SB Williams, who has always supported me and tried (with varying success) to encourage me, and kindly took me into his lab when I was stranded.

Members of the Harwood and Williams labs (past and present), who have given me valuable advice on both science and life.

My fellow PhD students, especially Karina McQuillan and Jason King for their help, stimulating discussions and fun nights out... and for letting me stay at their homes for a while.

My father, mother and sister for always being there.

Finally my wonderful husband Gabor for all his love, endless support and cheering-up ability. Without you this thesis would certainly never have been completed!
Chapter 2  

2.2.4 Transformation of bacteria and bacterial permanents ........................................47  
2.2.5 Maxiprep of bacterial plasmid DNA ..............................................................47  
2.2.6 Miniprep of bacterial plasmid DNA ..............................................................48  
2.2.7 Restriction enzyme digestion analysis of plasmid DNA ..................................49  
2.2.8 Gel purification of DNA fragments ...............................................................49  
2.2.9 Ligations ........................................................................................................49  
2.2.10 Sequencing and analysis of DNA .................................................................50  
2.2.11 Northern Blot analysis ................................................................................50  
2.2.12 Southern Blot analysis ................................................................................51  
2.2.13 Real time-PCR analysis ...............................................................................52  
2.2.14 Oligonucleotides ........................................................................................53  
2.2.15 Plasmids ......................................................................................................54  

2.3 Biochemistry ......................................................................................................55  
2.3.1 Protein separation and western blotting of whole cell extracts ......55  
2.3.2 Antisera .........................................................................................................56  
2.3.3 IMPase activity assay ....................................................................................57  
2.3.4 Ino1 activity assay ........................................................................................58  
2.3.5 DpoA activity assay .......................................................................................58  

2.4 Recipes and reagents .........................................................................................59  
2.4.1 Media .............................................................................................................59  
2.4.2 Cell biology .....................................................................................................61  
2.4.3 Molecular biology ..........................................................................................61  
2.4.4 Biochemistry ..................................................................................................63  

Chapter 3 Changes in gene expression are observed in the lithium resistant mutants LisA and LisG .................................................................66  

3.1 Introduction ........................................................................................................67  
3.2 Ino1 expression levels are increased in the LisA mutant ..................................67  
3.3 Ino1 expression levels are also increased in PO-Inhibitor treated wild type cells ..................................................................................................................69  
3.4 Establishing a Real Time-PCR assay to investigate gene expression ........71  
3.4.1 Genes examined ............................................................................................71  
3.4.2 Optimisation and transcript abundance .......................................................74  
3.5 RT-PCR confirms and extends data of northern blots ....................................81  
3.5.1 Gene expression in the LisA mutant ..............................................................81
3.5.2 Gene expression in wild type cells treated with PO-Inhibitor......83
3.5.3 Gene expression in the LisG mutant ..................................85
3.5.4 Gene expression in the LisG mutant treated with POI............87
3.5.5 Correlation of gene expression to protein activity...............89
3.6 Discussion.......................................................................91
3.6.1 Does PO up-regulate gene expression? Northern blotting. ....91
3.6.2 RT-PCR: the assay of choice to study gene expression ........92
3.6.3 Does PO up-regulate gene expression? RT-PCR .................94
3.6.4 Gene expression in the LisG mutant ...............................95
3.6.5 Does gene expression correlate with protein activity? .......96
3.6.6 Summary.......................................................................97

Chapter 4 The lithium resistant mutant LisG encodes a chromatin-remodelling factor .................................................................98
4.1 Introduction .......................................................................99
4.2 Transcript abundance of LisG in wild type cells .................103
4.3 Recapitulation of the LisG knock-out mutant.......................105
4.4 LisG is expressed in the LisG mutant .................................105
4.5 Basic motile behaviour of LisG compared to wild type and LisA: establishing a non-gradient assay ........................................108
4.6 Chemotactic analysis of LisG and LisA ...............................111
4.7 Discussion.......................................................................113
  4.7.1 LisG abundance in wild type cells ................................113
  4.7.2 Is the chromatin-remodelling factor LisG essential for cell viability? .................................................................114
  4.7.3 Is basic motile behaviour distinct in lithium resistant mutants compared to wild type cells? ........................................116
  4.7.4 Chemotactic behaviour of LisG and LisA ......................117
  4.7.5 Summary...................................................................117

Chapter 5 Over-expression of Ino1 and IMPase leads to lithium resistance ..............................................................................................118
5.1 Introduction .......................................................................119
5.2 The over-expressed proteins are functionally active ............119
5.3 Ino1 and IMPase over-expression in wild type cells ............123
8.4 Publications
### Table of Figures

- **Figure 1.1** Overview of Inositol phosphate-signalling ........................................... 26
- **Figure 1.2** The mechanism of Inositol depletion ................................................. 29
- **Figure 1.3** *D.discoideum* development .................................................................. 34
- **Figure 3.1** *Ino1* expression in the lithium resistant mutant LisA ............................ 68
- **Figure 3.2** *Ino1* expression in wild type cells treated with PO-inhibitor ............... 70
- **Figure 3.3** Dendrogram representing the phylogenetic relationship of the *D.discoideum* IMPase family ........................................................................ 73
- **Figure 3.4** Calibration curve generated using wild type cDNA and a plasmid standard for *lg7* .................................................................................. 75
- **Figure 3.5** Calibration curve generated using wild type cDNA and a plasmid standard for *Ino1* .................................................................................. 76
- **Figure 3.6** Calibration curve generated using wild type cDNA and a plasmid standard for *IMPase* ........................................................................... 77
- **Figure 3.7** Calibration curve generated using wild type cDNA and a plasmid standard *IPP1* and *IPP2* ........................................................................ 78
- **Figure 3.8** Calibration curve generated using wild type cDNA and a plasmid standard for the 5'-phosphatases 5P2, 5P3 and 5P4 ........................................... 79
- **Figure 3.9** Calibration curve generated using wild type cDNA and a plasmid standard for *dpoA* .................................................................................. 80
- **Figure 3.10** Gene expression in the lithium resistant mutant LisA ........................ 82
- **Figure 3.11** Gene expression in wild type cells treated with PO-Inhibitor .......... 84
- **Figure 3.12** Gene expression in the lithium resistant mutant LisG ........................... 86
- **Figure 3.13** Gene expression in LisG cells treated with PO-Inhibitor ..................... 88
- **Figure 3.14** PO-activity in the LisG mutant ......................................................... 90
- **Figure 4.1** LisG encodes a chromatin-remodelling factor ................................... 101/102
- **Figure 4.2** Calibration curve generated using wild type cDNA and a plasmid standard for *LisG* ................................................................. 104
- **Figure 4.3** *LisG* is expressed in the LisG mutant .............................................. 107
- **Figure 4.4** Basic motile behaviour of wild type, LisA and LisG cells towards lithium ........................................................................................................... 110
- **Figure 4.5** Chemotactic behaviour of Ax2, LisA and LisG cells ............................ 112
- **Figure 5.1** The IMPase-GFP protein is active ..................................................... 121
- **Figure 5.2** The *Ino1*-GFP protein is active ......................................................... 122
Figure 5.3 Phenotype of developing Ax2:IMPase-GFP cells ............................................. 124
Figure 5.4 Phenotype of developing Ax2:Ino1-GFP cells ............................................. 125
Figure 5.5 Paths illustrating basic motile behaviour of Ax2:IMPase and Ax2:Ino1 cells towards lithium ........................................................................................................ 128
Figure 5.6 Basic motile behaviour of Ax2:IMPase and Ax2:Ino1 cells towards lithium ......................................................................................................................... 129
Figure 5.7 Localisation of Ino1 and IMPase protein .................................................... 131
Figure 6.1 Effect of lithium on gene expression ......................................................... 142
Figure 6.2 Effect of VPA analogues on gene expression ............................................ 144/145
Figure 6.3 Effect of VGD on D.discoideum development ........................................... 147
Figure 6.4 Effect of VGD on Ax2:IMPase and Ax2:Ino1 development ....................... 148
Figure 8.1 Malachite green standard curve ............................................................... 164
Figure 8.2 RT-PCR output for Ig7 ............................................................................. 167
Figure 8.3 RT-PCR output for Ino1 ........................................................................... 168
Figure 8.4 RT-PCR output for IMPase ..................................................................... 169
Figure 8.5 RT-PCR output for IPP1 .......................................................................... 170
Figure 8.6 RT-PCR output for IPP2 .......................................................................... 171
Figure 8.7 RT-PCR output for 5P2 ............................................................................ 172
Figure 8.8 RT-PCR output for 5P3 ............................................................................ 173
Figure 8.9 RT-PCR output for 5P4 ............................................................................ 174
Figure 8.10 RT-PCR output for dpoA ....................................................................... 175
Figure 8.11 RT-PCR output for LisG ........................................................................ 176
Figure 8.12 Amino acid sequence alignment for IMPases .......................................... 179
Figure 8.13 Amino acid sequence alignment for IPPases ........................................... 180
Figure 8.14 Amino acid sequence alignment for PAPases ........................................ 181

List of Tables
Table 3.1 Transcript abundance in wild type cells .................................................... 81
Table 8.1 RT-PCR primers used to generate DNA standards .................................... 165
Table 8.2 RT-PCR efficiencies .................................................................................. 177
Chapter 1

Introduction
1.1 List of abbreviations

°C  degrees Celsius
µ  micro (10⁻⁶)
2M2P  2-methyl-2-pentynoic acid
³²P  Radioactive phosphorous
5P2  \textit{D.discoideum} Inositol 5'-phosphatase-2
5P3  \textit{D.discoideum} Inositol 5'-phosphatase-3
5P4  \textit{D.discoideum} Inositol 5'-phosphatase-4
ACA  Adenylyl cyclase A
AleA  Aimless
AMP  Adenosine monophosphate
ampR  Ampicillin resistant
APS  Ammonium persulphate
ATM  Ataxia telangiectasia mutated
BAF  Brahma associated factor
Bcl-2  B-cell lymphoma protein-2
bp  base pairs
BSA  Bovine serum albumin (Fraction V)
Ca²⁺  Calcium
cAMP  cyclic AMP
cAR  cAMP receptor
CBZ  Carbamazepine
cDNA  complementary DNA
CDP-DAG  Cytidine diphosphate-diacyl glycerol
cGMP  cyclic GMP
Ci  Curie
CRAC  Cytosolic regulator of adenylyl cyclase
Crl  cAMP-receptor-like
CsA  Contact site A
\textit{D.discoideum}  \textit{Dictyostelium discoideum}
dATP  2'-Deoxyadenosine 5'-triphosphate
dCTP  2'-Deoxyzycosine 5'-triphosphate
ddH₂O  Double distilled water (Millipore)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>dGTP</td>
<td>2'-Deoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>DIF</td>
<td>Differentiation inducing factor</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DmtA</td>
<td>de-methyl DIF-1 transferase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DpoA</td>
<td><em>D.discoideum</em> Prolyl oligopeptidase</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
</tr>
<tr>
<td>DTT</td>
<td>DL-Dithiothreitol</td>
</tr>
<tr>
<td>dTTP</td>
<td>Thymidine 5'-triphosphate</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Disodium Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EF</td>
<td>Elongation factor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>Gbp</td>
<td>cGMP binding protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>Glc-1-P</td>
<td>Glucose 1-phosphate</td>
</tr>
<tr>
<td>Glc-6-P</td>
<td>Glucose 6-phosphate</td>
</tr>
<tr>
<td>GMP</td>
<td>Guanosine monophosphate</td>
</tr>
<tr>
<td>Gsk-3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>GskA</td>
<td><em>D.discoideum</em> Glycogen synthase kinase A</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES-buffered saline</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2 Hydroxyethylpiperazine-N’2-ethanesulphonic acid</td>
</tr>
<tr>
<td>His-tag</td>
<td>6x histidine epitope tag</td>
</tr>
<tr>
<td>hrp</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IMPase</td>
<td>Inositol monophosphatase</td>
</tr>
<tr>
<td>IMPK</td>
<td>Inositol multiphosphate kinase</td>
</tr>
<tr>
<td>Ino1</td>
<td>Inositol 1-synthase</td>
</tr>
<tr>
<td>Ins(1)P</td>
<td>Inositol 1-phosphate (Ins1P)</td>
</tr>
<tr>
<td>Ins(1,3,4)P3</td>
<td>Inositol 1,3,4-trisphosphate (Ins(1,3,4)P3)</td>
</tr>
<tr>
<td>Ins(1,3,4,5)P4</td>
<td>Inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P4)</td>
</tr>
</tbody>
</table>
Ins(1,3,4,5,6)P₅  Inositol 1,3,4,5,6-pentakisphosphate (Ins(1,3,4,5,6)P₅)
Ins(1,3,4,6)P₄  Inositol 1,3,4,6-tetrakisphosphate (Ins(1,3,4,6)P₄)
Ins(1,4)P₂  Inositol 1,4-bisphosphate (Ins(1,4)P₂)
Ins(1,4,5)P₃  Inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃)
Ins(1,4,5,6)P₄  Inositol 1,4,5,6-tetrakisphosphate (Ins(1,4,5,6)P₄)
Ins(3)P  Inositol 3-phosphate (Ins3P)
Ins(3,4)P₂  Inositol 3,4-bisphosphate (Ins(3,4)P₂)
Ins(3,4,6)P₃  Inositol 3,4,6-trisphosphate (Ins(3,4,6)P₃)
Ins(3,6)P₂  Inositol 3,6-bisphosphate (Ins(3,6)P₂)
Ins(4)P  Inositol 4-phosphate (Ins4P)
Ins(4,5)P₂  Inositol 4,5-bisphosphate (Ins(4,5)P₂)
InsP  Inositol phosphate
InsP₆  Inositol hexakisphosphate (InsP₆)
InsP₇  Diphosphoinositol pentakisphosphate (InsP₇ or PPInsP₅)
InsP₈  Bis-diphosphoinositol tetrakisphosphate (InsP₈ or (PP)₂-InsP₄)
IPK  Inositol polyphosphate kinase
IPP1  D.discoideum Inositol polyphosphate-1-phosphatase
IPP2  D.discoideum protein homologous to PAP-phosphatases
IPPase  Inositol polyphosphate-1-phosphatase
kb  kilobase
kDa  kiloDalton
l  litre
Li  Lithium
LisA  Lithium suppressor A
LisG  Lithium suppressor G
m (prefix)  milli (10⁻³)
m  metre
M  molar
MARCKS  Myrostylelated alanine-rich C kinase substrate
MBq  Mega bequerels
MHCK  Myosine heavy chain kinase
min  minutes
MIPP  Multiple inositol polyphosphate phosphatase
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP-synthase</td>
<td><em>M. musculus</em> L-myo-inositol-1-synthase</td>
</tr>
<tr>
<td>mol</td>
<td>moles</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)propanesulphonid acid</td>
</tr>
<tr>
<td>MPP</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>n</td>
<td>nano ($10^{-9}$)</td>
</tr>
<tr>
<td>OD&lt;sub&gt;x&lt;/sub&gt;</td>
<td>Optical density at x nm</td>
</tr>
<tr>
<td>p</td>
<td>pico ($10^{-12}$)</td>
</tr>
<tr>
<td>PakA</td>
<td>p21-activated kinase-A</td>
</tr>
<tr>
<td>PAP</td>
<td>3'(2')-phosphoadenosine 5'-phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>PBS/0.1% Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositol dependent kinase-1</td>
</tr>
<tr>
<td>PGM</td>
<td>Phosphoglucomutase</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3'-kinase</td>
</tr>
<tr>
<td>Pia</td>
<td>Pianissimo</td>
</tr>
<tr>
<td>PIA</td>
<td>Propyl isopropyl acetic acid</td>
</tr>
<tr>
<td>PIns</td>
<td>Phosphatidylinositol (PtdIns)</td>
</tr>
<tr>
<td>PIns(3,4,5)P₃</td>
<td>Phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃)</td>
</tr>
<tr>
<td>PIns(4)P</td>
<td>Phosphatidylinositol 4-phosphate (PtdIns(4)P)</td>
</tr>
<tr>
<td>PIns(4,5)P₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂)</td>
</tr>
<tr>
<td>PITP</td>
<td>Phosphatidylinositol transfer protein</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PO</td>
<td>Prolyl oligopeptidase</td>
</tr>
<tr>
<td>PP-InsP₄</td>
<td>diphosphorylinositol tetrakisphosphate</td>
</tr>
<tr>
<td>PsA</td>
<td>Prespore-A</td>
</tr>
<tr>
<td>PSF</td>
<td>Prestarvation factor</td>
</tr>
<tr>
<td>Pst</td>
<td>Prestalk</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue deleted on chromosome ten</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein phosphatase</td>
</tr>
<tr>
<td>RLC</td>
<td>Regulatory light chain</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time Polymerase chain reaction</td>
</tr>
<tr>
<td>SDF-2</td>
<td>Spore differentiation factor-2</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>seconds</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2-containing inositol 5’-phosphatase</td>
</tr>
<tr>
<td>SMC</td>
<td>Structural maintenance of chromatin</td>
</tr>
<tr>
<td>SMIT</td>
<td>Sodium/myo-inositol transporter</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard sodium citrate</td>
</tr>
<tr>
<td>Swi/Snf</td>
<td>Switching/Sucrose non-fermenting</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Tris/EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N’N’N’N′Tetramethylethylenediamine</td>
</tr>
<tr>
<td>tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Tetracycline resistant</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) methylamine</td>
</tr>
<tr>
<td>USF</td>
<td>Upstream stimulating factor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>v/v</td>
<td>volume for volume</td>
</tr>
<tr>
<td>VGD</td>
<td>Valproyl glycaminamide (also known as Valrocemide)</td>
</tr>
<tr>
<td>VPA</td>
<td>Valproic acid</td>
</tr>
<tr>
<td>VPM</td>
<td>Valpromide</td>
</tr>
<tr>
<td>w/v</td>
<td>weight for volume</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich-syndrome-protein</td>
</tr>
<tr>
<td>ZAK-1</td>
<td>Zaphod kinase-1</td>
</tr>
<tr>
<td>ZW1</td>
<td>S-2-pentyl-4-pentynoic acid</td>
</tr>
<tr>
<td>ZW2</td>
<td>R-2-pentyl-4-pentynoic acid</td>
</tr>
</tbody>
</table>
1.2 Lithium and the molecular basis of bipolar disorder

Bipolar disorder, commonly known as manic-depression, afflicts approximately 1-3% of patients in primary health care worldwide (WHO, 1996). There are various psychological and sociocultural models to explain the causes underlying this disorder, but the biological basis is still unknown. Genetic studies, such as pedigree analysis, twin studies and linkage analysis, suggest that multiple genes are responsible for the inheritance of a predisposition to the disorder; however, no genes have yet been identified.

Lithium and valproic acid (VPA) are the two most commonly used mood-stabilising drugs in the treatment of bipolar depression, but varying potency and potentially severe side effects are often associated with the drugs, making their use less desirable. Although a number of molecular targets have been identified for these drugs, it is still unclear how they exert their therapeutic effects, impeding the design of new drugs.

Berridge et al. proposed the ‘inositol depletion’ hypothesis as a possible mechanism underlying the therapeutic effect of lithium (Berridge et al., 1989): in brief, lithium reduces the free pool of Inositol in the brain through inhibition of inositol monophosphatase (IMPase) and inositol polyphosphate-1-phosphatase (IPPase), and thereby attenuates second-messenger signalling. Subsequent pre-clinical studies have strengthened this hypothesis by extending its validity to other mood-stabilising drugs, such as VPA and Carbamazepine (CBZ); however, the variation in clinical results from magnetic resonance studies leads to some doubt over the validity of ‘inositol depletion’ in a clinical setting (Harwood, 2005).

Furthermore, the therapeutic time course of the mood-stabilising drugs suggests that changes in gene expression may occur as a result of drug treatment (Harwood, 2005); however, further research is needed to confirm that the changes in gene expression are due to ‘inositol depletion’.
To study the effects of lithium and other mood-stabilising drugs, *D.discoideum* has proven a useful model organism. *D.discoideum* can be grown in large quantities for biochemical assays and is easily open to genetic manipulation due to its haploid genome. In addition, several techniques are available to investigate basic cell motility and chemotaxis in *D.discoideum*. In this study, I have combined gene expression studies with movement analysis to test the hypothesis that lithium sensitivity is correlated with changes in gene expression in the model system *D.discoideum*.

1.3 Lithium affects a wide range of targets

Since its reintroduction by John Cade in 1949 (Mitchell, 1999), the mood-stabilising drug lithium has been shown to affect a wide range of targets: directly inhibited by lithium are (1) the enzyme phosphoglucomutase involved in glucose metabolism, (2) the serine/threonine protein kinase glycogen synthase kinase-3 (GSK-3), (3) the enzymes PAP-phosphatase, IMPase and IPPase, all of which are members of the super-family of structurally related phosphomonoesterases. In addition, lithium has been implicated in indirectly regulating PKC activity. (Please note that the targets IMPase and IPPase will be discussed together with the ‘inositol depletion’ theory in chapter 1.5.)

1.3.1 Phosphoglucomutase

Lithium has been shown to inhibit yeast and human phosphoglucomutase (PGM) *in vitro* with a $K_i$ of 0.2 mM and 1.5 mM, respectively. Although the $K_i$ is in the therapeutic range, it is unknown whether PGM is a therapeutic target of lithium in bipolar disorder. In yeast, PGM is essential for growth on galactose-minimal media, as inhibition of PGM by lithium leads to growth defects under these conditions, which is not seen in yeast cells over-expressing PGM (Masuda et al., 2001). Galactose is converted to Glucose-1-phosphate (Glc-1-P) via the Leloir pathway; subsequently, Glc-1-P is converted to Glc-6-P by PGM. Glc-6-P not only acts as entry point into glycolysis, but also as the substrate for Ino1 in the inositol *de novo* synthesis pathway; therefore, PGM
may in fact be a therapeutic target, as lithium, by inhibiting PGM activity, reduces the substrate for inositol de novo synthesis, which eventually may lead to depletion of the intracellular inositol pool.

1.3.2 PAP-phosphatase

3'(2')-phosphoadenosine 5'-phosphate (PAP) phosphatase, also belonging to the family of monophosphoesterases, hydrolyses PAP to adenosine 5'-phosphate (AMP) and inorganic phosphate. Lithium uncompetitively inhibits PAP phosphatase with a \( K_i \) of 0.3 mM (Yenush et al., 2000). In yeast, loss of PAP phosphatase leads to accumulation of PAP and is associated with defects in RNA processing and consequently stabilisation of mRNA (Dichtl et al., 1997). In addition to its PAP phosphatase activity, the mammalian homologue RnPIP also possesses inositol-polyphosphate-1-phosphatase activity (Lopez-Coronado et al., 1999). It is still unknown whether the therapeutic effect of lithium is a result of inhibiting PAP-phosphatase activity.

1.3.3 Glycogen synthase kinase-3

Glycogen synthase kinase-3 (Gsk-3), a serine/threonine kinase, was discovered and purified from skeletal muscle in 1980 (Embi et al., 1980). Two isoforms of mammalian Gsk-3, \( \alpha \) and \( \beta \), exist, sharing 97% sequence identity within their kinase domain (Woodgett, 1990). Although structurally similar, Gsk-3\( \alpha \) and Gsk-3\( \beta \) have distinct signalling functions, as Gsk-3\( \alpha \) is unable to rescue the embryonic lethal phenotype of Gsk-3\( \beta^{-} \) mice (Hoeflich et al., 2000). Gsk-3 is a key regulatory component of a number of intracellular signalling pathways, including signalling via insulin, growth factors and nutrients as well as cell proliferation, cytoskeletal dynamics and animal development, and its activity can be regulated through phosphorylation or through complex formation (Doble and Woodgett, 2003).

Lithium has been shown to directly inhibit Gsk-3 isolated from *Xenopus laevis* with a \( K_i \) of 1-2 mM (Klein and Melton, 1996). This work was based on previous findings that lithium induces duplication of the dorsal axis (Kao et al., 1986),
which is also seen with down-regulation of Gsk-3 (He et al., 1995). In *D.discoideum*, loss of GskA, the Gsk-3 homologue, leads to increased stalk cell formation at the expense of spore cells (Harwood et al., 1995), phenocopying the effect of lithium (Maeda, 1970). Subsequent work demonstrated that inhibition of Gsk-3 is due to competition of lithium ions with magnesium ions for binding in the active site; physiological magnesium concentrations bring the $K_i$ of lithium inhibition down to therapeutic levels (Ryves and Harwood, 2001).

In support of its putative role in bipolar disorder, increased Gsk-3 activity has been associated with apoptosis and neurodegeneration (Pap and Cooper, 1998; Takashima et al., 1996), as well as neuronal remodelling, via phosphorylating the microtubule binding protein MAP1B, and synaptic plasticity (Lucas et al., 1998; Lucas and Salinas, 1997). Conversely, lithium has been shown to protect cells from various apoptotic stimuli, such as ceramide, trophic withdrawal and 1-methyl-4-phenylpyridinium (MPP, a Parkinson’s disease model) (Hetman et al., 2000; King et al., 2001; Mora et al., 2002). In addition, chronic lithium treatment has been shown to increase the expression of the proapoptotic B-cell lymphoma protein-2 (Bcl-2) in the hippocampus of mice (Chen et al., 2000). Although not owing to lithium treatment, inhibition of Gsk-3 has been associated with increased cell proliferation, as Gsk-3 has been shown to phosphorylate cyclin D1 in vitro (Diehl et al., 1998).

### 1.3.4 PKC

Lithium has been suggested to indirectly affect PKC-signalling as a result of inositol depletion, as some members of the PKC family of serine/threonine kinases, known as conventional PKCs, require Ca$^{2+}$ and diacylglycerol (DAG) derived from an inositol-containing molecule for their activation and subsequent translocation to the plasma membrane. Among their many functions, members of the PKC family are implicated in neurotransmitter release, neuronal excitability, neuroplasticity and gene expression (Nishizuka, 1992). Supporting the hypothesis that PKC is a therapeutic target of lithium in the treatment of bipolar disorder, lithium has been found to inhibit PKC translocation, and therefore PKC activation, in cerebrocortical brain slices of rats (Wang et al.,
2001); furthermore, myostylated alanine-rich C kinase substrate (MARCKS), a positively activated PKC substrate, has also been demonstrated to be down-regulated in the immortalised hippocampal cell line HN33 in response to chronic lithium treatment; this effect can be reversed by lithium removal or addition of myo-Inositol (Watson and Lenox, 1996). The mood-stabiliser VPA has also been shown to down-regulate the PKC substrate MARCKS; in contrast to lithium, however, the VPA effect cannot be rescued by addition of myo-inositol. In addition, carbamazepine and other psychotropic drugs do not down-regulate MARCKS (Manji and Lenox, 1999). Taken together, these findings suggest that inositol depletion may not be the underlying cause for altered PKC activity.

1.4 Inositol phosphate signalling in yeast, *D.discoideum* and animals

Inositol phosphate (InsP) signalling is one of many pathways involved in translating extracellular stimuli into intracellular responses and is common to yeast, plants and animals (Figure 1.1). Extracellular stimuli activate phospholipase C-β (PLC-β) via the activation of G-protein coupled receptors, leading to the hydrolysis of phosphatidyl inositol-(4,5)-bisphosphate (PI(4,5)P_2), the formation of the second messengers DAG and inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3), and Ca^{2+}-release from the endoplasmatic reticulum (ER) (Berridge, 1982; Berridge *et al.*, 1989; Rhee and Bae, 1997). Subsequently, DAG, together with Ca^{2+}, activates conventional PKC and is then recycled back to cytidine diphosphodiacylglycerol (CDP-DAG), one of the two precursors for phosphatidyl inositol (PI(4,5)P_3, Ins(1,4,5)P_3 is sequentially dephosphorylated through the combined action of 5’phosphatase, inositol-1-polyphosphatase (IPPase) and inositol monophosphatase (IMPase) to inositol. In *D.discoideum*, dephosphorylation of Ins(1,4,5)P_3 to Ins(4)P not only proceeds via the intermediate Ins(1,4)P_2, but also via the intermediate Ins(4,5)P_2 (Campagne *et al.*, 1988).

PI(4,5)P_2 is formed from the precursors CDP-DAG and inositol in the ER and then transported to the active, caveolin-enriched sites in the plasma membrane (PM) via the phosphatidyl inositol transfer protein (PITP) (Pike and Casey, 1996).
Sustained elevation of Ins(1,4,5)P₃ has been suggested to inhibit PITP activity (Speed and Mitchell, 2000). At the plasma membrane, PIns is sequentially phosphorylated to phosphatidyl inositol-4-phosphate (PIns(4)P) and PIns(4,5)P₂ through PIns-4'-kinase and PIns(4)-5'-kinase activity respectively, restoring the pool of PIns(4,5)P₂. In addition to its function as precursor for Ca²⁺-release, PIns(4,5)P₂ has been shown to be involved in actin polymerisation and vesicular recycling (Toker, 1998).

Three classes of PIns-3'-kinases (PI3K) have been found in mammalian cells (Vanhaesebroeck and Waterfield, 1999): In addition to being a substrate for PLC-β, class I PI3Ks, consisting of a 110 kDa catalytic domain and a 85 kDa regulatory domain, phosphorylate PIns(4,5)P₂ to phosphatidyl inositol-(3,4,5)-trisphosphate (PIns(3,4,5)P₃), which acts as a docking site for Pleckstrin homology (PH)-domain containing proteins including protein kinase B (PKB) and phosphoinositol dependent kinase (PDK1), promoting cell survival and cell growth; class II PI3Ks have only been analysed in vitro; class III PI3K shows strong similarity to the yeast vesicular protein-sorting protein Vsp34p. Despite the lack of evidence for class II PI3Ks in D.discoideum, three genes encoding the catalytic subunit (PIns3K-1/2/3) with homology to mammalian class I PI3Ks and one gene encoding a class III PI3K (PIns3K-5) with homology to yeast Vsp34p have been found in D.discoideum (Zhou et al., 1995). In contrast, no class I homologues have been found in yeast or plants (Vanhaesebroeck and Waterfield, 1999), suggesting that D.discoideum is more closely related to mammalian cells. The function of PIns(3,4,5)P₃ as docking-site for PH-domain proteins also plays an important role in actin polymerisation, cell migration and secretion. PIns(3,4,5)P₃ can either be converted back to PIns(4,5)P₂ by the phosphatase and tensin homologue deleted on chromosome ten (PTEN) or dephosphorylated to PIns(3,4)P₂ by the SH2-containing inositol 5'-phosphatases SHIP-1 and SHIP-2. Mutations in both PTEN and SHIP-1/2 are frequently found in several types of cancer (Luo et al., 2003b; Sansal and Sellers, 2004).

The routes for the formation of higher inositol phosphates, however, are distinct between mammalian cells, D.discoideum and yeast. In mammalian cells, higher
inositol phosphates form via the following route: \( \text{Ins}(1,4,5)P_3 - \text{Ins}(1,3,4,5)P_4 - \text{Ins}(1,3,4)P_3 - \text{Ins}(1,3,4,6)P_4 - \text{Ins}(1,3,4,5,6)P_5 - \text{InsP}_6 \) (Sasakawa \textit{et al}., 1995). Although higher inositol phosphate formation follows a more direct route in yeast, from \( \text{Ins}(1,4,5)P_3 \) via \( \text{Ins}(1,4,5,6)P_4 \) and \( \text{Ins}(1,3,4,5,6)P_5 \) to \( \text{InsP}_6 \), \( \text{Ins}(1,4,5)P_3 \) remains the only precursor for higher inositol phosphates as seen in mammalian cells. \textit{D. discoideum} can form \( \text{InsP}_6 \) from \( \text{Ins}(1,4,5)P_3 \) via a nuclear pathway with the intermediates \( \text{Ins}(1,3,4,5)P_4 \) and \( \text{Ins}(1,3,4,5,6)P_5 \); this reaction is catalysed by Inositol multiphosphate kinase (IMPK), whereas \( \text{InsP}_6 \) to \( \text{Ins}(1,4,5)P_3 \) breakdown is catalysed by multiple inositol polyphosphate phosphatase (MIPP) (Van Dijken \textit{et al}., 1995; VanderKaay \textit{et al}., 1995). However, in contrast to yeast and mammalian cells, \textit{D. discoideum} can also form \( \text{Ins}(1,3,4,5,6)P_5 \) from inositol via cytoplasmic \textit{de novo} synthesis with the intermediates \( \text{Ins}(3)P - \text{Ins}(3,6)P_2 - \text{Ins}(3,4,6)P_3 \) and \( \text{Ins}(1,3,4,6)P_4 \) (Stephens and Irvine, 1990). Although the cytoplasmic \textit{de novo} synthesis pathway does not exist in yeast, it is still unclear whether this pathway exists in mammalian cells.

Several functions have been shown for \( \text{InsP}_6 \): modification of desensitisation through binding of \( \beta \)-arrestin (Sasakawa \textit{et al}., 1994); inhibition of clathrin cage assembly and receptor down regulation by binding to AP2/3 (Shears, 1998); inhibition of non-homologous DNA-end joining (Hanakahi \textit{et al}., 2000); and regulation of mRNA export (York \textit{et al}., 1999). \( \text{InsP}_6 \), however, is not the endpoint of the pathway. Recently, it has been shown that \( \text{InsP}_6 \) is converted to \( \text{InsP}_7 \) and \( \text{InsP}_8 \) (Huang \textit{et al}., 1998; Saiardi \textit{et al}., 2001). \( \text{InsP}_7 \) has been suggested to phosphorylate proteins (preferably nucleolar proteins) non-enzymatically (Saiardi \textit{et al}., 2004), whereas \( \text{InsP}_8 \) has been shown to play a role in hyperosmotic stress response (Pesesse \textit{et al}., 2004) and mild thermal stress response in mammalian cells (Choi \textit{et al}., 2005).

In addition to uptake from the diet, an alternative source for inositol is via \textit{de novo} synthesis through conversion of Glucose-6-phosphate (Glc(6)P) to \( \text{Ins}(3)P \) by inositol-synthase (Ino1) and subsequent dephosphorylation to inositol by IMPase (Culbertson \textit{et al}., 1976; Maeda and Eisenberg, 1980); this pathway is common to yeast, \textit{D. discoideum} and mammalian cells. In yeast, the levels of
inositol in the cell regulate inositol de novo synthesis: in the absence of inositol, binding of the transcriptional activators Ino2p and Ino4p to the upstream UAS_{INO} sequence derepresses \textit{ino1} expression; in the presence of inositol, the negative regulator Opi1p represses \textit{ino1}. An upstream stimulating factor (USF)-element, which possesses a similar core binding sequence to the UAS_{INO} sequence element in yeast, has been found for the mouse \textit{L-myo-inositol-1-synthase} (MIP) gene (Shamir \textit{et al.}, 2003). However, it is not yet understood how de novo synthesis is transcriptionally regulated in mammalian or \textit{D.discoideum} cells.
Figure 1.1 Overview of inositol phosphate signalling. External stimuli either activate PI3K (4) to form PIns(3,4,5)P$_3$ or PLC (1) to release DAG (not shown) and Ins(1,4,5)P$_3$. IPPase (2) and IMPase (3) dephosphorylate Ins(1,4,5)P$_3$ and thereby recycle inositol into the pathway. Ins(1,4,5)P$_3$ can also be phosphorylated to generate higher inositol phosphates; note that the routes are distinct in yeast, D.discoideum, and animals. In addition, higher order inositol phosphates can also be generated de novo in D.discoideum, with myo-inositol-3-hydroxykinase (5) synthesising the first step. MIPP (6) hydrolyses InsP$_6$ to Ins(1,4,5)P$_3$. In D.discoideum, the 5'-phosphatases (8) 5P2, 5P3 and 5P4 act on all the substrates indicated, but with varying degree of efficiency. Inositol can also be generated de novo from Glc(6)P via Ino1 (7) and IMPase (3).
1.5 The molecular mechanism of 'Inositol depletion'

Lithium has been shown to inhibit IMPase from bovine brain with a $K_i$ of 0.8 mM \textit{in vitro} and in rat brain slices \textit{in vivo} (Allison \textit{et al}., 1980; Hallcher and Sherman, 1980). Hallcher and Sherman determined that lithium acts via uncompetitive inhibition, thus only binding to the enzyme-substrate complex (Hallcher and Sherman, 1980). Structural analysis of mammalian IMPase determined a two-metal catalysed mechanism, in which an activated water molecule acts as a nucleophile; the same analysis found that lithium binds to the second metal-binding site and thereby prevents the release of the phosphate after hydrolysis from InsP$_1$, resulting in the formation of a stable lithium-enzyme-substrate complex (Attak \textit{et al}., 1995). Lithium also has been shown to inhibit IPPase isolated from calf brain with a $K_i$ of 0.46 mM for Ins(1,3,4)P$_3$-hydrolysis and with a $K_i$ of 9.63 mM for Ins(1,4)P$_2$-hydrolysis; similar to IMPase, lithium acts on IPPase via uncompetitive inhibition (Gee \textit{et al}., 1988). Based on these findings, Berridge \textit{et al}.
formulated the 'inositol-depletion' hypothesis (Figure 1.2): As the conversion from InsP$_1$ to inositol and inorganic phosphate is the last step in both Ins(1,4,5)P$_3$ recycling and inositol \textit{de novo} synthesis and requires IMPase, lithium, through inhibition of IMPase and also IPPase, depletes inositol and PI(4,5)P$_2$, and thereby attenuates inositol-based signalling in regions, such as in the brain, where external inositol is limiting. In mood disorders, where InsP signalling might be over-activated, lithium may restore the 'normal' state (Berridge \textit{et al}., 1989).

Lithium and valproic acid (VPA) have indeed been found to lower myo-inositol levels in yeast (Vaden \textit{et al}., 2001), mice frontal cortex (Shaltiel \textit{et al}., 2004), rat brain and humans (O'Donnell \textit{et al}., 2000; Silverstone \textit{et al}., 2002), although the drugs differ in their inositol-depleting mechanism. VPA does not deplete inositol by inhibiting IMPase, as seen with lithium, but by indirectly inhibiting Ino1 activity, and thereby inositol \textit{de novo} synthesis. This is seen in the decrease of InsP$_1$ with VPA treatment, in contrast to the increase of InsP$_1$ with lithium treatment (Vaden \textit{et al}., 2001). Furthermore, both lithium and VPA increase \textit{ino1} and \textit{ino2} expression in yeast (Vaden \textit{et al}., 2001), \textit{ino1} expression in \textit{D.discoideum} (Williams \textit{et al}., 2002) and MIP-synthase expression in mice.
hippocampus (Shamir et al., 2003). In yeast, ino1 and ino2 expression is only increased in the absence of inositol, confirming the inositol depletion effect of both drugs. Further support for the 'inositol-depletion hypothesis' comes from work by Williams et al. with dorsal root ganglia (DRG) from newborn rats, who showed that lithium, VPA and also CBZ increase the spread of sensory neuron growth cones; this effect could be reversed by supplementation with inositol (Williams et al., 2002).

Despite all the evidence in favour of the 'inositol-depletion' hypothesis, there is some evidence not consistent with it. Only the biologically active isomer myo-inositol would be expected to reverse the inositol depleting effect of lithium or VPA, but two studies showed that epi-inositol can also prevent lithium-pilocarpine-induced seizures in rats (Belmaker and Grisaru, 1998; Williams and Jope, 1995). Furthermore, Drosophila IPP1+ mutants have defects in synaptic vesicle function at the larval neuromuscular junction, and lithium can phenocopy this effect. However, IPP1− mutants are not deficient of inositol, because they can re-route their metabolism via Ins(1,3,4,5)P4, arguing against a role of inositol depletion in synaptic plasticity changes (Acharya et al., 1998). Similarly, a double gene disruption of the IMPase gene pair, imp1 and imp2, in yeast does also not lead to inositol auxotrophy, suggesting that inositol depletion is not an effect of lithium (Lopez et al., 1999). Recently, Berry et al. created mice that are deficient in the high affinity sodium/myo-inositol transporter (SMIT). These mice have a 92% reduction of brain inositol levels and a 84% reduction in body inositol levels and die shortly after birth due to central apnoea (Berry et al., 2003). Although these mice are severely deficient for inositol, they have normal PIIns levels compared to SMIT+/− mice, arguing against inositol depletion as the mechanism of lithium action (Berry et al., 2004); however, the authors suggested that a reduction in inositol may perturb the flux into PIIns without changing the concentration of PIIns. In addition, although this has not yet been observed in mammals, higher order inositol phosphates may be synthesised de novo from inositol and subsequently could be hydrolysed via MIPP to restore Ins(1,4,5)P3 levels, as seen in the D.discoideum dpoA− mutant (Williams et al., 1999).
Figure 1.2 The mechanism of inositol depletion. Lithium and VPA deplete the intracellular pool of inositol through inhibition of IMPase (1), IPPase (2) and PGM (5), and Ino1 (4) respectively, and thereby inhibit inositol-based signalling. In contrast, loss of PO in D.discoideum leads to increased higher inositol phosphate hydrolysis due to increased MIPP-activity (3), leading to lithium resistance.
1.6 Can 'inositol depletion' affect gene expression?

Since the therapeutic time courses of mood-stabilisers show a distinct lag phase prior to treatment response, mood-stabilisers have been suggested to exert their effects via modulation of gene expression. In eukaryotes, gene expression changes are strictly controlled via a complex process. In contrast to prokaryotes, the transcriptional ground state is restrictive in eukaryotes, as histones (H2A, H2B, H3 and H4) restrict the access of the transcription machinery to the DNA. The central domain of the histones is required for histone-histone interactions and wrapping of the DNA, whereas the lysine-rich amino-terminal domain of the histones is required to control transcription (i.e. acetylation by histone acetyl transferase promotes interaction with chromatin-remodelling factors). To activate gene expression, transcription factors interact with the DNA via sequence-specific DNA-binding domain(s) and recruit chromatin-remodelling complexes and histone deacetylases to the site. Chromatin-remodelling complexes are multi-subunit structures that hydrolyse ATP to change nucleosomal structure and increase DNA accessibility. Histone acetyltransferases and chromatin-remodelling complex function synergistically, thereby enabling the RNA polymerase machinery to form a functional initiation complex (Kadam and Emerson, 2002). In addition, as transcription and translation in eukaryotes is separated in time and space, changes in gene expression can also be regulated via translational repression of mRNAs (Nelson and Cox, 2000).

Supporting a direct link between ‘inositol depletion’ and gene expression, PIns(4,5)P₂ has been found to regulate the binding of the chromatin-remodelling complex Brahma associated factor (BAF) complex, to the nuclear matrix and chromatin in vitro (Zhao et al., 1998). In PIns(4,5)P₂ micelles and PIns(4,5)P₂-containing mixed lipid vesicles, PIns(4,5)P₂ enhances binding of the BAF complex to actin filaments, possibly via facilitating intramolecular uncapping of actin within the complex leading to actin filament assembly (Rando et al., 2002).

Furthermore, soluble inositol phosphates have also been implicated in the regulation of gene expression, mRNA export and telomere length. Odom et al.
demonstrated that inositol polyphosphate kinase-2 (IPK-2) and its product \( \text{Ins}(1,3,4,5)P_4 \) are required for assembly and functional activity of the ArgR-Mcm1-complex, controlling arginine-responsive gene expression in yeast (Odom et al., 2000). In addition, loss of PLC, IPK-1 or IPK-2 activity is associated with a defect in mRNA export, suggesting a requirement for \( \text{InsP}_6 \) (York et al., 1999). In a subsequent study, York et al. demonstrated that loss of IPK-1 leads to accumulation of diphosphorylinositol tetrakisphosphate (PP-\( \text{InsP}_4 \)) and shortened telomeres, whereas loss of Kcs-1 leads to accumulation of \( \text{InsP}_6 \) and elongated telomeres. DNA-dependent protein kinases such as Tel1, the yeast orthologue of mammalian ataxia telangiectasia mutated (ATM), are known to be important for telomere length and, as this study showed, are negatively regulated by PP-\( \text{InsP}_4 \) (York et al., 2005). Higher order inositol phosphates have also been shown to regulate gene expression via modulation of ATP-dependent chromatin-remodelling complexes in yeast. Steger et al. found that IPK-2 is required for the recruitment or stable binding of the chromatin-remodelling complexes Ino80 and Swi/Snf to the promoter of the phosphate-responsive \( \text{Pho5} \) gene in vivo (Steger et al., 2003). In an in vitro approach, Shen et al. tested the effect of inositol phosphates on chromatin-remodelling activity: \( \text{InsP}_6 \) inhibits the Ino80 complex, whereas \( \text{Ins}(1,3,4,5)P_4 \) activates the Swi/Snf complex. Furthermore, the authors showed that IPK-2 is required for \textit{ino1} expression in vivo (Shen et al., 2003). Taken together, these findings suggest an essential role for soluble and membrane-bound inositol phosphates in chromatin remodelling.

VPA has been shown to inhibit histone deacetylase (HDAC) activity \textit{in vitro} and \textit{in vivo} (Gottlicher et al., 2001; Phiel et al., 2001). Promoter activity is regulated in part by histone acetyltransferases (HATs) and HDACs: hyperacetylated histones are associated with increased gene expression and \textit{vice versa}. VPA, therefore, activates gene expression by inhibiting HDAC activity. Recently, Marchion et al. demonstrated that VPA alters chromatin structure by negatively regulating a group of proteins, known as structural maintenance of chromatin (SMC), which are important for chromatin condensation and gene silencing (Marchion et al., 2005).
Changes in gene expression as a result of drug treatment have been demonstrated: both lithium and VPA increase *ino1* and *ino2* expression in yeast (Vaden et al., 2001), *ino1* expression in *D.discoideum* (Williams et al., 2002) and MIP-synthase expression in mice hippocampus (Shamir et al., 2003). In addition, Shamir et al. found a lithium induced increase in *IMPA1* expression, but no change in *IMPA2* expression in the mice hippocampus (Shamir et al., 2003), which confirmed earlier studies in lymphoblastoid derived cell lines from bipolar patients (Shamir et al., 1998). Interestingly, two frequent single-nucleotide polymorphisms of *IMPA1* and *IMPA2* are associated with bipolar disorder (Sjoholt et al., 2004), suggesting that InsP signalling might be the underlying cause of the disease.

In summary, the findings presented here support the hypothesis that the mood-stabilisers lithium and VPA exert some of their therapeutic effects by regulating gene expression via indirectly affecting soluble and membrane bound InsPs. However, it is still unclear whether these effects are due to ‘inositol depletion’; further studies are needed to investigate this link.

1.7 The model system: *D.discoideum*

The cellular slime mould *D.discoideum* (*D.discoideum*) is a microbial eukaryote at the base of metazoan evolution. Modern phylogenetic trees, based on the comparison of 5279 orthologous proteins such as α-tubulin, β-tubulin, actin and elongation-factor-1α (EF-1α), suggest a divergence for the *Dictyostelidae* before the branching of the fungi and animals, but after the divergence of the plants (Baldauf et al., 2000; Eichinger et al., 2005). In comparison to yeast, *D.discoideum* shares more homologous genes to fungi and animals, and even possesses some genes that are missing in yeast (Eichinger et al., 2005).

The genome of *D.discoideum*, comprising 6 chromosomes and a palindromic extrachromosomal ribosomal RNA element, has a total size of 34 Mb and encodes for approximately 12,500 genes (Eichinger et al., 2005). In addition, *D.discoideum* possesses a mitochondrial genome with a total size of 55.5 kb,
encoding transfer RNAs and several other proteins, and which makes up 35% of the total DNA in the cell (Cole and Williams, 1994). With an AT-ratio of 77%, the genome is very AT-rich, especially in intergenic regions and introns. Some sequence characteristics of the *D.discoideum* genome are the long repeats of asparagine, glutamine and threonine, and a high frequency of transposable elements (Eichinger *et al.*, 2005).

Due to its evolutionary position, *D.discoideum* possesses signal transduction pathways similar to those of fungi and animals, which makes it an attractive model system for studying processes of chemotaxis and multicellular development. The use of *D.discoideum* as a model system is facilitated by the availability of fully sequenced genome (Eichinger *et al.*, 2005) as well as easy maintenance and manipulation of the organism under laboratory conditions, which make it suitable for any biochemical and cell biological analysis.

An overview of the life cycle of *D.discoideum* is shown in figure 1.3: When bacterial food is available, *D.discoideum* exists as unicellular amoebae; upon starvation, approximately 100,000 amoebae aggregate into a mound and subsequently enter a program of a multicellular development, similar to that of animal development, finally forming a fruiting body. A more detailed description of the processes involved is discussed below.
Figure 1.3 *D.discoideum* development. In the presence of bacteria, *D.discoideum* cells exist as amoebae. When the food source is depleted, cells aggregate into mounds, and subsequently undergo various morphological changes, finally forming the fruiting body. (Figure reproduced with permission of A. J. Harwood).
1.7.1 The role of Inositol phosphates in aggregation of D. discoideum

In the wild, D. discoideum live in the soil and usually feed on bacteria. When the bacteria become limited, the prestarvation factor (PSF) induces the gene expression of genes needed during the early period of aggregation, including adenylyl cyclase A (aca) and the cAMP receptor 1 (cAR1) (Rathi and Clarke, 1992; Schulkes and Schaap, 1995). Subsequently, aggregation is stimulated by extracellular cAMP emitted from cells in the centre of the future mound. A D. discoideum cell has the ability to sense a difference of less than 2% in the cAMP gradient between the front and the back of the cell (Firtel and Chung, 2000). The cAMP signal is detected via the cell surface G-protein coupled cAMP receptor cAR1 (Pitt et al., 1992). Three other cARs have been identified: cAR2 is required during tip formation; cAR3 is the second high affinity receptor during early development and can substitute for cAR1; cAR4 is required for cell-type specific gene expression during pattern formation (Johnson et al., 1993; Kim et al., 1998; Louis et al., 1994; Saxe et al., 1993; Saxe et al., 1991). Three cAMP-receptor-like proteins (CrlA-CrlC) have also been identified, that might play a role in growth and development (Raisley et al., 2004).

Stimulation of cAR1 leads to activation of a trimeric G-protein, which transmits the external signal to downstream effectors. D. discoideum contains eleven G-protein α-subunits with distinct cellular functions: Gα-2 is coupled to cAR1; Gα-4 lies downstream of the folate receptor; Gα-9 has been implicated as a negative regulator of development in the adaptation response (Brzostowski et al., 2002; Manahan et al., 2004). However, the cAMP signal is transmitted via the βγ-subunits. D. discoideum possesses only one β- and one γ-subunit, and loss of the β-subunit leads to defects in cGMP accumulation, adenylyl cyclase activation and actin polymerisation, all of which are correlated with a chemotaxis defect (Wu et al., 1995; Zigmond et al., 1997); reviewed in (Manahan et al., 2004).

Studies utilising GFP-tagged versions of cAR1 and the βγ-subunit demonstrated that both components are uniformly distributed across the cell surface (Jin et al., 2000; Xiao et al., 1997; Zhang et al., 2001), posing the question what
mechanism leads to polarisation of the cell and directional movement. Upon cAMP stimulation, PI3K-1 and PI3K-2 are translocated to the leading edge at the plasma membrane, correlating with localised PIns(3,4,5)P₃ accumulation. In addition, PTEN localises to the side and rear of the cells, degrading PIns(3,4,5)P₃, and thereby suppressing lateral pseudopod formation (Funamoto et al., 2001). Loss of PI3K leads to defects in polarity and chemotaxis (Huang et al., 2003), whereas loss of PTEN leads to formation of lateral pseudopods (Iijima and Devreotes, 2002). Thus, PI3K and PTEN establish polarity by controlling spatial distribution of PIns(3,4,5)P₃. The essential role of PIns(3,4,5)P₃ in chemotaxis is also conserved in neutrophils and cells derived from knockout mice (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000; Wang et al., 2002). D.discoideum also contains four PIns-5'-phosphatases (PI5P 1-4), which could be involved in down-regulating the local PIns(3,4,5)P₃ signal. However, only loss of PI5P-4 leads to a defect in growth and development, suggesting functional redundancy of the proteins (Loovers et al., 2003).

PIns(3,4,5)P₃ recruits Pleckstrin-homology containing (PH) proteins such as the cytosolic regulator of adenylyl cyclase (CRAC) and PKB to the leading edge of the plasma membrane (Lilly and Devreotes, 1995; Meili et al., 1999; Parent et al., 1998). Higher inositol phosphates, namely InsP₇ and InsP₈, have recently been shown to compete with PIns(3,4,5)P₃ for binding of PH-domain containing proteins, thereby possibly dampening the cAMP signal (Luo et al., 2003a).

During early development, CRAC is required for activation of adenylyl cyclase, which synthesises cAMP for secretion. This process also requires Pianissimo (Pia), the Ras exchange factor Aimless (AleA), Erk2 and possibly RasC. A prominent feature of D.discoideum aggregation is the formation of waves, associated with a periodic cycle of cAMP production and degradation. Elevated levels of cAMP lead to activation of PKA, and via inhibition of Erk2 to activation of the phosphodiesterase RegA. In turn, RegA degrades intracellular cAMP, leading to inactivation of PKA, activation of Erk2 and subsequently inhibition of RegA itself (Manahan et al., 2004). In later stages of development, CRAC is required for spore and prespore cell differentiation (Parent et al., 1998).
How is localised PIns(3,4,5)P₃ accumulation connected with pseudopod extension at the leading edge? Cells presented with a uniform cAMP stimulus show a biphasic actin-polymerisation response: a rapid peak after 5 sec and a later peak after 90 sec. The latter is thought to be PIns(3,4,5)P₃ dependent, as this peak is abolished in wild type or pten⁻ cells treated with a PI3K inhibitor (Chen et al., 2003). Recently, activation of the Rho-GTPase Rac1b via Rac-Gef-1 has been suggested to be dependent on PIns(3,4,5)P₃ (Affolter and Weijer, 2005; Chung et al., 2000; Lee et al., 2004). Downstream targets of activated Rac are members of the Wiskott-Aldrich-syndrome protein (WASP) family, including WASP and Scar/WAVE, which interact with Arp2/3 leading to actin polymerisation (Machesky and Insall, 1998; Rivero and Somesh, 2002).

It is essential for efficient chemotaxis that lateral pseudopod formation is suppressed. Regulation of myosin-II by cGMP is suggested to play an important role in this process. Upon cAMP stimulation, two guanylyl cyclases, GCA and sGC, are rapidly activated, leading to a transient rise in cGMP levels that peaks approximately 10 sec after stimulation. Subsequent activation of two cGMP-specific phosphodiesterases, PdeD and PdeE, returns cGMP levels to baseline by 30 sec after cAMP stimulation (Bosgraaf et al., 2002; Goldberg et al., 2002). Two novel cGMP-binding proteins, GbpC and GbpD, have been identified as the main downstream effectors, associated with (1) inhibition of lateral pseudopod formation by stabilising myosin-II filaments at the sides and rear of the cell and (2) uropod retraction by activating myosin light chain kinase-a (Mlck-a) that phosphorylates the regulatory light chain (RLC) (Bosgraaf et al., 2002). In addition, cAMP also activates p21-activated kinase-A (PakA) via activation of PI3K/PKB pathway; PakA inhibits MHCKs, and thereby facilitates myosin-II assembly (Chung et al., 2001). It has been proposed that cGMP stabilises myosin-II filaments globally, and only local activation of myosin heavy chain kinase-A (MHCK-A) leads disassembly of myosin-II filaments at the leading edge of the cell, thereby establishing cell polarity (Postma et al., 2004a). Indeed, destabilisation of myosin-II filaments is correlated with the translocation of MHCK-A to the leading edge; Translocation and activation of
MHCK-A has been suggested to be dependent on PI(3,4,5)P_3 and F-actin respectively (Egelhoff et al., 2005; Liang et al., 2002).

Each of these processes is required for the cells to coalesce into multicellular streams and to form a cell mass consisting of $10^5$ cells, known as the mound. The subsequent differentiation and culmination processes are regulated by changes in gene expression, as explained in the next sub-section.

1.7.2 Gene expression and *D.discoideum* development

Further differentiation starts with the generation of a tip on top of the mound, which acts as a co-ordinator, secreting cAMP throughout development (Williams et al., 1989). Under the influence of the tip, the mound elongates into a structure, known as the first finger, which can either culminate or fall over and migrate as a slug depending on environmental factors such as light intensity, humidity and ionic strength (Bonner et al., 1950). The slug consists of different cell types: prestalk A (pstA) and prestalk O (pstO) cells form the anterior fifth of the slug; prespore cells and a small population of anterior-like and prestalk B (pstB) cells form the posterior four-fifth of the slug. During culmination, the posterior of the slug moves under the tip, and then the prestalk cells pass through the open annulus, vacuolate and become stalk cells. Due to addition of new stalk cells the stalk elongates, lifting up the spore cells, eventually forming the sporehead. This process is regulated by several extracellular signals, including cAMP and the differentiation-inducing factor (DIF) (Harwood, 2001).

DIF-1, a chlorinated alkyl phenone, is the primary inducer of pstO cell differentiation. Secreted by the prespore cells, DIF-1 controls gene expression in adjacent pstO cells by regulating nuclear accumulation of the transcription factor StatC (Fukuzawa et al., 2003). However, DIF-1 only induces pstO, but not pstA cell differentiation, because loss of the enzyme that catalyses the final step in DIF synthesis, des-methyl DIF-1 transferase (dmtA), correlates with loss of pstO cells only (Thompson and Kay, 2000). The inducer for pstA cell differentiation has not been identified yet.
Although not essential for cell differentiation, regulation of GskA by cAMP is important for regulation of prestalk/prespore ratio in the slug, as GskA induces prespore A (psA) expression and represses prestalk B (pstB) expression (Harwood et al., 1995). Moderate levels of cAMP activate GskA via stimulation of cAR3 and subsequent activation of zaphod kinase-1 (ZAK-1) (Kim et al., 1999; Plyte et al., 1999); high levels of cAMP down-regulate GskA activity via stimulation of the low-affinity cAMP receptor cAR4 and subsequent activation of a protein phosphatase (PTP) (Ginsburg and Kimmel, 1997; Kim et al., 2002).

The final steps in culmination are dependent on the level of PKA activity: high levels of PKA are needed to proceed through terminal differentiation, as it phosphorylates target proteins that are necessary for the sporulation process, including the spore coat protein SpiA. Ammonia activates a two-component histidine kinase cascade, leading to activation of the cAMP phosphodiesterase RegA. Thus, following a decrease in ammonia concentration, cAMP concentrations rise, leading to activation of PKA, thereby inducing spore differentiation (Thomason et al., 1998; Thomason et al., 1999). In addition, RegA has been shown to be down-regulated by a negative feedback loop: upon PKA stimulation, the spore differentiation factor-2 (SDF-2) activates the two histidine kinases DhkA and DhkB, which in turn reduce RegA activity (Anjard et al., 1998).

1.8 ‘Inositol depletion’ in *D.discoideum*

Lithium has been shown to affect both aggregation and differentiation of *D.discoideum*. In the presence of 7 mM lithium, *D.discoideum* cells are able to aggregate, but form aberrant fruiting bodies; this has been shown to be due to inhibition of GskA (Harwood et al., 1995; Maeda, 1970); in the presence of 10 mM lithium, aggregation of *D.discoideum* cells is impaired; this, however, is independent of GskA (Maeda, 1970). As lithium has been shown to reduce intracellular InsP(1,4,5)P₃ levels by 20% (Peters et al., 1989), higher concentrations of lithium have been suggested to impair aggregation of *D.discoideum* by affecting InsP-signalling (Williams et al., 1999). Harwood et al.
used insertional mutagenesis to screen for mutants with reduced sensitivity to lithium; these mutants can be grouped as either affecting GskA-signalling or reversing the effect of lithium on InsP-signalling (Williams et al., 1999). The mutant lisA can aggregate in the presence of 10 mM lithium and is characterised by increased InsP(1,4,5)P₃ levels and increased Ins(1,4,5)P₃ hydrolysis. However, PLC activity in dpoA⁻ cells is similar to wild type cells, suggesting that increased levels of Ins(1,4,5)P₃ are not due to increased PIns(4,5)P₂ hydrolysis. The lisA gene encodes the enzyme prolyl oligopeptidase (PO) and affects InsP-signalling through inhibition of multiple polyphosphate phosphatase (MIPP) activity (Williams et al., 1999). MIPP is responsible for hydrolysis of higher inositol phosphate to Ins(1,4,5)P₃ (Van Dijken et al., 1995). Similarly, as loss of PO leads to reduced lithium sensitivity by increasing intracellular levels of Ins(1,4,5)P₃, conversely, over-expression of PO increases lithium sensitivity, by decreasing intracellular levels of Ins(1,4,5)P₃ (Williams et al., 1999). In addition, substance P stimulation in the presence of a PO-inhibitor or in PO antisense expressing cells leads to accumulation of Ins(1,4,5)P₃ in the astroglioma cell line U343 (Schulz et al., 2002), suggesting that this mechanism is conserved in mammals.

These findings are in contrast to some earlier studies that suggested that InsP-signalling may not be a significant target of lithium: First, Van Dijken et al. demonstrated that lithium decreases the total intracellular inositol pool by only 20%; the authors also reported three IMPase activities characterised by distinct InsP₁-hydrolytic specificity, with two of the enzymes being lithium resistant (Van Dijken et al., 1996). Second, loss of PLC in D.discoideum is not associated with a developmental phenotype (Drayer et al., 1994); this, however, has later been shown to be due to increased hydrolysis of higher InsPs (Van Dijken et al., 1997). However, as elevated levels of Ins(1,4,5)P₃ have subsequently been shown to lead to lithium resistance (Williams et al., 1999), this suggests that InsP-signalling is important for lithium sensitivity.
1.9 Aims of this thesis

In addition to affecting aggregation and differentiation in *D.discoideum*, lithium has also been shown to affect gene expression, as 10 mM lithium leads to loss of expression of the aggregation-specific csA (Williams *et al.*, 1999) and to loss of all developmentally induced gene expression (Peters *et al.*, 1989). Furthermore, investigating the effects of modulating PO activity on the complete spectrum of InsP species, Harwood *et al.* showed that PO activity regulates both the total mass of InsPs in the cell and the ratio of InsP$_6$ to InsP$_5$ and InsP$_4$ (A. J. Harwood, personal communication). Interestingly, higher order InsPs have been shown to regulate expression of *ino1* in yeast, via modulation of Swi/Snf related chromatin remodeling factors (Shen *et al.*, 2003; Steger *et al.*, 2003), suggesting that this mechanism may be conserved in *D.discoideum*.

Taken together, these findings suggest that lithium sensitivity may be associated with changes in gene expression. Thus, the aim of this work was to investigate whether lithium sensitivity in *D.discoideum* is indeed due to changes in gene expression; the following sub-questions were investigated to answer this hypothesis:

- Can changes in gene expression be observed?
- How does the mutant LisG relate to gene expression?
- Does increasing *IMPase* or *ino1* expression give lithium or VPA resistance?
- How do bipolar drugs affect gene expression?
Chapter 2

Materials and Methods
2.1 Cell biology

2.1.1 D.discoideum maintenance
Vegetative cells were grown at 22 °C in association with Klebsiella pneumoniae on SM, in a 9 cm cell culture dish (Falcon), or in shaking culture in axenic medium with 0.1 mg ml⁻¹ strep (Sigma) and appropriate antibiotic selection.

For long term storage, 10⁷ cells or more were resuspended in 1 ml of horse serum containing 5% DMSO, transferred to a 2 ml cryovial (Nunc), and frozen down slowly to –80 °C in a thick-walled airtight polystyrene container before transferring to liquid nitrogen stores.

2.1.2 Transformation of D.discoideum by electroporation
This method was used for generating G418 resistant gene over-expressing strains. Logarithmically (1-2 x 10⁶ cells ml⁻¹) growing wild type (Ax2) cells from axenic culture were harvested and washed twice in ice-cold, sterile electroporation buffer (KK₂, 50 mM sucrose) by centrifugation at 700 g for 3 min. 10⁷ cells were resuspended in 800 μl electroporation buffer per cuvette (Ø 4 mm, Equibio). 10 μg uncut plasmid DNA was added and the cell suspension was incubated on ice for 10 min. Electroporation was carried out at 1.6 kV, 3 μF using a BioRad Gene Pulser with time constants between 0.3 – 0.5 ms. The cells were allowed to recover on ice for a further 10 min before adding 8 μl of 1 mM MgCl₂/1 mM CaCl₂ and further incubation for 15 min at room temperature. The contents of each cuvette were split between four 96-well plates (Falcon). Media containing 20 μg ml⁻¹ streptomycin was added. After 24 hours, the media was replaced with media containing 20 μg ml⁻¹ strep (Sigma), appropriate selection (40μg ml⁻¹ G418; Gibco) and 1% heat-killed E. coli.

2.1.3 Development of D.discoideum
Cells were spun down from logarithmically growing culture (1-2 x 10⁶ cells ml⁻¹) and washed four times in KK₂. Cells were resuspended in 300 μl of KK₂ or KK₂ containing either LiCl (7.5 mM; 10 mM) or VPA (1 mM) respectively and spread evenly across black, nitrocellulose filters (Ø 4.7 cm, 0.45 μm pore size; Millipore) on a KK₂ - or KK₂-drug–soaked pre-filter. Filters were incubated at 22 °C in a humid atmosphere for the required length of time.
2.1.4 Immunofluorescent cell staining
Vegetative cells were taken from a log-phase culture and washed twice with KK₂. The cells density was adjusted with KK₂ to 5x 10⁵ cells ml⁻¹, and 300 μl of cells were allowed to settle on a glass coverslips (BDH) for 15 – 30 min. To fix cells, the media was removed and replaced with 200 μl 0.1% paraformaldehyde (w/v) in picric acid (pH 6.5) for 30 min. Cells were then washed in the following sequence (200 μl each): 10 mM PIPES (rinse once), PBS/glycin (rinse twice), 70% ethanol (incubate once for 10 min), and 100 mM PBS/glycin (incubate three times for 5 min each). Cells were blocked against non-specific binding by incubation with 200 μl of PBG for 15 minutes (twice), before incubation with 200 μl of the fluorescent dye DAPI (Sigma) for 15 min. Subsequently, cells were washed in the following sequence (200 μl each for 5 min): PBG (six times) and 100 mM PBS (three times), before mounting the coverslip with 20 μl PBS onto the slide, thereby avoiding air bubbles. Slides were viewed on a confocal microscope (Nikon).

2.1.5 Experimental set up for analysis of gene expression
For analysis of the gene expression profile in LisA and LisG cells, wild type (A×2) cells and mutant cells were taken from log phase cultures and 1x 10⁶ cells were transferred into a 9 cm cell culture dish (3 plates for each cell line). Cells were incubated overnight at 22 °C before preparing total RNA for LisG and mRNA for LisA.

To test the effect of the POI Z-prolinal, wild type (A×2) and LisG cells were taken from log phase cultures and 1x 10⁶ cells were transferred into a 9 cm cell culture dish. Cells were incubated in axenic media supplemented with 1.3 mM Z-prolinal or equal amount of DMSO (carrier control) at 22 °C for 24 hours before preparation of total RNA.

For analysis of the effect of mood-stabilising drugs or inositol on gene expression, 2x 10⁶ wild type (A×2) cells were taken from log phase cultures and set up in a total volume of 20 ml of axenic media supplemented with (a) VPA analogues or (b) myo-inositol or epi-inositol (1 mM and 5 mM) in a 100 ml flask and incubated shaking at 22 °C for 24 hours.
2.1.6 2D-DIAS

For analysis of *D.discoideum* cells in a spatial gradient of cAMP, cells from log phase cultures were dispersed on the bridge of a chemotaxis chamber, designed after that of Zigmond (Vamum and Soil, 1984; Zigmond, 1977), for bright-field analysis or differential interference contrast (DIC) microscopy. KK$_2$ alone was added to one of the two wells bordering the bridge and to the other KK$_2$ containing 1 µM cAMP. Cells were incubated at 22 °C for 5 min to facilitate cell adhesion and allow for the generation of a steep gradient of chemoattractant across the bridge, and then recorded for up to 15 min.

For analysis of basic motility of *D.discoideum* cells, cells were grown at low density (3x 10$^5$ cells per 9 cm cell culture dish (Falcon)) one day prior to the experiment. For the actual experiment, 40 µl from each resuspended plate were combined with 2 ml of KK$_2$ containing NaCl and LiCl. Cells were incubated at 22 °C for 1 hour, and then recorded for 10 min.

Subsequently video images were digitised onto the hard drive of a Macintosh computer at a rate of 4 frames min$^{-1}$ (15 sec intervals) for spatial-gradient analysis and at 6 frames min$^{-1}$ (10 sec intervals) for non-gradient analysis. 2D-DIAS software was then used for computer-assisted two-dimensional analyses. Cell perimeters were automatically outlined using the greyscale threshold algorithm. Outlines were converted to beta-spline replacement images. The following motility parameters were computed from the centroid positions and dynamic morphology parameters from contours of the replacement images.

Difference pictures were generated by superimposing frame $n-1$ over frame $n$. ‘Expansion zones’ were designated in green as regions in the cell outline in frame $n$ not overlapping the cell outline in frame $n-1$. ‘Contraction zones' were designated in red as regions in the cell outline in frame $n-1$ not overlapping the cell outline in frame $n$.

Experiments were set up in triplicates, with 40 cells analysed for each replicate. The Student's t-test (paired, unequal variance) was used for statistical analysis.
2.2 Molecular Biology

2.2.1 Agarose gel electrophoresis of DNA
Agarose gels were prepared in 0.5x TBE as described (Sambrook et al., 1989). The percentage of the gel was determined by the size of DNA fragments to be resolved. Ethidium bromide (0.5 µg ml⁻¹) was included in gels for visualisation of DNA under UV light. DNA was loaded in 10% v/v DNA loading buffer. 1 µg of size markers was also loaded (1 kb ladder, Bioline).

2.2.2 Polymerase chain reaction (PCR)
50 µl reactions were set up in thin-walled 200 µl tubes (Radleys), as follows:
- 0.1 µg genomic DNA from wild type (Ax2) cells
- 0.1 µl of 100 pmol µl⁻¹ 5'-primer (or ddH₂O control)
- 0.1 µl of 100 pmol µl⁻¹ 3'-primer (or ddH₂O control)
- 5 µl of dNTPs (200 µM dATP, dCTP, dTTP and dGTP; Amersham Pharmacia Biotech)
- 5 µl of 1x NH₄-buffer (Bioline)
- 2.5 U of Taq polymerase (Bioline)
Volume made up to 50 µl using ddH₂O.

For amplification of the RT-PCR plasmid standards, the DNA was denatured at 95 °C for 2 min, then subjected to 30 cycles of 95 °C for 30 sec, 55 °C for 30 sec, 68 °C for 2 min, followed by a final extension step at 68 °C for 10 min before storage at 4 °C (Perkin Elmer PTC-100 thermal cycler).

For the screening of putative LisG⁻ knock-out mutants, DNA was denatured at 95 °C for 2 min, then subjected to 30 cycles of 95 °C for 30 sec, 55 °C for 30 sec, 68 °C for 4 min, followed by a final extension step at 68 °C for 10 min before storage at 4 °C (Perkin Elmer PTC-100 thermal cycler). The following primer combinations were used: (1) LisG-KO-F1 and LisG-KO-R2 (endogenous 2 kb band should shift to 3.3 kb in a knock-out mutant); (2) LisG-KO-F1 and LisG-KO-screen (endogenous 2 kb band should shift to 3.3 kb in a knock-out mutant); (3) B2rev and LisG-KO-screen (0.9 kb band only visible when gene is knocked-out).
2.2.3 TOPO cloning of PCR fragments

All PCR fragments were cloned into pCR®II-TOPO vector (Invitrogen) according to manufacturer's instruction, and subsequently transformed into TOP10™ chemically competent E.coli (see 2.3.4). The TOPO cloning reactions utilise the following principle: Taq amplified PCR products contain overhanging 3'-deoxyadenosine, whereas the linearised pCR®II-TOPO vector contains overhanging 3'-deoxythymidine. DNA Topoisomerase-I, covalently bound to each 3'-phosphate of the linearised pCR®II-TOPO vector, ligates these overhangs efficiently with those of the PCR products. Resulting colonies were analysed by miniprep and digest analysis (see section 2.3.6 and 2.3.7).

2.2.4 Transformation of bacteria and bacterial permanents

TOP10™ chemically competent Escherichia coli (Invitrogen) were transformed as per the manufacturer's instructions. 50 μl of transformed cells were spread onto LB agar plates containing the appropriate selective antibiotic (100 μg ml⁻¹ ampicillin unless stated otherwise). Single transformed colonies were picked using a sterile loop and inoculated into L-Broth with appropriate selection. Cultures were shaken at 37 °C with 250 rpm in a tube or flask, at least 4 times the volume of the culture to ensure good aeration.

Bacterial permanents of positive clones were made by the addition of 0.5 ml of L-broth/glycerol (50:50 v/v) to 0.5 ml of the overnight culture. Stocks were stored at ~80 °C.

2.2.5 Maxiprep of bacterial plasmid DNA

For large-scale plasmid DNA preps, QIAGEN Plasmid Maxi kits (Qiagen Ltd) were used, which are based on a modified alkaline lysis procedure and utilise gravity flow anion-exchange resin columns to covalently bind plasmid DNA to immobilised diethylaminoethanol (DEAE) groups over a wide range of salt conditions.

Plasmid-containing E.coli cells were grown overnight in a 250 ml L-broth culture, supplemented with 100 μg ml⁻¹ amp. The cells were pelleted at 6000 g
for 15 min (Beckman J-25 centrifuge, JLA 10.5 rotor), resuspended homogeneously in 10 ml of buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 μg ml⁻¹ RNAseA) and lysed by adding 10 ml of buffer P2 (200 mM NaOH, 1% SDS) for 5 min with mixing by gentle inversion. Proteins, genomic DNA and cell debris were precipitated by adding 10 ml of high salt-containing buffer P3 (3 M potassium acetate pH 5.5), mixing gently, leaving on ice for 20 min, and spun out at 20 000 g for 30 min (Beckman J-25 centrifuge, JA 25.5 rotor). The supernatant containing the plasmid DNA were passed through the columns, previously equilibrated with 10 ml buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol, 0.15% TritonX-100), by gravity flow. The plasmid DNA remained tightly bound to the column, whereas the degraded RNA, proteins and small metabolites were washed from the column with 2x 30 ml of the low-salt buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 50% isopropanol). Subsequently, the plasmid DNA was eluted with 15 ml of the high-salt buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% isopropanol), desalted and concentrated by addition of 0.7 volumes of room temperature isopropanol and centrifugation at 15 500 g for 30 min. The DNA pellet was washed in 5 ml of 70% ethanol, recentrifuged for 10 min, air-dried and resuspended in 300 μl TE. Up to 500 μg plasmid DNA per column may be isolated by this method. Glycerol stocks were made as per section 2.3.4 and stored at −80 °C.

2.2.6 Miniprep of bacterial plasmid DNA

For small-scale plasmid preps, requiring sequencing quality DNA, spin miniprep kits (MoBio Laboratories Inc) were used. These utilise the same principle as the QIAGEN Plasmid Maxi kits to purify plasmid DNA. A typical yield from an individual prep is 5-10 μg plasmid DNA from a 1.5 ml bacterial culture.

For all applications other than sequencing, DNA was prepared from overnight bacterial cultures via the rapid boiling method (Harwood, 1996). 1.5 ml of Plasmid-containing E.coli were spun out at 12 000 g for 1 min in a benchtop centrifuge and the medium removed. The pellet was vortexed and the cells lysed in 200 μl STET containing 1 mg ml⁻¹ lysozyme in boiling water for exactly 45 sec. Bacterial genomic DNA and protein were precipitated as a fluffy white pellet by spinning the lysate at 12 000 g for 10 min and removed from the
supernatant using a toothpick. The remaining plasmid DNA and RNA was precipitated at room temperature by addition of 200 μl of isopropanol to the supernatant and centrifugation at 12 000 g for 5 min. The precipitate was washed in 100 μl of 70% ethanol, air-dried for 10 min and resuspended in 100 μl TE. Typical yields of 1-5 μg plasmid DNA may be obtained via this method.

2.2.7 Restriction enzyme digestion analysis of plasmid DNA
0.1-1 μg of DNA was usually digested with 1 μl of appropriate restriction enzyme, according to the manufacturers instructions. All restriction enzymes were obtained commercially from New England Biolabs and digests were performed in a 20 μl volume of 1x appropriate manufacturer’s enzyme buffer for 1 h at 37 °C. For analysis, half the digest was added to 1 μl of 10x DNA loading buffer and DNA fragments separated on TBE agarose gels.

2.2.8 Gel purification of DNA fragments
DNA fragments were separated on 0.8% TBE/agarose gels, the relevant bands excised and the DNA purified using QIAquick Gel Extraction Kit (Quiagen Lmt.). In brief, gel slices were melted in > 3 gel volumes of buffer QG (6 M NaI, 0.12 M Na₂SO₃, 0.3 M sodium acetate) for 10 min at 50 °C. The supernatant was transferred onto a QIAquick spin column and the DNA bound to a silica resin column by spinning at 12000 g for 1 min (Note that guanidine thiocyanate chelates the water and forces the DNA to bind to the column). The DNA was washed with 750 μl buffer PE (contains ethanol), sped dry for an additional 1 min and eluted with 50 μl of buffer EB (10 mM Tris-Cl, pH 8.5). 4 μl were run out on a 0.8% agarose gel to determine the yield.

2.2.9 Ligation
Ligation reactions were set up with molar ratios of 2:1 and 10:1 (insert:vector) in a total volume of 20 μl. 0.2 μl of T₄ DNA ligase (New England Biolabs) was added to each ligation reaction and incubated at room temperature for 2 hours.

For the creation of double-stranded oligonucleotides, 0.1 μg of each strand of oligonucleotides were annealed at 60 °C for 1 hour in a volume of 20 μl of 1x T₄ DNA ligase (New England Biolabs). For the insertion of double-stranded
oligonucleotide into the vector, ligation reactions were set up with molar ratios
of 50:1 and 100:1 (double-stranded oligo:vector) in a total volume of 10 µl. 0.2
µl of T₄ DNA ligase (New England Biolabs) was added to each ligation reaction
and incubated at room temperature for 2 hours.

2.2.10 Sequencing and analysis of DNA
Sequences were obtained from 1 µg plasmid DNA or from 5 µg PCR products.
PCR fragments were purified away form the PCR reaction using spin columns
(S400, Amersham Pharmacia Biotech), according to the manufacturer’s
instructions. All DNA was sequenced by MWG Biotech Ltd. The raw sequence
data was analysed using the Lasergene software (DNASTar™).

2.2.11 Northern Blot analysis
Total RNA was prepared from wild type (Ax2) cells treated with the POI Z-
prolinal or DMSO (carrier control) for 24 hours using the High Pure RNA
isolation kit (Roche) according to the manufacturer’s instructions; mRNA was
prepared from wild type (Ax2) and LisA cells using the mRNA isolation kit
(Roche) according to the manufacturer’s instructions. RNA concentration was
determined by measuring the Aₒ₆₀ on a spectrophotometer. RNA was prepared
for electrophoresis in 1x MOPS buffer, 0.2 M formaldehyde, 50% v/v formamide
and 10% v/v sterile RNA loading dye, denatured at 60 °C for 15 min and cooled
on ice.

5 µg of total RNA per lane (or the equivalent of mRNA) was loaded on a 1%
agarose gel containing 1x MOPS buffer, 0.65 M formaldehyde and ethidium bromide to a final concentration of 0.25 µg ml⁻¹. The gel was run in 1x MOPS buffer at 120 V for 3.5 hours. After electrophoresis, RNA was visualised under UV light and photographed before equilibrating in 10x SSC (see section 2.4 for recipe) and blotting onto Hybond N nylon membrane (Amersham Pharmacia Biotech) by capillary transfer in 10x SSC. The RNA was UV-crosslinked to the membrane (Stratalinker, Stratagene).

Filters were prehybridised at 42 °C for 2 hours in RNA hybridisation buffer and
then hybridised with the denatured probe. For this, Ino1 or Ig7 PCR products
were labelled with $[\alpha^{32}\text{P}]$ dATP using the Megaprime DNA labelling kit (Amersham Pharmacia Biotech). Unincorporated nucleotides were removed by purifying the probe on MicroSpin™ S-400 HR columns (Amersham Pharmacia Biotech). The purified probes were denatured by boiling for 5 min before being added to RNA hybridisation buffer, and incubated with filters overnight at 42 °C. Unbound probe was removed with 2x SSC/0.1% SDS washes (2x 15 minutes), followed by 0.5x SSC/0.1% SDS (2x 15 minutes). Filters were sealed in plastic and exposed against a phosphorimaging screen (K-Screen, Kodak), before imaging in BioRad phosphorimagger.

2.2.12 Southern Blot analysis
Genomic DNA was prepared from *D.discoideum* cells as follows: $10^7$ cells were spun down at 700 g for 2 min, washed once in KK$_2$ and resuspended in 500 μl of DNAzol (Invitrogen) and transferred to an eppendorf tube. Cell debris was pelleted at 12000 g for 10 min, and the supernatant transferred to a fresh eppendorf tube. 250 μl of 100% ethanol was added, and the mixture incubated at room temperature for 5 min before precipitation of the genomic DNA by centrifugation at 12000 g for 5 min. The pellet was washed in 500 μl of 70% ethanol, air-dried for 10 min and resuspended in 20 μl of 8 mM NaOH.

10 μl of the genomic DNA preparation was digested in a total volume of 20 μl at 37 °C with appropriate restriction enzyme (New England Biolabs) in 1x manufacturer's buffer. The enzyme volume in the reaction was kept at less than 5% of the total volume. The whole digest was run out on a 20 cm long 0.7% TBE/agarose gel. The gel was visualised under UV light to confirm digestion of the DNA. The gel was first soaked in depurination solution for 10 min, then in denaturation solution for 30 min and finally in neutralisation solution for 30 min. The DNA was transferred and fixed onto charged nylon membrane (Hybond N*, Amersham Pharmacia Biotech) by capillary transfer in 10x SSC as described previously (Sambrook *et al.*, 1989). After transfer the membrane was rinsed in 2x SSC, air-dried and the DNA was UV-linked to the membrane (Stratalinker, Stratagene).
High stringency probing of Southern blots was carried out as described previously (Church and Gilbert, 1984): In brief, filters were prehybridised at 63 °C for 30 min in Church and Gilbert buffer, and then hybridised with the denatured probe. For this, a gene specific PCR product was labelled with [α-32P] dATP using the Megaprime DNA labelling kit (Amersham Pharmacia Biotech). Unincorporated nucleotides were removed by purifying the probe on MicroSpin™ S-400 HR columns (Amersham Pharmacia Biotech). The purified probes were denatured by boiling for 5 min before being added to 20 ml of Church and Gilbert buffer, and incubated with filters overnight at 63 °C. Unbound probe was removed with 2x SSC/0.1% SDS washes (2x 15 minutes), followed by 0.5x SSC/0.1% SDS (2x 15 minutes). Filters were sealed in plastic and exposed against a phosphorimaging screen (K-Screen, Kodak), before imaging in BioRad phosphorimager.

2.2.13 Real time-PCR analysis

*Dictyostelium* cells were set up for analysis of gene expression as described (see section 2.2.2), and RNA was prepared using the High Pure RNA isolation kit (Roche) according to the manufacturer's instructions. RNA concentration was determined by measuring the A260 on a spectrophotometer. 1 μg of total RNA was converted into cDNA using the First strand synthesis kit (Roche) according to manufacturer's instructions. The cDNA was then diluted 1:10 (equals 100 ng of total cDNA) for use as template in the RT-PCR reaction. Subsequently, 25 μl reactions were set up in thin-walled clear 200 μl 96-well plates (Abgene), as follows:

- 1 μl of 100 ng cDNA dilution (or ddH2O control)
- 0.1 μl of 100 pmol μl⁻¹ 5'-primer
- 0.1 μl of 100 pmol μl⁻¹ 3'-primer
- 12.5 μl Sybr Green master mix (Abgene)

Volume made up to 25 μl using ddH2O.

The polymerase was activated at 95 °C for 15 min, and subsequently the cDNA was subjected to one cycle of 95 °C for 15 min (enzyme activation), then 30 cycles of 95 °C for 15 sec, 57 °C for 20 sec, 68 °C for 25 sec, plate read,
followed by the generation of a melting curve (50 – 90 °C, read every 0.2 °C for 1 sec) before storage at 4 °C (DNA Engine Opticon®-2 System; MJ Research Inc.). The data was analysed using the 2-ΔΔCT-method as described (Livak and Schmittgen, 2001). RT-PCR reactions were set up in triplicates and paired Student’s t-test was used for the statistical analysis.

Note that the RT-PCR assay conditions were optimised by gradient PCR (between 50 and 60 °C) in order to run all PCR reactions analysing transcript abundance for Ino1, IMPase, IPP1/2, 5P2/3/4, dpoA, LisG and Ig7 simultaneously. The melting temperature of RT-IG7-F2 lies above the chosen annealing temperature by only 0.1 °C. As the probability of primer annealing is reduced when the annealing temperature is too close to the melting temperature, product amplification for Ig7 would be expected to be less than ideal. Therefore, this primer should be optimised in future assay designs.

2.2.14 Oligonucleotides

The following oligonucleotides were used in (1) the RT-PCR reaction and to construct the DNA plasmid standards, and used for (2) construction of the LisG knockout construct and (3) screening of putative LisG knock-outs:

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Used for</th>
<th>Sequence (5'-3')</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-Ino1-For</td>
<td>(1)</td>
<td>GACACCGTCGTCGTTATGTGGTGTCGG</td>
<td>67.9</td>
</tr>
<tr>
<td>RT-Ino1-Rev</td>
<td>(1)</td>
<td>GCAATCATATCATCGACAACATTGG</td>
<td>58.1</td>
</tr>
<tr>
<td>RT-IMPase-For2</td>
<td>(1)</td>
<td>GGTAATGAACCAACATGGGTAATTG</td>
<td>59.7</td>
</tr>
<tr>
<td>RT-IMPase-Rev</td>
<td>(1)</td>
<td>GCTGTACCTGAGAAATCTTAATGCTTGAC</td>
<td>63.9</td>
</tr>
<tr>
<td>RT-IPP1-For</td>
<td>(1)</td>
<td>AGAGGAAACATGTGAAATACCAAC</td>
<td>57.6</td>
</tr>
<tr>
<td>RT-IPP1-Rev</td>
<td>(1)</td>
<td>TTGAACCTTTATCATGAAATGAGGCC</td>
<td>58.9</td>
</tr>
<tr>
<td>RT-IPP2-For</td>
<td>(1)</td>
<td>GATCAAGAGGAACCAATTAAGG--TTTC</td>
<td>60.1</td>
</tr>
<tr>
<td>RT-IPP2-Rev</td>
<td>(1)</td>
<td>CCTTTTGAGTATCTAATTTGGTGTTC</td>
<td>55.3</td>
</tr>
<tr>
<td>RT-Dd5P2-For</td>
<td>(1)</td>
<td>GGTTCAATCAGTATGGTCATTGGGATTG</td>
<td>64.0</td>
</tr>
<tr>
<td>RT-Dd5P2-Rev</td>
<td>(1)</td>
<td>GTGAACATCTTGTTCCCTTTGTGAAGG</td>
<td>64.0</td>
</tr>
<tr>
<td>RT-Dd5P3-For</td>
<td>(1)</td>
<td>AGATTGGTGTGCTGCCAAATATAACAGG</td>
<td>62.2</td>
</tr>
<tr>
<td>RT-Dd5P3 Rev</td>
<td>(1)</td>
<td>TGCAATTGAAGGCTGGTTGGTGGTCGC</td>
<td>63.2</td>
</tr>
<tr>
<td>RT-Dd5P4-For</td>
<td>(1)</td>
<td>GTATTATCAATTTAGGATCAATCAACACC</td>
<td>59.6</td>
</tr>
<tr>
<td>Oligo name</td>
<td>Used for</td>
<td>Sequence (5'-3')</td>
<td>Tm (°C)</td>
</tr>
<tr>
<td>--------------------</td>
<td>----------</td>
<td>------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>RT-Dd5P4-Rev</td>
<td>(1)</td>
<td>CTGGTTGTGTCAACCACATAGCAATG</td>
<td>63.0</td>
</tr>
<tr>
<td>RT-dpoA-For</td>
<td>(1)</td>
<td>GTGGTCAATCTACAATTGCTCAATCG</td>
<td>64.8</td>
</tr>
<tr>
<td>RT-dpoA-Rev</td>
<td>(1)</td>
<td>GCCATTAAAGGTTTCTTAACGTGG</td>
<td>58.9</td>
</tr>
<tr>
<td>RT-LisG-For</td>
<td>(1)</td>
<td>GCCAGGTTCATGATGGGAAAGAGATTG</td>
<td>61.6</td>
</tr>
<tr>
<td>RT-LisG-Rev</td>
<td>(1)</td>
<td>ACATATAGATTATGCTCAACATTGCAG</td>
<td>59.3</td>
</tr>
<tr>
<td>RT-Ig7-F2</td>
<td>(1)</td>
<td>GTACTTAACACCGACACTGGTTATTG</td>
<td>57.1</td>
</tr>
<tr>
<td>RT-Ig7-R2</td>
<td>(1)</td>
<td>CGCTACCTTAGAACGCAGTCAATGTTAC</td>
<td>64.8</td>
</tr>
<tr>
<td>LisGKO-F1-66</td>
<td>(2);(3)</td>
<td>ATGGGTCTGGGTAAGACCCATTC</td>
<td>58.4</td>
</tr>
<tr>
<td>LisGKO-F2-72</td>
<td>(2)</td>
<td>CATATGATTTACATAATCGTAATATACCTACG</td>
<td>62.2</td>
</tr>
<tr>
<td>LisGKO-R1-68</td>
<td>(2)</td>
<td>CCATGGCCTCTAAAATATTCAAGCATTCAACC</td>
<td>64.4</td>
</tr>
<tr>
<td>LisGKO-R2-64</td>
<td>(2);(3)</td>
<td>ATGAAGAGCTGAACCCACTTTGC</td>
<td>58.4</td>
</tr>
<tr>
<td>LisG KO screen</td>
<td>(3)</td>
<td>CTGGAACCAATTGGCCAGGAGATTCTTC</td>
<td>62.1</td>
</tr>
<tr>
<td>B2rev</td>
<td>(3)</td>
<td>CGAGGTGTATCATATGCGCATGG</td>
<td>60.6</td>
</tr>
<tr>
<td>5'Clal-KpnI-Pstl3'</td>
<td>(2)</td>
<td>CGATGGTACCCTGCA</td>
<td>N/A</td>
</tr>
<tr>
<td>3'Clal-KpnI-Pstl5'</td>
<td>(2)</td>
<td>GGGTACCAT</td>
<td>N/A</td>
</tr>
</tbody>
</table>

2.2.15 Plasmids

The plasmid constructs used in this thesis were generated as follows:

- **pTX-GFP**: G418-resistance vector allowing transactivated Ddp2-based extrachromosomal replication and expression of GFP-tagged cDNA due to insertion of cDNAs into multiple cloning site (Levi et al., 2000).

- **pTX-IMPase-GFP**: The coding region of IMPase was amplified from the cDNA clone FCBP15 obtained from the Japanese *D discoideum* cDNA project (Morio et al., 1998)\(^1\) using the primer pair IMP5 and M13-forward and cloned into the BluntII Topo vector (Invitrogen). The IMPase insert was cut out from the pBluntII-IMPase vector with *B*glI- *XhoI* and ligated into the pTX-GFP vector cut with *BamH*I- *XhoI* (gift from M. Capur).

- **pTX-Ino1-GFP**: The coding region of Ino1 was amplified from the cDNA clone SLB678 obtained from the Japanese *D discoideum* cDNA project (Morio et al., 1998)\(^1\) using the primer pair INO5 and M13-forward and cloned

---

\(^1\) Supported by the Japan Society for the Promotion of Science (RFTF96L00105) and the Ministry of Education, Science, Sports and Culture of Japan (08283107).
http://www.csm.biol.tsukuba.ac.jp/cDNAPrOJECT.html
into the BluntII Topo vector (Invitrogen). The Ino1 insert was cut out from the pBluntII-IMPase vector with BglII-XhoI and ligated into the pTX-GFP vector cut with BamHI-XhoI (gift from M.Capur).

- **pLPBLP-LisG-KO-mut#1**: The 5’-part of the LisG knock-out construct was amplified from genomic DNA using the primer pair LisG-F1 and LisG-R1 and cloned into pCR-TOPO-2.1 vector (Invitrogen); the 3’-part of the LisG knock-out construct was amplified from genomic DNA using the primer pair LisG-F2 and LisG-R2 and cloned into pCR-TOPO2.1 vector (Invitrogen). Then, the 5’-part was cut out of the pCR-TOPO-2.1 with KpnI and NcoI, and ligated into the pLPBLP vector (gift from L. Kreppel), also cut with KpnI and NcoI; next, the 3’-part was cut out of the pCR-TOPO-2.1 with NdeI and NotI, and ligated into the pLPBLP vector containing the 5’part of the knock-out construct, also cut with NdeI and NotI; last, a KpnI-site was introduced at the 3’-end of the knock-out construct by ligating a KpnI-site containing oligo (5’-Clal-KpnI-Pstl-3’/3’-Clal-KpnI-Pstl-5’) into the with Clal and Pstl cut vector. The pLPBLP vector contains an ampicillin resistance gene for bacterial selection and the blasticidin resistance cassette for selection in *D.discoideum*; the blasticidin gene is expressed from an Actin 6 promoter and is also flanked on either side with P-lox sequences, allowing excision of the blasticidin cassette.

### 2.3 Biochemistry

#### 2.3.1 Protein separation and western blotting of whole cell extracts

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins for western blotting. In brief, approximately $10^7$ logarithmically growing cells were pelleted at 2000 g for 2 min, lysed by boiling for 5 min in Laemmli buffer, and debris pelleted at 20 000 g for 5 min before loading on a polyacrylamide gel. $10^3$ to $10^6$ cell equivalents per lane were loaded on to a SDS-gel (Invitrogen).

Electrophoresis was at 150 V for 1.5 hours, and coloured molecular weight standards (Rainbow markers, Amersham) were run alongside samples for size determination. After electrophoresis, some gels were stained with Coomassie
Blue for 30 min and destained over several hours with 4-5 changes of destain to visualise total protein. All other gels were equilibrated in western transfer buffer for 10 min.

Protein was electrophoretically transferred from gels to Hybond C-extra nitrocellulose membrane (Amersham Pharmacia Biotech) by semi-dry blotting in a BioRad transfer cell according to the manufacturer's instructions. The membrane was rinsed in phosphate buffered saline (PBS) and proteins were visualised using 2% PonceauS (w/v) in 1% acetic acid (v/v) to ensure even transfer and loading. Membranes were blocked for 1 hour at room temperature in 5% dried skimmed milk (w/v) containing 0.1% Tween-20 (v/v). After three 5 min washes in PBS containing 0.1% Tween-20 (v/v) (PBST) membranes were incubated in primary antibody diluted in PBST for 1 hour at room temperature. After incubation, the filters were washed three times in PBST for 5 min, and subsequently incubated with an HRP-conjugated secondary antibody diluted in PBST for a further hour. After another three washes, the secondary antibody was detected using enhanced chemoluminescence reagent (Supersignal™, Pierce & Warringer Ltd) according to the manufacturer's instructions and exposure to Biomax film (Kodak).

2.3.2 Antisera

The following antibodies were used in this work:

Primary

- **GFP**: Rabbit polyclonal IgG, which recognises the epitope corresponding to amino acids 1-238 of green fluorescent protein (GFP) of Aequorea victoria.
  Used at 1: 2000 for western blotting (gift of J. Faix).

Secondary

- **Anti-rabbit IgG (H+L)**: Horseradish peroxidase-conjugated antibody, raised in goats. Used at 1:10 000 for western blotting (Cell signalling Inc.).
2.3.3 IMPase activity assay

The IMPase activity assay described by Kaya et al. was adapted for the use on Macs microbead columns (Kaya et al., 2004). In brief, approximately 10⁶ cells were harvested by centrifugation at 2000 g for 3 min and lysed by incubation with 1 ml of Lysis buffer (150 mM NaCl, 1% TritonX-100, 50 mM Tris-HCl pH 8.0) on ice for 30 min. Cell debris was pelleted by centrifugation at 10 000 g at 4 °C for 10 min. The GFP-tagged IMPase protein was bound via the epitope to MACS microbead columns (Miltenyi Biotech, UK) by incubating the supernatant with 50 µl of Anti-Tag Microbeads at 4 °C for 30 min. After the labelling procedure has finished, the cell lysate was applied onto with Lysis buffer equilibrated µ column, which was placed in the magnetic field of the µMACS Separator beforehand. Subsequently, columns were washed four times with 200 µl of Wash buffer 1 (150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0), once with 100 µl of the IMPase assay buffer and incubated with 25 µl of IMPase assay buffer (0.7 mM myo-inositol-1-phosphate, 50 mM Tris-HCl pH 7.8, 250 mM KCl, 3 mM MgCl₂, 10 mg ml⁻¹ BSA) at 37 °C for 30 min. After the enzyme reaction has finished, the released phosphate was eluted from the column with 100 µl of IMPase assay buffer.

Phosphate release was determined via a colorimetric assay as described (Itaya and Ui, 1966). 40µl of the eluate containing the released phosphate was added to 120 µl of ddH₂O and 40 µl of Malachite green dye reagent, incubated at room temperature for exactly 10 min before measuring absorbance at 630 nm. 1 µM to 40 µM of sodiumhydrogen phosphate was used to construct the malachite green standard curve (see Appendix 8.1).

Columns were washed free of residual salt and detergent with 100 µl of Wash buffer 2 (20 mM Tris-HCl pH 7.5) and incubated with 20 µl of pre-heated 95 °C hot elution buffer (50 mM Tris-HCl pH 6.8, 50 mM DTT, 1% SDS, 1 mM EDTA, 0.005% bromophenol blue, 10% glycerol) at room temperature for 5 min. The bound IMPase protein was eluted with 50 µl of pre-heated elution buffer (95 °C) and analysed by SDS-PAGE.
2.3.4 Ino1 activity assay

Approximately 10^8 cells were harvested by centrifugation at 2000 g for 3 min and lysed by incubation with 1 ml of Lysis buffer (150 mM NaCl, 1% TritonX-100, 50 mM Tris-HCl pH 8.0) on ice for 30 min. Cell debris was pelleted by centrifugation at 10 000 g at 4 °C for 10 min. The GFP-tagged IMPase protein was bound via they epitope to MACS microbead columns (Miltenyi Biotech, UK) by incubating the supernatant with 50 µl of Anti-Tag Microbeads at 4 °C for 30 min. After the labelling procedure has finished, the cell lysate was applied onto with Lysis buffer equilibrated µ column, which was placed in the magnetic field of the µMACS Separator beforehand. Subsequently, columns were washed four times with 200 µl of Wash buffer 1 (150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0), once with 100 µl of the IMPase assay buffer and incubated with 25 µl of Ino1 assay buffer (0.7 mM glucose-6-phosphate, 50 mM Tris-HCl pH 7.8, 250 mM KCl, 3 mM MgCl2, 10 mg ml^-1 BSA) at 37 °C for 30 min. After the enzyme reaction has finished, the produced InsP_1 was eluted from the column with 100 µl of Ino1 assay buffer. 2.5-10 units of recombinant IMPase from bovine brain was added to the collected eluate, the reaction incubated at 37 °C for a further 30 min and phosphate release was measured using the malachite green method as described in section 2.3.3.

Columns were washed free of residual salt and detergent with 100 µl of Wash buffer 2 (20 mM Tris-HCl pH 7.5) and incubated with 20 µl of pre-heated 95 °C hot elution buffer (50 mM Tris-HCl pH 6.8, 50 mM DTT, 1% SDS, 1 mM EDTA, 0.005% bromophenol blue, 10% glycerol) at room temperature for 5 min. The bound IMPase protein was eluted with 50 µl of pre-heated elution buffer (95 °C) and analysed by SDS-PAGE.

2.3.5 DpoA activity assay

DpoA activity was measured as described (Williams et al., 1999). In brief, wild type (Ax2) and LisG cells were harvested and washed in KK_2, and resuspended to give 2x 10^8 cell ml^-1 in homogenisation buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (DTT), 5 µg ml^-1 aprotinin, 1 mM benzamidine, 10 µg
µl⁻¹ leupeptin, 0.1 mM AEBSF). Cells were lysed by sonication, and soluble fractions were prepared by centrifugation (100 000 g, 30 min, 4 °C).

Total protein content was determined via a dye-binding method, set up in triplicate (Bradford, 1976): 2 µl of the prepared soluble fraction was added to 198 µl of Bradford reagent (Biorad Laboratories GmbH), and absorbance was measured at 595 nm. 2.5 to 10 mg of bovine serum albumin (BSA) was used to calibrate the protein assay.

DpoA assays were carried out in triplicate by combining calculated 2.5 or 5 µg of total protein with 60 µl of assay buffer (50 mM HEPES pH 7.8, 1 mM EDTA, 1 mM DTT, 0.25% dimethylsulfoxide (DMSO), 141 µM Z-Gly-Pro-pNA) with or without the presence of 1.3 mM POI Z-prolinal and incubating at 37 °C for 2 hours. Units of enzyme activity were calculated from the increase in absorbance at 405 nm in 2 hours, measured in duplicate using a microtitre plate reader.

2.4 Recipes and reagents
Standard laboratory chemicals were from BDH. Other chemicals were from Sigma Chemical Company unless otherwise stated. All chemicals were reagent grade or better.

2.4.1 Media
Axenic medium
- 1.43% peptone (Oxoid, L34)
- 0.72% yeast extract (Oxoid L21)
- 3.6 mM Na₂HPO₄, 3 mM KH₂PO₄
- 30% glucose
- 0.5 mg ml⁻¹ vitamin B12, 1 mg ml⁻¹ folic acid pH 9.0
Final pH 6.4 (sterile).

HEPES-HL5
- 20 mM HEPES pH 7.05
- 0.5% yeast extract (Oxoid L21)
1% peptone (Oxoid L34)
1% glucose

**HBS**
- 270 mM NaCl
- 10 mM KCl
- 1.2 mM Na$_2$HPO$_4$
- 40 mM HEPES pH 7.05
- 0.2% glucose

**KK$_2$**
- 15.5 mM KH$_2$PO$_4$
- 3.8 mM K$_2$HPO$_4$
Final pH 6.2.

**L-Broth**
- 1% bactotryptone (Difco)
- 0.5% bacto-yeast extract (Difco)
- 17 mM NaCl
Final pH 7.0 (sterile).

**LB agar**
- L-broth
- 1.5% Bactoagar (Difco)

**SM (Sussman's medium)**
- 1% glucose
- 1% peptone (Oxoid L34)
- 0.1% yeast extract (Oxoid L21)
- 2% agar (Difco)
- 4 mM MgSO$_4$, 4 mM KH$_2$PO$_4$, 6 mM K$_2$HPO$_4$
2.4.2 Cell biology

PBG
In 1L PBS:
- 5 g BSA
- 1 g fish gelatin
Sterile filter.

PBS/Glycin
- 500 ml PBS
- 3.75 g glycin
Sterile filter.

Picric acid
- 0.4 g paraformaldehyde
- 5 ml ddH$_2$O
- drops 1 M NaOH
Heat to 40 °C until solution becomes translucent, then make up to 7ml
Add:
- 10 ml 20mM PIPES
- ml saturated picric acid solution
Final pH 6.5.

2.4.3 Molecular biology

50x Denhardt's solution
- 1% BSA (FractionV)
- 1% Ficoll
- 1% Polyvinylpyrrolidione

DNA loading buffer
- 0.25% bromophenol blue
- 0.5% xylene cyanol
- 15% Ficoll-400
Depurination solution
- 0.25 M HCl

Denaturation solution
- 1.5 M NaCl
- 1.5 M NaOH

Church and Gilbert buffer
- 500 mM Na₂HPO₄/NaH₂PO₄ pH 7.2
- 7% SDS

MOPS buffer
- 20 mM MOPS pH7.0
- 5 mM Na Acetate
- 1 mM EDTA

Neutralisation solution
- 1.5 M NaCl
- 0.5 M Trizma base

RNA buffer
- 100 mM Tris-HCl, pH 7.4
- 200 mM NaCl,
- 20 mM EDTA

RNA hybridisation buffer
- 43% v/v formamide
- 5x SSC
- 10x Denhardt's solution
- 10 mM Na₂HPO₄/NaH₂PO₄ pH 6.8
- 200 mg ml⁻¹ denatured, sheared herring sperm DNA
- 0.1% SDS
**RNA loading dye**
- 50% v/v glycerol
- 1 mM EDTA
- 0.4% w/v bromophenol blue
- 0.4% w/v xylene cyanol

**SSC**
- 150 mM NaCl
- 15 mM Na$_3$citrate

**STET**
- 50 mM Tris-HCl pH 8.0
- 50 mM EDTA
- 8% sucrose
- 5% Triton-X 100

**0.5x TBE**
- 45 mM Tris-HCl
- 45 mM Boric acid
- 1 mM EDTA

**TE**
- 10 mM Tris-HCl pH 7.4
- mM EDTA

**2.4.4 Biochemistry**

**Coomassie destain**
- 25% methanol
- 16% acetic acid

**Coomassie stain**
- 0.25% Coomassie Brilliant Blue
- 10% acetic acid
- 45% methanol
DpoA assay buffer
- 50 mM HEPES pH 7.8
- 1 mM EDTA
- 1 mM DTT
- 0.25% dimethylsulfoxide (DMSO)
- 141 µM Z-Gly-Pro-pNA

DpoA homogenisation buffer
- 50 mM Tris-HCl pH 7.4
- 1 mM EDTA
- 1 mM dithiothreitol (DTT)
- 5 µg µl⁻¹ aprotinin
- 1 mM benzamidine
- 10 µg µl⁻¹ leupeptin
- 0.1 mM AEBSF

Laemmli buffer
- 10% glycerol
- 100 mM DTT
- 2% SDS
- 50 mM Tris-HCl pH 6.8
- 0.1% bromophenol blue

IMPase assay buffer
- 0.7 mM myo-inositol-1-phosphate
- 50 mM Tris-HCl pH 7.8
- 250 mM KCl
- 3 mM MgCl₂
- 10 mg ml⁻¹ BSA

Ino1 assay buffer
- 0.7 mM glucose-6-phosphate
- 50 mM Tris-HCl pH 7.8
- 250 mM KCl
- 3 mM MgCl₂
- 10 mg ml⁻¹ BSA

**Malachite green stock solution**
- 0.44 g malachite green
- 360 ml 3 M sulphuric acid
  Stable for at least 1 year.

**Malachite green dye reagent**
- 2.5 ml 7.5% (w/v) ammonium molybdate
- 10 ml malachite green stock solution
  To be prepared fresh on the day when used.

**PBS**
- 137 mM NaCl
- 2.68 mM KCl
- 7.98 mM Na₂HPO₄
- 1.47 mM KH₂PO₄
  Final pH 7.2.
Chapter 3

Changes in gene expression are observed in the lithium resistant mutants LisA and LisG
3.1 Introduction

As lithium has been shown to lead to an increase in the expression of *ino1* (Shamir *et al*., 2003; Vaden *et al*., 2001; Williams *et al*., 2002), it has been suggested that lithium could exert some of its effect in the cell via modulating gene expression. If this were the case, it would be expected that changes in gene expression also occur in mutants that are resistant to the drug. Therefore, the first aim of my thesis was to investigate whether changes in gene expression can be observed in lithium resistant mutants.

In this chapter I describe the use of northern blot analysis to study changes in *ino1* expression in the LisA mutant. These findings were extended to other key enzymes of the InsP-pathway by establishing a Real-Time PCR assay. Finally, changes in gene expression were investigated in the lithium resistant mutant LisG.

3.2 *ino1* expression levels are increased in the LisA mutant

Wild type (Ax2) and LisA mutant cells were taken from log phase culture and plated into three plates of 10⁶ cells. The cells were incubated for 24 hours before harvest for mRNA extraction. The equivalent of 5 μg of total RNA as mRNA per lane was separated by gel electrophoresis and transferred to nitrocellulose by northern blotting. Blots were probed with a [α-³²P] dATP labelled PCR product for *ino1* and the loading control *lg7*, and expression was quantified using a BioRad phosphorimager screen. Changes in *ino1* expression were normalised to expression of the loading control *lg7* for the individual cell type, before comparing expression between LisA mutant and wild type (Ax2) cells.

As seen in Figure 3.1, *ino1* expression was increased in the LisA mutant by about 75% as compared to wild type (Ax2) cells (p-value < 0.05; paired Student’s t-test). The percentage increase depicts the average increase including SEM, calculated from three independent repeats.
Figure 3.1 *ino1* expression in the lithium resistant mutant LisA. mRNA was isolated from wild type (Ax2) and LisA cells and analysed by northern blotting for *ino1* expression (displayed as percentage of the loading control *Ig7*). *ino1* expression is increased in the LisA mutant by 75%. Averages from three independent repeats including SEM are displayed. * p-value < 0.05 (Paired student’s t-test).
3.3 *ino1* expression levels are also increased in PO-Inhibitor treated wild type cells

To confirm this result, wild type (Ax2) cells were set up as before in the presence of 1.3 mM of the prolyl oligopeptidase inhibitor Z-prolinal or DMSO as carrier control respectively for 24 hours. 5 μg of total RNA was separated by gel electrophoresis and transferred to nitrocellulose by northern blotting. Blots were probed with a [α-32P] dATP labelled PCR product for *ino1* and the loading control *lg7*, and expression was quantified using a BioRad phosphorimager screen. Changes in *ino1* expression were normalised to expression of the loading control *lg7* for the individual cell type, before comparing expression between POI treated and carrier control (DMSO) treated wild type (Ax2) cells.

As seen in Figure 3.2, treating wild type cells with the inhibitor also increased *ino1* gene expression by 41% as compared to cells treated with DMSO (p-value < 0.01; paired Student’s t-test). The percentage increase depicts the average increase including SEM, calculated from three independent repeats.
**Figure 3.2 Ino1 expression in wild type cells treated with a PO-Inhibitor.** mRNA was isolated from wild type cells treated with DMSO (carrier control) and the PO-Inhibitor Z-prolinal (POI) and analysed by northern blotting for ino1 expression (displayed as percentage of the loading control Ig7). Ino1 expression is increased in cells treated with Z-prolinal by 42%. Averages from three independent repeats inculding SEM are displayed. *** p-value < 0.001 (Paired student's t-test).
3.4 Establishing a Real Time-PCR assay to investigate gene expression

A variety of methods can be used for studying gene expression such as Northern Blot and semi-quantitative PCR. Recently new developments in fluorescent PCR led to the establishment of Real-time PCR (RT-PCR), which is easier to set up in greater numbers and more accurate than Northern Blot or semi-quantitative PCR. RT-PCR data obtained during this project was analysed using the $2^{\Delta\Delta CT}$ method as described by Livak et al. (Livak and Schmittgen, 2001). This method is based on relative quantification and relates the PCR signal of the target to that of the control (i.e. untreated sample).

3.4.1 Genes examined

Inositol signalling is required for aggregation and PO is known to affect this. To get a more complete picture of how different enzymes in the InsP pathway are regulated, the following enzymes were chosen for the RT-PCR assay: IMPase, ino1, PO, IPP1, IPP2 and the 5'phosphatases 5P2, 5P3 and 5P4. The sequences for the genes were downloaded from the *D.discoideum* database (Chisholm et al., 2006)$^2$.

The amino acid sequence of ino1 (DDB0231710), identified by Fischbach et al., showed 45 – 60% identity to inositol-1-synthases from other eukaryotic organisms (Fischbach et al., 2006). The three *D.discoideum* inositol 5'-phosphatases 5P2, 5P3 and 5P4 were cloned and identified by Loovers et al. (Loovers et al., 2003). Their catalytic domains are highly conserved with the catalytic domains of type II inositol 5'-phosphatases from human and yeast: (1) 5P2 encodes a homologue of mouse Inositol polyphosphate-5-phosphatase B (41% identity, 59% similarity); (2) 5P3 encodes a homologue of human synaptojanin-1 (37% identity, 59% similarity); (3) 5P4 encodes a homologue of human Inositol polyphosphate-5-phosphatase B (44% identity, 60% similarity) (Loovers et al., 2003). DpoA (DDB0185041), identified as a prolyl

---

oligopeptidase by Williams et al., shows 41% identity to its human homologue, with the highest homology occurring within the catalytic domain (Williams et al., 1999). The Ig7 gene (DDB0169550) is located on the mitochondrial genome of D.discoideum, and is used as an internal standard in the RT-PCR analysis.

D.discoideum possesses three enzymes with IMPase-like activity, only one of which is lithium sensitive (Van Dijken et al., 1996). A homology search of the D.discoideum genome database using the Blast algorithm and subsequent sequence alignment analysis using the ClustalW algorithm confirmed the identity of the family of IMPase proteins (Chenna et al., 2003) (Figure 3.3): DDB0204100 was identified as the closest homologue to human, mouse and rat IMPase (38% identity, 61% similarity), and therefore referred to as IMPase in this study; DDB0189923 was identified as being homologous to the PAP-phosphatase-like proteins in yeast, known as 3'(2''),5'-bisphosphate nucleotidase and Hal2 (35% identity, 52% similarity), and was referred to as IPP2; DDB0167248 was identified as homologue of mouse and human IPPase (29% identity, 45% similarity), and as such referred to as IPP1. Amino acid sequence alignments for individual gene families can be found in the appendix (Figure 8.12 to 8.14).
Figure 3.3 Dendrogram representing the phylogenetic relationship of the *D.discoideum* IMPase family. The clustalW program was used to align the following protein sequences: AtSal1 (*A.thaliana*, GeneBank™ Q42546), AtSal2 (*A.thaliana*, GeneBank™), ScHal2 (*S.cerevisiae*, GeneBank™ P32179), SpDPNP (*S.pombe*, GeneBank™ Q094505), DdIPP2 (*D.discoideum*, Dictybase DDB0189923), RnIMP (*R.norvegicus*, GeneBank™ P97697), HsIMP (*H.sapiens*, GeneBank™ P29218), MmIMP (*M.musculus*, GeneBank™ Q55023), DdIMP (*D.discoideum*, Dictybase DDB0204100), HsIMP2 (*H.sapiens*, GeneBank™ Q14732), MmIPP (*M.musculus*, GeneBank™ P49442), HsIPP (*H.sapiens*, GeneBank™ P49441), BtIPP (*B.taurus*, GeneBank™ P21327), DdIPP1 (*D.discoideum*, Dictybase DDB0167248), AtFBP (*A.thaliana*, GeneBank™ P25851) and BnFBP (*B.nappus*, GeneBank™ Q07204). Evolutionary distance, expressed as ((100% - % Identity)/100) is displayed, as calculated by the clustalW software.
3.4.2 Optimisation and transcript abundance

Primers were designed to the coding region of the individual genes to yield a PCR product between 300 and 600 bp. After generation of plasmid standard curves for each gene, 1 µg of total RNA from wild type (Ax2) cells was converted to cDNA and then serial diluted from 10 ng to 0.01 ng (10, 1, 0.5, 0.2, 0.1, 0.05 and 0.01 ng) to establish the transcript numbers of each gene in a cell. Mean C_t-values of cDNA dilutions for each gene were displayed as part of the plasmid calibration curves (Figure 3.4 to Figure 3.9). The 10 ng dilution was the only concentration in the linear range of the plasmid calibration curve resulting in a consistent signal. Therefore, this concentration was used to calculate the approximate transcript abundance of each gene in the cell (Table 3.1). As Ig7 is a mitochondrial gene, it is highly abundant with approximately 700 transcripts per cell. In comparison, there are about 100 and 50 transcripts each of Ino1 and IMPase in the cell, whereas there are less than 10 transcripts of the 5'-phosphatases, IPP1 and dpoA in the cell.
Figure 3.4 Calibration curve generated using wild type cDNA and a plasmid standard for Ig7. $C_T$-values of plasmid dilutions (20, 2, 0.2, 0.02, 0.002 and 0.0002 pg) were plotted against log transcript numbers for Ig7 (green diamonds). $C_T$-values for cDNA dilutions (10, 1, 0.5, 0.2, 0.1, 0.05 and 0.01 ng) were displayed in the same graph (blue squares). All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising three repeats of the same sample. The $C_T$-value is the cycle number at which the fluorescence in the sample increases above a defined threshold fluorescence, which is calculated by the software.
Figure 3.5 Calibration curve generated using wild type cDNA and a plasmid standard for Ino1. $C_t$-values of plasmid dilutions (20, 2, 0.2, 0.02, 0.002 and 0.0002 pg) were plotted against log transcript numbers for Ino1 (green diamonds). $C_t$-values for cDNA dilutions (10, 1, 0.5, 0.2, 0.1, 0.05 and 0.01 ng) were displayed in the same graph (blue squares). All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising three repeats of the same sample. The $C_t$-value is the cycle number at which the fluorescence in the sample increases above a defined threshold fluorescence, which is calculated by the software.
Figure 3.6 Calibration curve generated using wild type cDNA and a plasmid standard for IMPase. Cₜ-values of plasmid dilutions (20, 2, 0.2, 0.02, 0.002 and 0.0002 pg) were plotted against log transcript numbers for IMPase (green diamonds). Cₜ-values for cDNA dilutions (10, 1, 0.5, 0.2, 0.1, 0.05 and 0.01 ng) were displayed in the same graph (blue squares). All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising three repeats of the same sample. The Cₜ-value is the cycle number at which the fluorescence in the sample increases above a defined threshold fluorescence, which is calculated by the software.
Figure 3.7 Calibration curve generated using wild type cDNA and a plasmid standard for IPP1 and IPP2. $C_T$-values of plasmid dilutions (20, 2, 0.2, 0.02, 0.002 and 0.0002 pg) were plotted against log transcript numbers for (A) IPP1 and (B) IPP2 (green diamonds). $C_T$-values for cDNA dilutions (10, 1, 0.5, 0.2, 0.1, 0.05 and 0.01 ng) were displayed in the same graph (blue squares). All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising three repeats of the same sample. The $C_T$-value is the cycle number at which the fluorescence in the sample increases above a defined threshold fluorescence, which is calculated by the software.
Figure 3.8 Calibration curve generated using wild type cDNA and a plasmid standard for the 5'-phosphatases 5P2, 5P3 and 5P4. C<sub>t</sub>-values of plasmid dilutions (20, 2, 0.2, 0.02, 0.002 and 0.0002 pg) were plotted against log transcript numbers for (A) 5P2, (B) 5P3 and (C) 5P4 (green diamonds). C<sub>t</sub>-values for cDNA dilutions (10, 1, 0.5, 0.2, 0.1, 0.05 and 0.01 ng) were displayed in the same graph (blue squares). All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising three repeats of the same sample. The C<sub>t</sub>-value is the cycle number at which the fluorescence in the sample increases above a defined threshold fluorescence, which is calculated by the software.
Figure 3.9 Calibration curve generated using wild type cDNA and a plasmid standard for dpoA. $C_v$-values of plasmid dilutions (20, 2, 0.2, 0.02, 0.002 and 0.0002 pg) were plotted against log transcript numbers for dpoA (green diamonds). $C_v$-values for cDNA dilutions (10, 1, 0.5, 0.2, 0.1, 0.05 and 0.01 ng) were displayed in the same graph (blue squares). All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising three repeats of the same sample. The $C_v$-value is the cycle number at which the fluorescence in the sample increases above a defined threshold fluorescence, which is calculated by the software.
Table 3.1 Transcript abundance in wild type cells.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Transcripts per cell*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ino1</td>
<td>101</td>
</tr>
<tr>
<td>IMPase</td>
<td>50</td>
</tr>
<tr>
<td>IPP1</td>
<td>9</td>
</tr>
<tr>
<td>IPP2</td>
<td>9</td>
</tr>
<tr>
<td>5P2</td>
<td>3</td>
</tr>
<tr>
<td>5P3</td>
<td>5</td>
</tr>
<tr>
<td>5P4</td>
<td>3</td>
</tr>
<tr>
<td>DpoA</td>
<td>4</td>
</tr>
<tr>
<td>Ig7</td>
<td>669</td>
</tr>
</tbody>
</table>

* Transcript numbers were calculated with the assumption that 1% of total RNA is mRNA.

3.5 RT-PCR confirms and extends data of northern blots

3.5.1 Gene expression in the LisA mutant

Wild type (Ax2) and LisA mutant cells were taken from log phase culture and plated into three plates of 10^6 cells. The cells were incubated for 24 hours before harvest for mRNA extraction. mRNA equal to 1 μg of total RNA was converted into cDNA. 1 μl of the 1:100-dilution was used for the Real-time PCR assay.

Confirming the trend from the northern blot analysis (Figure 3.1), ino1 expression was increased by 280% in the LisA mutant compared to wild type (Ax2) cells. DpoA expression was decreased to nearly zero in the LisA mutant, validating the use of this RT-PCR assay. Expression of IMPase was increased by 1550%. In addition, expression of IPP1 and IPP2 were both increased by 30%. Note that as the signal of the amplified mRNA was weak, resulting in distorted increased percentages and great variation between the individual RT-PCR repeats, only the data for one repeat is displayed in Figure 3.10.
Figure 3.10 Gene expression in the lithium resistant mutant LisA. mRNA was isolated from wild type (Ax2) and LisA cells, reverse transcribed and used in the RT-PCR reaction. Relative change in expression of individual genes in wild type and LisA cells is displayed as percentage to the internal standard Ig7, calculated using the \(2^{-\Delta\Delta CT}\)-method. Averages of three repeats of the same sample including SEM for a single experiment are displayed.
3.5.2 Gene expression in wild type cells treated with PO-Inhibitor

Wild type (Ax2) cells were taken from log phase culture and plated into three plates of $10^6$ cells in the presence of 1.3 mM of the prolyl oligopeptidase inhibitor Z-prolinal or DMSO respectively. The cells were incubated for 24 hours before harvest for RNA extraction. 1 µg of total RNA was converted into cDNA. 1 µl of the 1:100-dilution was used for Real-time PCR.

As seen in Figure 3.11, *ino1* and *IMPase* expression were increased significantly by about 40% ± 8% (paired Student's t-test; p-value < 0.05) and 100% ± 12% (paired Student's t-test; p-value < 0.01) respectively. In addition, *5P2* and *5P3* expression were also increased significantly by about 60% ± 11% (paired Student's t-test; p-value < 0.1) and 100% ± 20% (paired Student's t-test; p-value < 0.05) respectively. As the POI only affects protein activity and not *dpoA* expression, *dpoA* expression was not changed in POI treated wild type cells compared to wild type cells treated with the carrier control DMSO. *5P4* expression was also not changed in POI treated cells. Expression of both *IPP1* and *IPP2* was increased by 100% compared to wild type cells treated with the carrier control DMSO.
Figure 3.11 Gene expression in wild type cells treated with the PO-Inhibitor. Total RNA was isolated from wild type (Ax2) cells treated with DMSO (carrier control) or the PO-Inhibitor Z-prolinal (POI) for 24 hours, reverse transcribed and used in the RT-PCR reaction. Relative change in expression of individual genes in wild type cells treated with DMSO or POI is displayed as percentage to the internal standard Ig7, calculated using the $2^{-\Delta\Delta CT}$ method. All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising three repeats of the same sample. Paired student's t-test was used for statistical analysis.
3.5.3 Gene expression in the LisG mutant

LisG is a lithium resistant mutant, identified from the same genetic screen that identified LisA. To test whether the changes in gene expression observed in the LisA mutant are genuine and can be correlated to lithium resistance in general, changes in gene expression were investigated in the LisG mutant.

Wild type (Ax2) and LisG mutant cells were taken from log phase culture and plated into three plates of $10^6$ cells. The cells were incubated for 24 hours before harvest for RNA extraction. 1 μg of total RNA was converted into cDNA. 1 μl of the 1:100-dilution was used for Real-time PCR.

As shown in Figure 3.12, ino1 and IPP1 expression was elevated significantly by 35% ± 10% and 52% ± 29% respectively (paired Student’s t-test; p-value < 0.05), whereas IMPase expression reduced by about 40% ± 7% (paired Student’s t-test; p-value < 0.01) in LisG mutant cells compared to wild type. Furthermore, dpoA expression decreased by 45% ± 2% (paired Student’s t-test; p-value < 0.001) in the LisG mutant. A change in gene expression could not be seen for all other genes in the LisG mutant.
Figure 3.12 Gene expression in the lithium resistant mutant LisG. Total RNA was isolated from wild type (Ax2) and LisG cells, reverse transcribed and used in the RT-PCR reaction. Relative change in expression of individual genes in wild type and LisG cells is displayed as percentage to the internal standard Ig7, calculated using the $2^{-\Delta\Delta C_{t}}$-method. All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising three repeats of the same sample. Paired student’s t-test was used for statistical analysis.
3.5.4 Gene expression in the LisG mutant treated with POI

To analyse whether PO and LisG could be genetically linked, gene expression was investigated in LisG cells treated with the PO-Inhibitor (POI). In brief, LisG mutant cells were taken from log phase culture and plated into three plates of $10^6$ cells in the presence of 1.3 mM of the Prolyl oligopeptidase inhibitor Z-prolinal or DMSO respectively. The cells were incubated for 24 hours before harvest for RNA extraction. 1 μg of total RNA was converted into cDNA. 1 μl of the 1:100-dilution was used for Real-time PCR.

Gene expression in LisG cells treated with POI was unchanged compared to LisG cells treated with DMSO as carrier control, suggesting that LisG cells do not respond to PO, with one exception: Expression of dpoA was further reduced by about 56% ± 4% (paired Student’s t-test; p-value < 0.01) in cells treated with POI compared to LisG cells treated with DMSO (Figure 3.13).
Figure 3.13 Gene expression in LisG cells treated with the PO-Inhibitor. Total RNA was isolated from LisG cells treated with DMSO (carrier control) or the PO-Inhibitor Z-prolinal (POI) for 24 hours, reverse transcribed and used in the RT-PCR reaction. Relative change in expression of individual genes in LisG cells treated with DMSO or POI is displayed as percentage to the internal standard Ig7, calculated using the $2^{\Delta\Delta Ct}$ method. All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising three repeats of the same sample. Paired student’s t-test was used for statistical analysis.
3.5.5 Correlation of gene expression to protein activity

Understanding how protein abundance is related to mRNA transcript levels is essential for interpreting gene expression data. As dpoA gene expression was decreased in the LisG mutant, a decrease in PO activity in the LisG mutant would also be expected. To assess whether changes in gene expression correlated with changes in protein activity, PO activity was investigated in the LisG mutant.

The PO-assay determines the hydrolytic activity towards the synthetic substrate pNA-Gly-Pro. In brief, wild type (Ax2) and LisG cells were harvested as 2x 10^8 cells per pellet, lysed and total protein content determined using the Bradford assay. 2.5 and 5 µg of total protein was used in the PO-assay; recombinant dpoA tagged with His-V5 (provided by K. McQuillan in the lab) and no protein was used as a positive and negative control respectively. Data presented here was the mean of three independent repeats.

PO-activity in the LisG mutant was mildly decreased by 21% (paired Student’s t-test; p-value < 0.05) compared to that of wild type cells (Figure 3.14). The recombinant DpoA protein had similar PO-activity to that of wild type cell extracts. Adding the PO-Inhibitor Z-prolinal to the assay buffer decreased PO-activity in wild type and LisG cell extracts to near background levels, as seen in the negative (no protein) control. This was also seen for the recombinant DpoA protein, confirming that the assay was functional.
Figure 3.14 PO-activity in the LisG mutant. Extracts from growing cells of wild type (Ax2) or LisG cells were assayed for dpoA activity with or without the presence of 1.3 mM POI Z-prolinal in the assay buffer. 2.5 μg of total protein isolated from LisG and wild type (Ax2) cells, 2.5 μg of recombinant dpoA protein (control) or lysis buffer (blank) were used in the assay. All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising two repeats of the same sample. * p-value < 0.05 (Paired Student’s t-test).
3.6 Discussion

I used northern blot analysis and established a Real-time PCR assay to investigate whether the expression of genes involved in the InsP-pathway was altered in the lithium resistant mutants LisA and LisG. Indeed, I found that lithium resistance correlated with changes in gene expression: an increase in \textit{Ino1} expression was found in the lithium resistant mutant LisG, and as a result of loss of PO in both LisA mutants and POI treated wild type cells; in addition, elevated \textit{IMPase} expression was found as a result of loss of LisA, whereas loss of LisG led to reduced \textit{IMPase} expression; furthermore, cells did not respond to POI in the absence of LisG.

3.6.1 Does PO up-regulate gene expression? Northern blotting.

The data presented here suggests for the first time that PO not only regulates higher polyphosphate inositol breakdown by regulating MIPP activity, but also could regulate inositol \textit{de novo} synthesis by regulating \textit{Ino1} expression. Using northern blotting, I found that loss of PO activity, either through gene disruption or through PO-inhibitors, led to an increase in \textit{Ino1} expression. However, a difference in expression levels could be observed: \textit{Ino1} expression was increased by 75\% in the LisA mutant compared to the 41\% increase in POI treated wild type cells. As it was difficult to get a strong and consistent signal for LisA using total RNA extracts, mRNA extracts were used for LisA, whereas total RNA extracts were used for POI treated wild type cells. Thus, the ratios of expression may have been amplified by the use of mRNA, leading to a more pronounced increase of \textit{Ino1} expression in the LisA mutant. Alternatively, the concentration of POI used may not have been sufficient to inhibit PO completely, resulting in the smaller increase in Ino1 levels in wild type cells treated with POI. Furthermore, as the time of POI incubation did not allow for second site mutations, the use of POI treated wild type cells to investigate gene expression was more accurate, which can be seen at the statistical significance for POI treated cells.
3.6.2 RT-PCR: the assay of choice to study gene expression

Since its introduction in 1996, RT-PCR has become the method of choice for the measurement of mRNA concentration, as it allows monitoring amplification of PCR products during each cycle of a PCR reaction (Valasek and Repa, 2005). RT-PCR is less time consuming and less labour intensive, compared to alternative methods to measure levels of mRNA in the cell, such as northern or in situ hybridisations. Most importantly, RT-PCR can measure nucleic acids over a wide range of abundance, thereby allowing comparison between RNAs that differ widely in their abundance. However, as it is a PCR based approach, the same limitations exist as seen for conventional PCR. Contamination with genomic DNA, nuclease or RT-PCR inhibitors carried over from RNA preparations can inhibit RT-PCR reaction. Furthermore, if RNA extractions and Reverse Transcriptase reactions are not carried out carefully, RNA integrity is damaged, thereby leading to false results.

Non-specific binding of the fluorescent dyes to DNA is a specific problem of RT-PCR. SybrGreen, the dye used in the present studies, binds to the minor groove of DNA, leading to a 1000-fold increase in fluorescence compared to when it is free in solution (Wilhelm et al., 2003). As the dye binds to any double stranded DNA, amplification of a non-specific product will give misleading results. Therefore, it is essential to assess specificity of the primers with a melting curve. For the genes in this assay, melting curve analysis showed that the primers only amplify the desired product. However, for the Ino1 melting curve, it was initially observed that increasing cycle number led to primer dimer formation (plateau effect). Therefore, the cycle number was decreased to 30 cycles as part of the optimisation process; subsequently, no primer dimers were recorded in the no-DNA reaction for all the genes studied.

Plotting Cycle threshold against transcript numbers semi-logarithmically, most data points for the cDNA dilution series were outside the linear range of the plasmid standard curves, with only the highest concentration (10 ng) lying on the low concentration end of the plasmid standard curves. Therefore, higher cDNA concentrations should be used in future assays. PCR efficiencies,
calculated from the slope of the plasmid standard curves, were similar among the individual genes. However, for some standard curves the PCR efficiency was greater than 100%. This was probably due to differences in the kinetics of primer annealing and eventual enzyme limitations; furthermore, inhibitors may have interfered with the amplification process (Trapnell, 1993).

Using cDNA titrations, the transcript abundance of the individual genes in the cell was investigated. Not surprisingly, as Ig7 is a mitochondrial gene, its transcript was highly abundant in the cell, up to 70-fold more than the transcripts of other genes. Expression of Ino1 was approximately twice that of IMPase and ten times that of the remaining investigated transcripts, indicating that the cell has no requirement to change expression of Ino1 to the same extent as that of the other genes to achieve the same effect.

A major problem observed with the RT-PCR assay is the great variability between repeats. This could be due to the 'Monte Carlo' effect, describing a correlation between gene abundance and variation during the RT-PCR analysis as followed: when random priming is used during the reverse transcriptase reaction, ribosomal RNA will be amplified most; thus, if the gene of interest is present at low levels, it might not be effectively primed by random primers and amplification is not quantitative (Bustin and Nolan, 2004; Stahlberg et al., 2004). High variation was observed with the 5'-phosphatases and IPP1/2. Interestingly, the abundance of these genes in the cell is low, supporting the 'Monte Carlo' effect as the reason for this variation.

Another possibility for the high variation observed may be the use of a mitochondrial gene as the internal standard. Transcript numbers of the mitochondrial genome may vary between cells, and this may mask some changes in gene expression of less abundant genes. Therefore, to avoid uncontrolled variation of a single marker, it may be more accurate to use a chromosomall gene instead of the highly abundant mitochondrial gene Ig7 as the internal standard. However, the two following criteria would need to be ascertained before using a chromosomal gene as the internal standard: (1) its
expression is not changed during development and (2) its expression is not changed during drug treatments.

3.6.3 Does PO up-regulate gene expression? RT-PCR.

As could be observed with the northern analysis, the expression profile of the LisA mutant showed greater variation compared to POI treated wild type cells. As described above, this was probably due to the incomplete inhibition of PO in POI treated wild type cells, leading to a smaller increase of \textit{Ino1} expression in these cells. However, the same trend in changes in gene expression was found and only background levels of expression for \textit{dpoA} could be observed in the LisA mutant, validating the assay.

Confirming the results obtained from the northern analysis, RT-PCR analysis found that \textit{Ino1} expression was increased in wild type cells treated with the POI. Furthermore, expression of other genes involved in the InsP-signalling pathway, such as \textit{IMPase} and the 5'-phosphatases 5P2 and 5P3, was also increased, thereby extending the results from the northern analysis. Although the present study was unable to find a significant increase in \textit{IPP1} expression due to high variation of amplified product between RT-PCR repeats, elevated \textit{IPP1} expression levels due to loss of PO are most likely genuine as \textit{IPP1} expression was also increased by 75\% in wild type cells over-expressing MIPP (J. King, personal communication). Overall these findings correlate well with the fact that the flux through the InsP-pathway is increased in the LisA mutant (Williams \textit{et al.}, 1999). Loss of PO leads to increased breakdown of higher inositol phosphates to Ins(1,4,5)P$_3$, thereby feeding more substrate into the InsP-cycle. As a consequence, the cell requires more metabolic enzymes to handle the substrate over-load.

Furthermore, increased \textit{Ino1} expression found in the LisA mutant also correlates well with studies by Shen \textit{et al.} and Steger \textit{et al.} who found that InsPs regulate \textit{Ino1} expression in yeast (Shen \textit{et al.}, 2003; Steger \textit{et al.}, 2003). The authors demonstrated that InsP$_4$ and InsP$_5$ can activate Swi/Snf type
chromatin-remodelling complexes leading to increased \textit{Ino1} expression and conversely that \textit{Ino6} can inhibit the \textit{Ino80} chromatin-remodelling complex leading to decreased \textit{Ino1} expression. Interestingly, when examining \textit{Ino}P concentrations in LisA and wild type cells overexpressing PO compared to wild type cells with HPLC analysis, decreased levels of \textit{Ino}P$_6$ and increased levels of \textit{Ino}P$_3$, \textit{Ino}P$_4$ and \textit{Ino}P$_6$ are observed in LisA cells, and vice versa in wild type cells overexpressing PO (A. J. Harwood, personal communication). Taking these results together with the observations from the present study, one can conclude that PO can regulate \textit{Ino1} expression and thereby inositol \textit{de novo} synthesis through regulation of the ratio of \textit{Ino}P$_4$/\textit{Ino}P$_6$. The finding that total mass of \textit{Ino}Ps, when analysed by HPLC, is increased by 33% in the LisA mutant supports this hypothesis (A. J. Harwood, personal communication). However, whether and how loss of PO leads to lithium resistance due to increased inositol \textit{de novo} synthesis remains to be investigated.

3.6.4 Gene expression in the LisG mutant

The lithium resistant mutant LisG was identified from the same lithium suppressor screen that identified LisA. Loss of PO resulted in an increase in \textit{Ino1} and \textit{IPP1} expression; similarly, an increase in \textit{Ino1} and \textit{IPP1} expression was found in the LisG mutant, suggesting that an increase in either \textit{Ino1} or \textit{IPP1} expression or an increase in both is sufficient to confer lithium resistance. Loss of LisG led to decreased expression of IMPase; this is likely to be a distinct effect due to loss of LisG, independent of PO and probably unrelated to the lithium resistance phenotype, as elevated \textit{IMPase} expression was found in the LisA mutant and POI treated wild type cells. In addition, no change in expression of the 5'-phosphatase or \textit{IPP2} was found in the LisG mutant; this observation is in contrast to the finding of increased 5P2/5P3 and \textit{IPP2} expression seen in the LisA mutant, suggesting that these changes are characteristic for the LisA mutant.

How are LisA and LisG connected? \textit{DpoA} expression was decreased in the LisG mutant and further reduced when LisG cells were treated with the POI,
suggesting that LisG could lie upstream of PO in a genetic pathway. However, the observation that *ino1* expression was not increased in LisG cells treated with the POI, contradicts the hypothesis of LisG lying upstream of LisA, because a further reduction in *dpoA* expression, as seen in the LisG mutant due to treatment with the POI, should have resulted in a further elevation of *ino1* expression. But regardless of how LisG and LisA are connected, LisG does ablate changes in gene expression caused by PO, because cells that have lost LisG do not respond to the POI, supporting the existence of a connection between PO and LisG; however, how they are linked remains to be investigated.

### 3.6.5 Does gene expression correlate with protein activity?

Understanding how protein abundance is related to mRNA transcript levels is essential for interpreting gene expression data. A study comparing 156 protein spots analysed by capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS) to mRNA levels calculated from serial analysis of gene expression (SAGE) frequency tables, suggested that mRNA and protein abundance do not always correlate (Gygi *et al.*, 1999). As RT-PCR analysis identified a 45% decrease in *dpoA* expression in the LisG mutant, a PO-activity assay was used to investigate whether changes in gene expression also correlated with changes in protein activity. Indeed, PO-activity was decreased in the LisG mutant by 21%. These findings suggest that mRNA transcript levels and protein abundance do correlate and are in accordance with a more recent study in yeast. Comparing different types of gene expression data with its corresponding protein abundance data from yeast, the authors concluded that, as a whole, a correlation between mRNA and protein abundance exists (Greenbaum *et al.*, 2002).

The observed difference in magnitude between 45% mRNA and 21% protein abundance suggests that a direct translation of mRNA abundance into protein activity is not possible. Enzyme activity is not equivalent to copy number of the enzyme, but gives the rate of substrate conversion; as an increase in activity does not necessarily have a linear correlation with enzyme copy number, and
may be subject to allostERIC regulation, a linear correlation with mRNA copy
number may also not be expected.

3.6.6 Summary

- *Ino1* expression was increased in both LisA and LisG.
- *IMPase* expression was increased in LisA, but decreased in LisG.
- Cells did not respond to PO in the absence of LisG.
Chapter 4

The lithium resistant mutant LisG encodes a chromatin-remodelling factor
4.1 Introduction

As shown in the previous chapter, cells do not respond to PO in the absence of LisG. Therefore, the aim of this chapter was to characterise the mutant LisG.

What is LisG? LisG was isolated from a genetic screen for its ability to aggregate in the presence of 10 mM lithium, similar to LisA. The original LisG mutant was generated by restriction enzyme mediated mutagenesis (REMI) (Kuspa and Loomis, 1992), and a 182 bp genomic fragment of the LisG gene was cloned by inverse PCR rescue (Keim et al., 2004). Screening of the *D.discoideum* genome database (Chisholm et al., 2006)\(^3\) using the Blast-server determined that LisG (DDB0220643) encodes the ATPase subunit of a Swi/Snf-type chromatin-remodelling complex (Figure 4.1) (Altschul et al., 1997; Keim, 2002; Keim et al., 2004). The LisG cDNA contains four open reading frames with a total size of 9216 bp. The insertion site of the blasticidin-REMI cassette lies in the intron between the first and second open reading frame. Note that the first intron is only 15 bp in size and that the second ORF also contains a methionine; however, no characteristic promoter sequences, such as a translation initiation consensus sequence (Kozak sequence: CCRCCAUGG with R=A/G), TATA-box, CAAT-box or GC-box, upstream of the ATG codon were identified; furthermore, the ORF was not immediately preceded by a long run of deoxyadenosine residues, which is characteristic of the 5’ untranslated region of *D.discoideum* genes; in addition, conserved donor (AGGTAtxxx), acceptor (xTAG) and branch (CTNA) sites required for the splicing process were identified (Rivero, 2002); taken together, these findings suggest that this second methionine is not the start of the gene or the start of a splice variant as seen in *Drosophila melanogaster*.

The following conserved domains were present in the translated LisG protein sequence: two chromo domains, one SNF2-domain, one Helicase_C domain and one Myb-lik/telobox DNA binding domain. LisG shows strong homology to the family of chromo-ATPase/helicase-DNA-binding (CHD) proteins, in

---

particular CHD-1, as well as to the *D. melanogaster* kismet protein, which is an important regulator in the early segmentation process (Daubresse *et al*., 1999). The two chromodomains have recently been shown to bind to the Lysine 4-methylated histone H3 tail (Flanagan *et al*., 2005). Gene organisation and homology are displayed in Figure 4.1.
(A)

(B)

splice donor site
ATGACTGTGTTCAAAGGTATTATTCAATAACATCATTTTTTTTTATTATT
M T V S K>

REMI-insertion site
C TTTATTTTTTTTTTTTTTTAAAAATAAAATTTTTTTTAAGATCAAT

splice branch site
ATTACTCATATTTAATTAATATTITTTTTTTTTTTTTTTTTTTTTTAATCTCTC

splice acceptor site
TAAAAATTTTTTTTTTTAAAAATTTTCACTTTATTATTTAGAAATCAAAAACCTATT

K S K T I

GCAAAAAAAGGATAATAATATGAAAAAGATGATGATGATAATAATTTATGAATGAA

A K K D N N M K D D D T I M N E

GAATTTAAATAAATAATAGTAATAATAATAATAATAATAAT>

E L N N N S N S N N N N N N N N>

- 101 -
Figure 4.1 LisG encodes a chromatin-remodelling factor. (A) Genome organisation. The LisG protein is encoded by four exons. The REMI-cassette containing the blasticidin (Bs+) resistance gene inserted into a DpnI-site into the first intron; (B) The first 292 bp of the genomic sequence and its corresponding amino acid sequence. The insertion site of the REMI cassette is highlighted in red. The 182 bp fragment obtained via inverse PCR to identify the LisG mutant is outlined in red. Splice-sites are highlighted in yellow; (C) Overview and comparison of the structural domains of LisG (D.discoideum, Dictybase DDB0220643) with its closest orthologues Kismet-L (D.melanogaster, GeneBank™ AAF43002) and HsCHD-1 (H.sapiens, GeneBank™ AAB87381). Identical residues are highlighted in grey.
4.2 Transcript abundance of LisG in wild type cells

To investigate transcript abundance of LisG in wild type (Ax2) cells, a primer pair specific for the LisG gene was optimised for use in the RT-PCR assay (see Appendix 8.2). The PCR product was cloned into a TOPO vector and a plasmid standard curve was generated using a serial dilution of the plasmid from 200 pg to 0.0002 pg. Furthermore, RNA from wild type (Ax2) cells was converted to cDNA and then serial diluted from 10 ng to 0.01 ng (10, 1, 0.5, 0.2, 0.1, 0.05 and 0.01 ng) to establish the transcript numbers of each gene.

Mean C_t-values of plasmid dilutions for each gene were plotted against log transcript numbers resulting in a linear regression with a good R^2 value (Figure 4.2). In addition, mean C_t-values of the cDNA dilutions were displayed in the same graph. The 10 ng dilution was used to calculate the approximate abundance of LisG transcripts in wild type cells: assuming that 1% of total RNA is mRNA, there are about 30 transcripts of LisG mRNA in the cell.
**Figure 4.2 Calibration curve generated using wild type cDNA and a plasmid standard for LisG.** (A) Properties of primers used for RT-PCR; (B) Cₚ-values of plasmid dilutions (20, 2, 0.2, 0.02, 0.002 and 0.0002 pg) were plotted against log transcript numbers for LisG (green diamonds). Cₚ-values for cDNA dilutions (10, 1, 0.5, 0.2, 0.1, 0.05 and 0.01 ng) were displayed in the same graph (blue squares). All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising three repeats of the same sample. The Cₚ-value is the cycle number at which the fluorescence in the sample increases above a defined threshold fluorescence, which is calculated by the software.
4.3 Recapitulation of the LisG- knock-out mutant

In order to confirm that loss of the chromatin-remodelling factor is responsible for lithium resistance, a construct was designed in which a 1.8 kb *KpnI*-NotI fragment from the ATPase domain region was replaced with a 3.2 kb *KpnI* fragment containing the blasticidin-resistance cassette. Thus, homologous genomic recombinants will be blasticidin resistant and unable to express the full length LisG mRNA or resulting protein. The vector was linearised via digestion with *KpnI* and transformed into *D.discoideum* wild type (Ax2) cells by electroporation. Blasticidin resistant colonies were grown to confluence in 96-well culture dishes and a range of independent colonies were screened by PCR. No knockout mutant was obtained with this process (data not shown).

In addition, rearrangement of the gene encoding the chromatin-remodelling factor in the original LisG mutant was analysed by Southern Blot analysis. LisG and wild type cells were taken from log phase cultures for genomic DNA extraction. Genomic DNA was digested with *XmnI*, separated by gel electrophoresis and transferred to nylon membrane. Blots were probed with a [α-32P] dATP labelled PCR product, obtained with the primer set LisGKO-F1-66 and LisGKO-R1-68, and expression was visualised using a BioRad phosphorimager screen. No rearrangement of the gene encoding the chromatin-remodelling factor was observed in the original LisG mutant compared to wild type cells (data not shown).

4.4 LisG is expressed in the LisG mutant

As several attempts to recapitulate the LisG mutant in wild type (Ax2) cells failed, expression levels of LisG were investigated in the original LisG mutant. Wild type (Ax2) and LisG mutant cells were taken from log phase culture and plated into three plates of 10^6 cells. The cells were incubated for 24 hours before harvest for RNA extraction. 1 μg of total RNA was converted into cDNA. 1 μl of the 1:100-dilution was used for Real-time PCR. As shown in Figure 4.3a,
expression of *LisG* in the LisG mutant was comparable to that of wild type cells, as examined by RT-PCR.

To confirm that *LisG* expression was not due to non-specific binding of the primer pair used, several PCR reactions were set up using different primer combinations amplifying different parts of the coding region and cDNA as template. cDNA made without reverse transcriptase was used as control to test for genomic background contamination. Figure 4.3b shows that *LisG* was expressed and that the signal in the RT-PCR assay was not due to genomic contamination of the cDNA sample.

The fact that the size of the *LisG* cDNA is about 9216 bp and the REMI cassette integrated into the first intron, with the size of the first exon only being 15 bp (equals 5 amino acids), made it impossible to confirm that the cDNA used was LisG cDNA, because a shift of wild type and LisG cDNA would have been indistinguishable using northern blotting. To test if the LisG cDNA was accidentally substituted for wild type cDNA, the cDNAs were screened for blasticidin expression. The *blasticidin* resistance gene was expressed in the LisG cDNA, but not in the wild type cDNA, suggesting that the cDNA used for LisG was that of a blasticidin resistant mutant (Figure 4.3c).
Figure 4.3 LisG is expressed in the LisG mutant. (A) Total RNA was isolated from wild type (Ax2) and LisG cells, reverse transcribed and used in the RT-PCR reaction. Relative change in expression of LisG is displayed as percentage to the internal standard Ig7, calculated using the $2^{-\Delta\Delta CT}$-method. All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising three repeats of the same sample. (B) Total RNA was isolated from wild type (Ax2) and LisG cells and reverse transcribed with RTase. Reactions without RTase were set up as control. The generated cDNA was subsequently used as template in a PCR reaction with two sets of LisG specific primers. Genomic DNA isolated from wild type and LisG cells was used as control. The LisG mutant expresses the LisG gene. (C) Total RNA was isolated from wild type (Ax2) and LisG cells, reverse transcribed and subsequently used as template in a PCR reaction with pBlast specific primers. Plasmid DNA encoding blasticidin gene (pBlast) and no DNA were used as positive and negative control.
4.5 Basic motile behaviour of LisG compared to wild type and LisA: establishing a non-gradient assay

A non-gradient motility assay was designed to examine basic motile behaviour of LisG compared to wild type (Ax2) cells. LisA cells were used as lithium resistant control. Cells were grown in inositol-free media prior to the experiment to exclude the possibility that the inositol found in the axenic media suppressed the effect of lithium. For the assay, cells were set up in duplicates in 2-well dishes at low density and then starved for one hour; the first well contained buffer including 2, 4 and 6 mM NaCl and the second well contained buffer including 2, 4 and 6 mM LiCl. After a one-hour incubation, the cells were recorded for 10 min using a time-lapse microscopy system. The data was analysed using the DIAS 3.1 software. In brief, all cells in a single frame for every frame in the movie were outlined individually, subsequently all of the frames were superimposed resulting in the path of an individual cell, and the parameters were computed. The parameters total path length and speed were chosen to describe basic motile behaviour. The data was analysed using the Student’s t-test (two-tailed, unequal variance).

The parameter ‘total path length’ describes the path a cell has taken from the starting point to the ending point. The parameter ‘speed’ as calculated by the DIAS software takes into account the frame-to-frame changes in speed, and therefore is a more accurate method of describing speed than dividing total path length by the elapsed time. However, both methods will give similar results.

In the presence of 2 and 4 mM NaCl, wild type (Ax2) cells moved with a speed of $3.8 \pm 0.09 \ \mu m \ min^{-1}$ and covered $47 \pm 1 \ \mu m$ in total path length. Increasing the concentration of NaCl to 6 mM improved basic motile behaviour, as wild type (Ax2) cells now moved with a speed of $5 \pm 0.24 \ \mu m \ min^{-1}$ and covered on average $58 \pm 2.4 \ \mu m$ of total path length. In the presence of 2 to 6 mM LiCl, however, speed was reduced to $2.1 \pm 0.06 \ \mu m \ min^{-1}$ (Student’s t-test; p-value < 0.001) and total path length was reduced to $30 \pm 0.8 \ \mu m$ (Student’s t-test; p-value < 0.001) (Figure 4.4a).
In the presence of 2 mM NaCl, LisA cells moved with a speed of 4.5 ± 0.29 μm min⁻¹ and covered 58 ± 3.7 μm of total path length. Increasing the concentration of NaCl did not improve basic motile behaviour as seen in wild type (Ax2) cells. Furthermore, 2 mM LiCl did not affect motility of LisA cells, as these cells moved with similar speed compared to its sodium control; however, increasing the concentration of lithium to 4 or 6 mM LiCl reduced speed and total path length (Figure 4.4b), as seen with wild type (Ax2) cells (Figure 4.4a).

LisG cells moved with a speed of 5.8 ± 0.45 μm min⁻¹ and covered 65 ± 2.4 μm of total path length in the presence of 2 mM NaCl. Increasing concentrations of NaCl to 4 or 6 mM improved the speed of LisG cells, but did not increase total path length covered. As observed for LisA cells, basic motility of LisG cells was not impaired in the presence of 2 mM LiCl, and LisG cells moved with similar speed compared to its sodium control; however, basic motility of LisG cells was reduced with increasing lithium concentrations (Figure 4.4c).

In comparison, total path length and speed of LisA and LisG cells were decreased on average by 12% ± 8% in the presence of 2 mM LiCl, whereas total path length and speed of wild type (Ax2) cells were decreased on average by 46% ± 2%. Increasing lithium concentrations to 4 or 6 mM led to a percentage decrease in total path length and speed in LisA and LisG cells, similar to that of wild type (Ax2) cells.
Figure 4.4 Basic motile behaviour of wild type (Ax2), LisA and LisG cells towards lithium. Cells were starved for 1 hour in buffer supplemented with either 2, 4 or 6 mM LiCl or 2, 4 or 6 mM NaCl and subsequently videoed using time-lapse microscopy. 2D-DIAS analysis software was used to analyse the parameters total path length and speed for (A) wild type (Ax2), (B) LisA and (C) LisG cells. Basic motile behaviour of the lithium resistant mutants LisA and LisG is impaired only at concentrations greater than 4mM LiCl. The average including SEM for one replicate comprising 40 cells is displayed. *** p-value < 0.001 (Paired student's t-test).
4.6 Chemotactic analysis of LisG and LisA

The 2D-DIAS software was used to address the question whether the lithium resistant mutants show a different behaviour in response to a chemotactic stimulus. For this experiment, the lithium resistant mutants LisA and LisG were starved for 4 hrs in shaking culture and then set up in a Zigmond chamber with a cAMP gradient. After the establishment of the cAMP gradient, the cells were recorded using a time-lapse microscopy system. The data was analysed using the DIAS 3.1 software, as described in the previous sub-section. Representative paths for each of the mutants and wild type (Ax2) cells are displayed in Figure 4.5a, and the corresponding computed data is displayed in Figure 4.5b. Data was analysed using the Student’s t-test (two-tailed, unequal variance).

Comparing the paths for each cell type, no differences in the chemotactic behaviour between wild type (Ax2) and LisG cells were observed, as both cell types sensed and moved towards the chemotactic source. LisA cells also sensed the cAMP source; however, LisA cells formed fewer lateral pseudopods compared to wild type (Ax2) and LisG cells (Figure 4.5a).

Ax2, LisA and LisG cells moved with a similar speed of approximately 10 μm min⁻¹. Speed is calculated as total path length divided by elapsed time, with the DIAS software taking into account frame-to-frame changes in speed (Figure 4.5b). No change in total directionality was observed for LisG cells compared to wild type (Ax2) cells; however, a 28% increase in total directionality was observed for LisA compared to wild type (Ax2) cells. The parameter ‘total directionality’ is expressed as net path length over total path length by the DIAS software; thus, for a cell moving in a straight line, total path length will equal net path length, thereby total directionality will be 1, whereas total directionality will be smaller than 1 for a cell following a meandering path. Therefore, the increase in total directionality for LisA cells indicates that the cells followed a more direct path to the cAMP source compared to wild type (Ax2) cells; this can probably be attributed to the observation that LisA cells formed fewer lateral pseudopods (Figure 4.5b).
Figure 4.5 Chemotactic behaviour of Ax2, LisA and LisG cells. Wild type (Ax2) and lithium resistant mutants LisA and LisG were starved for 4 hours and subsequently set up in a cAMP chemotaxis assay. 2D-DIAS analysis was used to (A) construct paths and (B) analyse parameters. The lithium resistant mutants show normal chemotactic behaviour when compared to wild type cell, although LisA forms less lateral pseudopods than either wild type or LisG. Student's t-test (two-tailed, unequal variance) was used for statistical analysis. *Ax2 data was provided by E Dalton; †Total directionality expressed as net path length divided by total path length.
4.7 Discussion

In this chapter, I described the analysis of the lithium resistant mutant LisG: (1) LisG encodes a chromatin-remodelling factor and is highly abundant in the cell, with 30 copies per cell; (2) Loss of LisG improves basic cell motility, but does not change chemotactic behaviour, as determined by 2D-DIAS analysis; (3) the LisG mutant could not be recapitulated and expression of LisG was found in the LisG mutant. Taken together, these findings support the hypothesis that changes in gene expression are associated with lithium sensitivity, but the nature of the LisG mutant remains to be confirmed.

4.7.1 LisG abundance in wild type cells

Wild type cells possess three times the copy number of LisG compared to the other genes studied, such as IPP1/2, the 5'-phosphatases or dpoA; this finding is in agreement with the function of LisG as the ATPase subunit of a chromatin-remodelling complex, which indicates a high abundance in the cell. Several families have been identified based on their ATPase subunit; LisG belongs to the Swi/Snf family. All families have in common that the multi-subunit chromatin-remodelling complexes hydrolyse ATP to disrupt nucleosomal structure and increase DNA accessibility, thus their recruitment to DNA is required in the early steps in eukaryotic gene regulation (Kadam and Emerson, 2002).

Compared to other genes, the LisG gene is large; thus, transcription, processing, export and subsequent translation will delay the appearance of a functional LisG protein. In eukaryotes, genes are not only regulated by intercellular signal (i.e. hormones) or phosphorylation of transcription factors (i.e. β-adrenergic activation of CREB), but also by translational repression of mRNA. The principle of translational repression is often used for long genes, as translational repressed mRNA that is already in the cytoplasm can be activated without delay when the protein is needed (Nelson and Cox, 2000); owing to its
size, LisG is a good candidate for translational regulation, although this remains to be confirmed.

Several studies suggest that distinct chromatin-remodelling complexes may associate with specific genes (Kadam and Emerson, 2002; Kal et al., 2000). What subset of genes does LisG regulate? As LisG is involved in the lithium resistance response, LisG may be implicated in regulating expression of enzymes of the InsP-pathway. However, it is unlikely that LisG is involved in positively regulating dpoA expression, as gene expression studies, described in the previous chapter, argue against this hypothesis. However, LisG could positively regulate expression of IMPase, which is indicated by the finding that loss of LisG led to decreased IMPase expression; however, this hypothesis needs to be confirmed, i.e. by isolating the promoter of IMPase and conducting ATPase nucleosome remodelling assay in the presence or absence of recombinant LisG protein.

4.7.2 Is the chromatin-remodelling factor LisG essential for cell viability?

The findings that several attempts to knock out LisG failed and subsequently LisG was found to be expressed in the original LisG REMI mutant suggest that loss of LisG could be lethal, and that expression of a truncated form, as seen in the REMI mutant, is sufficient to give rise to the lithium resistance phenotype. However, there is no evidence in the literature associating loss of the CHD-family of proteins with lethality. To confirm whether disruption of LisG is lethal, one could (1) try to disrupt the gene in diploids, (2) try to repeat the original REMI-mutation, or (3) use a knock-in technique to rescue the gene in the LisG mutant. Furthermore, to establish whether there is a truncated protein one could use techniques such as rapid amplification of cDNA ends (RACE) or primer extension. In addition, ATPase nucleosome remodelling assays should be conducted to establish whether the expressed LisG protein is functional, which might not be the case.
Furthermore, although the reverse transcriptase reaction showed that the RT-PCR sample was not contaminated by genomic DNA and that the LisG mutant was expressing a blasticidin-resistance cassette, this does not exclude the possibility that (1) a second blasticidin-resistance cassette inserted into a different part of the genome or (2) that a second site in the genome is mutated, giving rise to the lithium resistant phenotype. No second product was amplified during the inverse PCR reaction used to identify the gene (Keim et al., 2004), arguing against the existence of a second blasticidin-cassette in the genome. However, as Southern Blot analysis showed that the gene encoding the chromatin-remodelling factor had not been rearranged in the original LisG mutant, a second site mutation as the cause for lithium resistance cannot be excluded until the mutant has been recapitulated, confirming the phenotype.

Alternatively, it could also be possible that the inserted blasticidin-resistance cassette initiated expression of the truncated LisG protein; nonetheless, as the truncated LisG protein gives rise to the lithium resistant phenotype, the question arises how this truncated form is responsible for this phenotype. Chromatin-remodelling complexes consist as multi-subunit complexes: e.g. the BAF (Brg- or Brm-associated factors)-complex consists of the Swi2-like ATPase core unit (either Brg1 or Brm) and up to 12 other proteins; furthermore, chromatin-remodelling complexes interact with a variety of transcription factors, and this interaction is critical for targeted promoter interaction and transcriptional specificity (Mohrmann and Verrijzer, 2005). Therefore, by clipping off the first 5 amino acids of LisG as in the REMI mutant, putative regulatory elements (e.g. a InsP-binding site, DNA-binding or transcription factor binding motifs) may have been destroyed, rendering the LisG protein non-functional; this may again be confirmed by ATPase nucleosome remodelling assays. However, no specific protein-protein interaction motifs have been reported for the N-terminus, therefore it remains to be investigated whether the N-terminal first 5 amino acids of LisG are essential for protein-protein interactions. In conclusion, it remains to be confirmed whether loss of the chromatin-remodelling factor LisG is lethal, as suggested by the data presented here.
4.7.3 Is basic motile behaviour distinct in lithium resistant mutants compared to wild type cells?

A non-gradient assay was designed to test basic motile behaviour of the lithium resistant cells. Treating wild type (Ax2) cells with 2 mM LiCl was sufficient to impair basic motile behaviour, characterised by a 50% reduction of total path length and speed compared to the corresponding sodium control, whereas the basic motile behaviour of the lithium resistant mutants was not impaired at this concentration.

Pseudopods are thought to be self-organising structures. However, when cells become more polarised, only one pseudopod is formed at a time, close to the previous one. Although these processes are G-protein independent, they are enhanced by Plns(3,4,5)P3, localising to the site of pseudopod formation, and require a functional actin-myosin machinery (Postma et al., 2004b). Therefore, owing to the lithium-induced inositol depletion, a decrease in the frequency of random pseudopod formation and polarisation in wild type (Ax2) cells, which was measured as the parameters total path length and speed, was to be expected, as less Plns(3,4,5)P3 can be formed due to low concentrations of the precursor inositol present in the cell; conversely, increased InsP-signalling, as found in the LisA mutant, was expected to confer resistance to the inositol-depleting effect of lithium, as more Plns(3,4,5)P3 can be formed due to high concentrations of inositol present in the cell. Furthermore, mRNA expression analysis found that LisG possesses elevated expression of enzymes of the InsP-pathway similar to LisA (see chapter 3), suggesting that increased flux through the InsP-pathway may be the underlying cause for this resistance.

In agreement with this explanation that lithium resistance in the lithium resistant mutants is dose-dependent, increasing the concentration of lithium led to inositol depletion even in the lithium resistant mutants LisA and LisG, thereby impairing random pseudopod formation and polarised movement. In addition, increasing the extracellular concentration of salt higher than 6 mM resulted in cell lysis.
4.7.4 Chemotactic behaviour of LisG and LisA

In the chemotactic assay, no difference in speed or direction towards the cAMP source was observed for the lithium resistant mutants LisA and LisG compared to wild type (Ax2) cells; this observation suggests that directional sensing was not altered in the lithium resistant mutants. However, LisA cells took a more direct path towards the cAMP source, most likely due to formation of fewer lateral pseudopods compared to wild type or LisG cells. But what could be the cause for a decrease in lateral pseudopod formation? As levels of Ins(1,4,5)P$_3$ are increased in the mutant, more substrate may be available for (1) generation of PIns(3,4,5)P$_3$ at the front of the cell, thereby increasing activation of the actin-cytoskeleton, and for (2) activation of PakA, thereby increasing suppression of lateral pseudopods. To test this hypothesis, one could compare PIns(3,4,5)P$_3$ levels between LisA and LisG cells by measuring binding of a GFP-tagged Ph-domain to PIns(3,4,5)P$_3$, or follow activation of PakA and myosin by expressing GFP-tagged PakA or myosin in LisA and LisG cells.

As lateral pseudopod formation of LisG cells was comparable to that of wild type (Ax2) cells, this suggests that the effects of LisG are distinct compared to LisA. This is in accordance with the findings from the RT-PCR analysis described in the previous chapter: although *ino1* and *IPP1* expression was increased in both LisA and LisG cells, IMPase expression decreased in LisG and increased in LisA. Taken together, these findings suggest that IMPase may have a less prominent role in lithium resistance than suggested by the inositol depletion theory. The question how increasing the flux through InsP-pathway without increasing IMPase protein and activity levels should overcome the uncompetitive inhibition of lithium is intriguing and remains to be elucidated.

4.7.5 Summary

- LisG encodes a chromatin-remodelling factor.
- Loss of LisG improved basic cell motility, but not chemotaxis.
- The nature of LisG remains to be confirmed, as LisG was expressed in the LisG mutant.
Chapter 5

Over-expression of *Ino1* and *IMPase* leads to lithium resistance
5.1 Introduction

Having established that changes in gene expression occur in the lithium resistant mutants LisA and LisG, the subsequent aim of my thesis was to investigate whether increasing the expression of *ino1* and IMPase conversely can overcome the ‘inositol depletion’ effect of VPA and lithium respectively.

Functional activity of the expressed proteins was assessed in *in vitro* enzyme assays. Comparison of the morphology of cells aggregating on filters and analysis of basic motile behaviour was used to investigate my hypothesis. Furthermore, *ino1* and IMPase were fused to a sequence encoding a C-terminal green fluorescent protein (GFP) “tag” to permit visualisation of the localisation of the proteins in fixed cells.

5.2 The over-expressed proteins are functionally active

Each of the over-expression constructs was transformed into wild type (Ax2) cells. Cells expressing the GFP-tagged vector only were used as control. As the epitope-tag could impair enzyme activity, it was important to assess functional activity of the over-expressed proteins. In addition, the presence of IMPase activity confirms whether the Blast search identified the correct enzymes.

Endogenous IMPase could mask the activity of non-functional GFP-tagged-proteins. Therefore, the GFP-tagged proteins were purified via binding to MACS micro bead columns (Miltenyi, UK). An *in vitro* IMPase assay has recently been described (Kaya *et al*., 2004) and was adapted, so that enzyme activity could be assessed with the proteins bound to the micro bead columns. The enzyme assay assessed the ability to hydrolyse Ins(1)P1 to inositol and inorganic phosphate.

The over-expressed IMPase protein was functionally active and hydrolysed InsP1. No IMPase activity was found for assays set up without the substrate in the assay buffer or for lysates of Ax2:GFP cells. The concentrations of protein
bound to the columns were not different between the samples, as determined by western blotting using antisera directed against GFP; this confirmed that the difference in measured phosphate release was not due to different amounts of protein present (Figure 5.1a). Furthermore, the over-expressed IMPase protein was lithium sensitive, because addition of 30 mM LiCl to the reaction buffer inhibited its activity by 80%. The concern that 30 mM LiCl, as used by Kaya et al. (Kaya et al., 2004), would impair functional activity not due to the presence of lithium but due to high salt concentration was not proven to be the case, as 30 mM NaCl did not impair function (Figure 5.1b).

Owing to the fact that a direct assay to test Ino1 activity did not exist, enzyme activity was measured indirectly using a two-step reaction (Figure 5.2a). In brief, columns with bound Ino1-GFP were incubated with buffer containing Glc-6-P as substrate. The flow through containing InsP$_1$ was collected and recombinant IMPase was added to complete the reaction. Phosphate release was measured with the malachite green method. Figure 5.2b shows an increase in phosphate release, suggesting that the ino1 protein is active. Again, this difference was not due to different amount of protein bound to the column, as determined by western blotting. Note that the background in the presence of substrate in the Ino1 assay (Figure 5.2b) is comparable to the background in the IMPase assay (Figure 5.1a).
Figure 5.1 The IMPase-GFP protein is active. GFP alone or a fusion protein of GFP and IMPase was purified by binding to GFP beads and subjected to activity assay. Phosphate release was measured with malachite green. Subsequently, the proteins were eluted and concentration bound to the beads were assessed by western blotting using antisera directed against GFP. The IMPase-GFP fusion protein is functionally active (A) and can be inhibited by lithium (B). A single assay each is displayed.
Figure 5.2 The Ino1-GFP protein is active. (A) Description of two-step process to assess ino1 activity. (B) GFP alone or a fusion protein of GFP and Ino1 was purified by binding to GFP beads and subjected to activity assay. Phosphate release was measured with malachite green. Subsequently, the proteins were eluted and concentration bound to the beads were assessed by western blotting using antisera directed against GFP. The Ino1-GFP fusion protein is functionally active. A single assay is displayed.
5.3 *Ino1* and *IMPase* over-expression in wild type cells

Having established that the over-expressed proteins are functionally active, the next step was to address the question whether over-expression of *ino1* and *IMPase* gives rise to VPA and lithium resistance respectively. To do this, two independent clones for each over-expressing mutant were starved and allowed to develop on nitrocellulose filters with and without lithium and VPA respectively. Morphology of the fruiting body was examined after 30 hours by light microscopy. Figure 5.3 and Figure 5.4 are representative of two repeats.

All cells aggregated well on 7.5mM NaCl. Wild type (Ax2) cells failed to aggregate in the presence of 10 mM LiCl, but aggregated in the presence of 7.5mM LiCl and formed aberrant fruiting bodies that mostly resembled those of *gskA*− mutant cells, characterised by formation of an increased basal disc at the expense of spore cells (Harwood *et al.*, 1995; Maeda, 1970). Furthermore, aggregation of wild type (Ax2) cells was impaired in the presence of 1 mM VPA, as characterised by a delay in aggregation and formation of few and small mounds. The morphology of Ax2:GFP cells in response to lithium or VPA resembled that of wild type cells (Figure 5.3 and Figure 5.4).

Over-expression of IMPase led to lithium resistance, as Ax2:IMPase-GFP cells aggregated and formed fruiting bodies in the presence of 10 mM LiCl; compared to those formed in the presence of 7.5 mM NaCl, most of the developed fruiting bodies were smaller and resembled the *gskA*− mutant phenotype, although some smaller wild type fruiting bodies were found interspersed. In addition, over-expression of IMPase led to VPA resistance; the fruiting bodies were characterised by short, thin stalks and large spore heads (Figure 5.3).
Figure 5.3 Phenotype of developing Ax2:IMPase-GFP cells. Ax2, Ax2:GFP-2 and Ax2:IMPase-GFP-2 were developed on nitrocellulose filters in the presence of 7.5 mM NaCl, 7.5 mM and 10 mM LiCl, and 1 mM VPA for 30 hours. Ax2:IMPase-GFP-2 cells develop into mature fruiting bodies in the presence of 10 mM LiCl and 1 mM VPA, whereas Ax2 and Ax2:GFP-2 fail to aggregate and develop. Scale bar represents 0.5 mm.
**Figure 5.4 Phenotype of developing Ax2:ino1-GFP cells.** Ax2, Ax2:GFP-2 and Ax2:ino1-GFP2 were developed on nitrocellulose filters in the presence of 7.5 mM NaCl, 7.5 mM and 10 mM LiCl, and 1 mM VPA for 30 hours. Ax2:ino1-GFP2 cells develop into mature fruiting bodies in the presence of 10 mM LiCl and 1 mM VPA, whereas Ax2 and Ax2:GFP-2 fail to aggregate and develop. Scale bar represents 0.5mm.
Cells over-expressing Ino1 formed wild type-like fruiting bodies with shorter stalks in the presence of 1 mM VPA. Cells over-expressing Ino1 were also cross-resistant to lithium, as they formed wild type fruiting bodies in the presence of 10 mM LiCl; compared to the fruiting bodies formed in the presence of 7.5 mM NaCl, the fruiting bodies formed were smaller (Figure 5.4).

5.4 Analysis of basic motile behaviour for Ax2:Ino1-GFP and Ax2:IMPase-GFP

To investigate whether lithium resistance was due to increased InsP-signalling, wild type (Ax2) cells over-expressing the enzymes Ino1 (Ax2:Ino1-GFP) and IMPase (Ax2:IMPase-GFP) were set up in the non-gradient assay. As the greatest difference between wild type (Ax2) and lithium resistant mutants LisA and LisG was observed at a concentration of 2 mM lithium, this concentration was used to examine basic motile behaviour of the over-expressers. However, as these mutants did not grow in inositol-free media (data not shown), the cells were grown in axenic media prior to the experiment. In addition, wild type (Ax2) and LisG cells were set up under the same conditions as controls to confirm the functionality of the assay. The data was analysed as before using the DIAS 3.1 software. Paths for each cell type tested are displayed in Figure 5.5, and the corresponding computed data is displayed in Figure 5.6.

Lithium impaired random movement of wild type (Ax2) cells, but did not change basic motility of LisG. In the presence of 2 mM LiCl, total path length and speed of wild type (Ax2) cells were decreased by 25% each (for all: Student's t-test; p-value < 0.01). Compared to cells grown in inositol-free media prior to conducting the experiment, basic motility was improved when cells were grown in axenic media: in the presence of 2 mM NaCl, wild type (Ax2) cells moved with a speed of 7.5 ± 0.6 μm min⁻¹ and covered 78 ± 6 μm of total path length, whereas LisG cells moved with a speed of 9 ± 0.7 μm min⁻¹ and covered 87 ± 8 μm of total path length.
Ax2:GFP control cells behaved like wild type (Ax2) cells: in the presence of 2 mM NaCl, Ax2:GFP cells moved with a speed of $6.9 \pm 0.7 \ \mu m \ min^{-1}$ and covered $73 \pm 4 \ \mu m$ of total path length; in the presence of 2 mM LiCl, Ax2:GFP cells moved with a speed of $5.1 \pm 0.5 \ \mu m \ min^{-1}$ and covered $58 \pm 5 \ \mu m$ of total path length; thus, in the presence of 2 mM LiCl, total path length and speed of Ax2:GFP cells were decreased by 21% (Student’s t-test; p-value < 0.05) and 28% (Student’s t-test; p-value < 0.01) respectively.

Wild type cells over-expressing *Ino1* or *IMPase* were both lithium resistant, as basic motile behaviour of these cells in the presence of 2 mM LiCl was not different from basic motile behaviour of these cells in the presence of 2 mM NaCl. In the presence of either 2 mM NaCl or 2 mM LiCl, Ax2:*Ino1-GFP moved with a speed of $6.4 \pm 0.5 \ \mu m \ min^{-1}$ and covered $68 \pm 5 \ \mu m$ of total path length, and Ax2:*IMPase-GFP moved with a speed of $7.3 \ \mu m \ min^{-1}$ and covered $75 \pm 3 \ \mu m$ of total path length (for all: Student’s t-test; p-value > 0.5).
Figure 5.5 Paths illustrating basic motile behaviour of Ax2:IMPase and Ax2:Ino1 cells towards lithium. Cells were starved for 1 hour in buffer supplemented with either 2 mM LiCl or 2 mM NaCl and subsequently videoed using time-lapse microscopy. 2D-DIAS analysis software was used to display the paths of individual cells. Basic motile behaviour of Ax2:IMPase and Ax2:Ino1 cells is not impaired at 2mM LiCl similar to LisG, when compared to wild type (Ax2) and Ax2::GFP cells.
Figure 5.6 Basic motile behaviour of Ax2:IMPase and Ax2:Ino1 cells towards lithium. Cells were starved for 1 hour in buffer supplemented with either 2 mM LiCl or 2 mM NaCl and subsequently videoed using time-lapse microscopy. 2D-DIAS analysis software was used to analyse the parameters (A) total path length and (B) speed. Basic motile behaviour of Ax2:IMPase and Ax2:Ino1 cells is not impaired at 2mM LiCl similar to LisG, when compared to wild type (Ax2) and Ax2:GFP cells. The average including SEM for one replicate comprising 40 cells is displayed. * p-value < 0.05; ** p-value < 0.01 (paired student’s t-test).
5.5 Cellular localisation of ino1 and IMPase

The localisation of Ino1 and IMPase has not been determined in *D.discoideum*. Therefore, wild type (Ax2) cells over-expressing GFP-tagged ino1 and IMPase respectively were fixed, stained with DAPI and observed by fluorescence microscopy.

As seen from the diffuse pattern of fluorescence in the control (Ax2:GFP), the GFP protein was not restricted to specific locations within the cell, although it showed enrichment in the nucleus of some cells; this observation has been reported by others (Fukuzawa *et al.*, 2003; Ginger *et al.*, 2000). The staining pattern of the IMPase protein (Ax2:IMPase-GFP) was similar to the diffuse staining pattern of the GFP protein (Ax2:GFP), suggesting that the IMPase protein also was not restricted to specific localisations in the cell. In contrast, the ino1 protein was excluded from the nucleus, as seen at the missing fluorescence in the nucleus in wild type cells expressing ino1-GFP (Ax2:Ino1-GFP); this observation was confirmed via co-staining of the nucleus with the fluorochrome DNA dye DAPI (Figure 5.7).

Neither the uniform distribution of IMPase nor the cytoplasmic localisation of Ino1 changed during cell movement, as was observed by time-lapse videomicroscopy (data not shown).
Figure 5.7 Localisation of Ino1 and IMPase protein. C-terminal GFP fusion proteins of Ino1 and IMPase were expressed in wild type *D.discoideum* cells. Localisation was observed with fluorescence microscopy: the IMPase protein localises uniformly throughout the cell, whereas the Ino1 protein is excluded from the nucleus as confirmed by staining with DAPI. Scale bars represent 10 μm.
5.6 Discussion

Filter development studies and 2D-DIAS analysis was used to investigate whether over-expression of \textit{ino1} and \textit{IMPase} suppresses the inositol-depletion effect of the mood-stabilisers VPA and lithium in the model organism \textit{D.discoideum}. Indeed, I found that over-expression of both \textit{ino1} and \textit{IMPase} was associated with VPA and lithium resistance respectively; however, over-expression of \textit{ino1} also led to lithium resistance, suggesting that inhibition of IMPase by lithium may not be the only determining factor for lithium sensitivity in \textit{D.discoideum}. In addition, using fluorescence microscopy I found that Ino1 was only localised in the cytoplasm whereas IMPase was localised throughout the cell.

5.6.1 Over-expression of \textit{Ino1} and \textit{IMPase}

It was a pre-requisite for subsequent experiments to confirm that the over-expressed proteins were functionally active. In addition, earlier studies observed three IMPase activities with different substrate specificity and lithium sensitivity: enzyme I requires the co-factor Mg$^{2+}$, hydrolyses all three metabolically derived isomers of InsP$_1$ and is inhibited by lithium; enzyme II hydrolyses only Ins(4)P$_1$ and does neither require Mg$^{2+}$ nor is it inhibited by lithium; enzyme III is also Mg$^{2+}$ independent and lithium resistant, but hydrolyses all three InsP$_1$, preferably Ins(3)P$_1$ (Van Dijken et al., 1996); therefore, it was important to establish whether the over-expressed IMPase is lithium sensitive. Although the assay did not allow for calculating kinetics of the enzyme, it demonstrated nonetheless that the IMPase-GFP protein was unaffected by the GFP, hydrolysed Ins(1)P$_1$ into inositol and inorganic phosphate, and was inhibited by lithium. As only the lithium resistant enzyme activity III is known to also hydrolyse Ins(1)P$_1$, this suggests that the over-expressed protein was the correct IMPase. Sequence alignments, described in chapter 3.4.1, support this finding, as the sequence of the over-expressed IMPase protein was closely related to the sequence of the IMPase protein from other organisms.
Owing to the fact that a direct assay to test Ino1 activity did not exist, the IMPase assay was adapted by a preceding step. Although this strategy was successful and showed that the Ino1-GFP protein was active, the observed enzyme activity was very low compared to the activity levels observed for IMPase-GFP. The following reason may account for this: Ino1 has been shown to require NAD$^+$ as a co-factor (Park and Kim, 2004), which was not present in the assay buffer; the observed activity of Ino1-GFP was probably due to residual NAD$^+$ bound to the purified enzyme.

Both the IMPase and the Ino1 assay were characterised by the same amount of background in the ‘no substrate control’. The individual enzyme activities were detected by measuring phosphate release via the malachite green method; this detection method is not specific for the enzymes, as the malachite green reagent will react with any free phosphate present in the reaction, leading to an increase in absorbance measurements. Therefore, a contaminating phosphatase present in the reaction may have given rise to an increase in absorbance, leading to the observed background.

5.6.2 Does lithium resistance correlate with increased flux through the InsP-pathway?

Lithium has been shown to inhibit IMPase through an uncompetitive mechanism, by trapping the enzyme product in the active site, thereby preventing its release (Hallcher and Sherman, 1980). This study showed that over-expression of IMPase leads to lithium resistance, which was analysed by examining aggregation and development in the model system D.discoideum. This observation is in accord with a study in yeast demonstrating that over-expression of IMPase also leads to lithium resistance (Lopez et al., 1999); in addition, the authors showed that over-expression of IMPase leads to increased intracellular free Ca$^{2+}$ which is required for activation of the Ena1 cation-extrusion ATPase; therefore, cells over-expressing IMPase are lithium resistant, because increased activation of Ena1 leads to increased reduction in the
cytoplasmic lithium concentration. Whether this mechanism is present in
*D.discoideum* remains to be investigated. Note that although cells over-
expressing IMPase are lithium resistant, the formed fruiting bodies resemble
those of the *gskA* mutants, suggesting that lithium’s effects of alternative
targets (i.e. inhibition of GskA) in the cell remain.

The finding that over-expression of Ino1 resulted in lithium resistance seems
counterintuitive. Owing to the uncompetitive mode of inhibition, it should be
difficult to overcome the effect of lithium through increasing the concentration of
the substrate InsP₁, as a result of *ino1* over-expression, and thereby giving rise
to lithium resistance. This observation may be explained by the existence of
three IMPase activities with distinct specificity and lithium sensitivity in
*D.discoideum*. Van Dijken *et al.* found that two of the three IMPase activities
present in *D.discoideum* are unaffected by lithium and hydrolyse all InsP₁
isomers; the authors also showed that 10 mM lithium reduces the inositol
content in the cell by only 20% (Van Dijken *et al.*, 1996). Taken together, the
findings by van Dijken *et al.* and the observation that over-expression of *ino1*
resulted in lithium resistance, as described in the present study, suggest that, in
*D.discoideum*, due to the existence of non-specific phosphatases acting on the
increased amount of InsP₁ caused by overexpression of *ino1*, lithium may not
act via depletion of the intracellular inositol pool through inhibition of IMPase, as
suggested for animal systems. Supporting this theory is the finding that double
gene disruption of *imp1* and *imp2*, encoding the yeast IMPase orthologues, did
not result in inositol auxotrophy (Lopez *et al.*, 1999). What could be the identity
of these IMPase activities? Sequence homology searches identified two other
genes closely related to IMPase, as described in chapter 3.4.1: IPP1
(DDB0167248), closely related to human and mouse IPPase, and IPP2
(DDB0189923), encoding a PAP-phosphatase-like protein. So far the genes
correlating to the IMPase enzyme activities have not been identified, although
the *D.discoideum* genome has been fully sequenced (Eichinger *et al.*, 2005);
therefore, as only IPP1 and IPP2 have been identified as being homologous to
IMPase, this suggests that IPP1 and IPP2 may encode the IMPase enzyme
activities II and III described by van Dijken *et al.* (Van Dijken *et al.*, 1996),
although this seems counterintuitive as IPPases are also inhibited by lithium;
enzyme activity assays should determine the $K_i$ of lithium for these enzymes and test whether IPP1 or IPP2 or both hydrolyse InsP$_1$, and thereby substitute for IMPase. Should neither show IMPase activity, this would suggest that an as yet not identified non-specific phosphatase exists in *D. discoideum*.

Similarly counterintuitive was the finding that over-expression of IMPase resulted in VPA resistance; as IMPase acts on a different part of the InsP-signalling pathway than VPA, over-expression of IMPase should not lead to VPA resistance. A possible explanation is the following: overexpression of IMPase may have led to an increased hydrolysis of InsP$_1$ to inositol and inorganic phosphate, thereby depleting InsP$_1$ in the cell; as a consequence of this, more Glc-6-P may have been converted to InsP$_1$, and subsequently to meet the demand, *Ino1* expression may have been increased, thereby giving rise to VPA resistance.

### 5.6.3 Does InsP-signalling regulate basic motile behaviour?

As the lithium resistant mutant LisA is characterised by increased levels of Ins(1,4,5)P$_3$ (Williams *et al.*, 1999), I hypothesised that lithium resistance may be due to increased flux through the InsP-pathway, as described in chapter 4.7.3. The data presented in the present chapter further supports this hypothesis of the flux through the InsP-pathway playing an important role in random pseudopod formation and polarisation: Studying the basic motile behaviour of wild type cells over-expressing *Ino1* (Ax2:*Ino1-GFP*) and IMPase (Ax2:IMPase-GFP) demonstrated that both of these cell types were lithium resistant, whereas the transformation control (Ax2:GFP) behaved similar to wild type (Ax2) cells, which are characterised by lithium sensitivity. The signalling molecule PIns(3,4,5)P$_3$ has been shown to localise to the site of pseudopod formation, thereby playing an important part in the process of random pseudopod formation and polarisation in *D. discoideum* cells (Postma *et al.*, 2004a). As PIns(3,4,5)P$_3$ is generated from inositol, lithium’s inositol depletion effect will impair random pseudopod formation. Conversely, over-expression of
IMPase will overcome the inositol depletion effect of lithium, ensuring the production of the PIns(3,4,5)P$_3$ precursor PIns(4,5)P$_2$.

However, similar to the lithium-resistant phenotype when analysed by filter developments, cells over-expressing Ino1 were also resistant to lithium when basic motility was analysed in a non-gradient assays; this further supports the theory that lithium may not deplete inositol through inhibition of IMPase as seen in animal systems. The importance of two other lithium-resistant IMPase activities, which may substitute for the by lithium inhibited IMPase protein, was discussed in the previous sub-section (see chapter 5.6.2).

In comparison, these results show that the non-chemotactic assay is easy and more reliable for screening lithium resistant mutants compared to filter developments; in addition, the non-chemotactic assay can also be adapted to investigate resistance of *D.discoideum* to other drugs.

### 5.6.4 Localisation of Ino1 and IMPase

Localisation studies using GFP-fusion proteins of IMPase and Ino1 showed for the first time that the IMPase protein is located uniformly throughout cell, whereas the Ino1 protein is excluded from the nucleus. Although no change in intracellular localisation of either IMPase or Ino1 due to cell movement were found in the present study, localisation of either protein might change with progression through the developmental process; this, however, remains to be investigated.

Although no study from other organisms exists so far demonstrating that IMPase resides in the nucleus, the finding of intranuclear localisation of IMPase in *D.discoideum*, as described in the present study, is in accord with emerging evidence of the existence of InsP-signalling in the nucleus: PLC-β1 has been found to localise to the nucleus in Swiss 3T3 cells (Martelli *et al.*, 1992); activation of PLC-β1 by MAPK-signalling leads to generation of the signalling molecule DAG, which has been shown to recruit PKCα (Martelli *et al.*, 2000; Xu
et al., 2001a; Xu et al., 2001b); PKC\(\alpha\) interferes with the G\(\beta\)\(\gamma\)-binding of PLC-\(\beta 1\), thereby leading to deactivation of PLC-\(\beta 1\) (Xu et al., 2001b). Taken together, the presence of a PLC isoform, the production of DAG and the inactivation of PLC-\(\beta 1\) by PKC\(\alpha\) in the nucleus, imply that the cell also requires components of the recycling machinery such as IMPase in the nucleus. As the activation of the nuclear InsP-pathway is distinct from the activation of its cytoplasmic counterpart, this suggests that the function of nuclear InsP-signalling is also distinct. What could be the role of nuclear InsP-signalling? PIns(4,5)P\(_2\) has been shown to be involved in pre-mRNA splicing (Osborne et al., 2001), as well as in facilitating the interaction between the chromatin-remodelling factor BAF-complex and the nuclear matrix by recruiting actin (Rando et al., 2002; Zhao et al., 1998). In addition, higher order inositol phosphates have been shown to be involved in mRNA export (York et al., 2001), and modulation of the activity of chromatin-remodelling complexes (Shen et al., 2003; Steger et al., 2003). Although Ins(1,4,5)P\(_3\) has not been shown in the nucleus so far, the existence of Ins(1,4,5)P\(_3\) receptors at the inner nuclear membrane (Humbert et al., 1996; Leite et al., 2003) suggests that Ins(1,4,5)P\(_3\) does have an as yet unidentified role.

The Ino1 protein is a cytoplasmic protein that converts Glc-6-P to Ins(3)P\(_1\), which is the first step in the inositol de novo synthesis (Donahue and Henry, 1981); this process requires NAD\(^+\) and consists of an oxidation reaction where NAD\(^+\) serves as H\(^+\)-acceptor, a condensation reaction to a cyclic product, and a reduction reaction where NADH serves as H\(^+\)-donor (Park and Kim, 2004; Stein and Geiger, 2002). Therefore, the solely cytoplasmic localisation for Ino1 in *D. discoideum* is in agreement with these earlier studies.

### 5.6.5 Summary

- Overexpression of IMPase or Ino1 in wild type cells led to lithium and VPA resistance.
- Ino1 was localised only to the cytoplasm, whereas IMPase was localised throughout the cell.
Chapter 6

Bipolar drugs affect gene expression of enzymes in the InsP-pathway
6.1 Introduction

The therapeutic time course of the mood-stabilising drugs during the treatment of bipolar disorder suggests that the mood-stabilisers may affect gene expression, as a short lag-phase between the first dose of the drug and the onset of the effect was found (Harwood, 2005). Therefore, using the RT-PCR assay, the aim of this chapter was to investigate whether bipolar drugs such as lithium, VPA and VPA-analogues also affect gene expression in wild type (Ax2) cells. VPA analogues used in this study were valpromide (VPM), N-valproyl glycinamide (VGD; also known as valrocemide), propyl isopropyl acetic acid (PIA), 2-methyl-2-pentynoic acid (2M2P), S-2-pentyl-4-pentynoic acid (ZW1) and R-2-pentyl-4-pentynoic acid (ZW2). Most of these VPA analogues have only recently been developed; the available information on their mechanism of action is limited, as summarised below.

VPA has been suggested to directly inhibit Gsk-3 activity in vitro (Chen et al., 1999), but this could not be substantiated. Phiel et al. showed that VPA inhibits Histone deacetylase (HDAC1) directly with a Ki of 0.4 mM (Phiel et al., 2001). In D.discoideum, VPA treatment does not phenocopy loss of GskA, further strengthening the argument against direct inhibition of Gsk3 by VPA (Williams et al., 2002).

VPM is the primary amide derivative of VPA and has been found to be more potent in the treatment of epilepsy than VPA. Furthermore, it is non-cytotoxic and non-teratogenic, as it does no inhibit HDAC activity (Eyal et al., 2005). However, it is known that VPM can act as a pro-drug for VPA (Bialer, 1991). PIA is an isomer of VPA and, like VPM, is non-teratogenic and non-cytotoxic (Eyal et al., 2005).

VGD is a conjunction product between VPA and glycaminamide, originally produced in an attempt to produce VPA analogues that penetrate the blood-brain barrier more easily than VPA. VGD's non-teratogenic and non-mutagenic properties were tested in animal models and are most likely due to the substitution of the free carboxyl group with an amide group and also due to
missing inhibition of HDAC activity. Furthermore, VGD was mainly metabolised via amide hydrolysis (non-oxidative pathway) to the inactive metabolite, valproyl glycine, with only a minor portion being converted to VPA (Hovinga, 2004). Anti-epileptic properties of VGD, which are thought to be due to the branched structure, were confirmed in phase II clinical trials (Hovinga, 2004; Isoherranen et al., 2001). Although VGD has not yet been tested for its efficacy in bipolar disorder, VGD reversed the effect of amphetamine-induced hyperactivity in rats (Bialer et al., 2004). The mechanism of action is still unknown.

2M2P is non-teratogenic, as it does not inhibit HDAC activity (Eyal et al., 2005). Furthermore, 2M2P has been shown to possess inositol depleting efficacy, which correlated with an increased spreading of growth cone from DRG; this could be reversed by addition of myo-inositol (Eickholt et al., 2005).

ZW1, a saturated derivative of VPA, leads to increased spreading of growth cones in DRGs, correlating to its strong effect of inositol depletion. ZW1 was also found to be highly teratogenic. In contrast, its non-inositol depleting enantiomer ZW2 did not affect growth cone spreading, although the effect on teratogenicity remained (Eickholt et al., 2005).
6.2 Lithium affects gene expression of recycling enzymes of the InsP-pathway

Wild type (Ax2) cells were set up shaking in axenic media containing either 7.5 mM NaCl or 7.5 mM LiCl for 24 hrs for RNA extraction. Before centrifugation, the cells were examined under the microscope to establish whether the drugs affected cell viability. cDNA was made from 1 μg of RNA, and the 100 ng of cDNA-dilution was used as template in the RT-PCR reaction. The results for the investigated genes are shown in Figure 6.1.

Treating wild type (Ax2) cells with 7.5 mM LiCl did not affect their viability (data not shown). Changes in gene expression due to lithium treatment were observed as followed: Expression of *Ino1* increased by 100% ± 22% in cells treated with 7.5 mM LiCl compared to cells treated with 7.5 mM NaCl (paired Student’s t-test; p-value < 0.05). Although lithium treatment did not change *IMPase* gene expression, expression of *IPP1* increased by 60% ± 12% in the presence of lithium compared to its sodium control (paired Student’s t-test; p-value < 0.01). Furthermore, expression for the 5’phosphatases 5P2, 5P3 and 5P4 was also elevated by 145% ± 50% (paired Student’s t-test; p-value < 0.05), 80% ± 5% (paired Student’s t-test; p-value < 0.01) and 47% ± 2% (paired Student’s t-test; p-value < 0.001) respectively, when cells were treated with lithium compared to cells treated with sodium.
Figure 6.1 Effect of lithium on gene expression. Total RNA was isolated from wild type (Ax2) cells treated with NaCl (salt control) or LiCl for 24 hours, reverse transcribed and used in the RT-PCR reaction. Relative change in expression of individual genes in wild type cells treated with NaCl or LiCl is displayed as percentage to the internal standard Ig7, calculated using the $2^{-\Delta\Delta CT}$ method. All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising three repeats of the same sample. Paired student’s t-test was used for statistical analysis.
6.3 Effect of VPA analogous on gene expression

Wild type (Ax2) cells were set up shaking in axenic media containing 1 mM of
VPA, VPM, VGD and PIA, or in media containing 0.5 mM of 2M2P, 1 mM ZW1
and ZW2 for 24 hrs for RNA extraction; in addition, untreated wild type (Ax2)
cells and wild type cells set up shaking in axenic media containing ethanol or
DMSO (carrier controls) were set up as controls. Before centrifugation, the cells
were examined under the microscope to investigate whether the drugs had any
effect on cell viability. cDNA was made from 1 µg of RNA, and 100 ng of cDNA
was used as template in the RT-PCR reaction. The results for the investigated
genes are shown in Figure 6.2.

Treating wild type (Ax2) cells with the mood-stabilising drugs did not affect their
viability (data not shown). Changes in gene expression due to drug treatment
were observed as followed: Expression of ino1 increased in wild type cells
treated with VPA and VPM by 44% ± 18% (paired Student’s t-test; p-value = 0.06) and 26% ± 10% (paired Student’s t-test; p-value = 0.06) respectively.
Expression of the remaining genes investigated was unchanged compared to
the carrier control. In addition, no change in gene expression was observed in
wild type (Ax2) cells treated with the VPA analogues PIA, 2M2P, ZW1 and
ZW2.

Expression in the wild type (Ax2) cells treated with VGD was as follows: IMPase
and IPP1 expression was decreased by 55% ± 1% and 48% ± 3% respectively
(for both: paired Student’s t-test; p-value < 0.001); in addition, dpoA expression
was decreased by 22% ± 6% (paired Student’s t-test; p-value < 0.05); no
change in expression of ino1 and the 5’-phosphatases were found.
(A) relative change in gene expression [%]

(B) relative change in gene expression [%]

(C) relative change in gene expression [%]

(D) relative change in gene expression [%]
Figure 6.2 Effect of VPA analogues on gene expression. Total RNA was isolated from wild type (Ax2) cells treated with (A) VPA, (B) VPM, (C) VGD, (D) PIA, (E) 2M2P, (F) ZW1, (G) ZW2 or the carrier controls KK$_2$ (A), EtOH (B-D) and DMSO (E-G) for 24 hours, reverse transcribed and used in the RT-PCR reaction. Relative change in expression of individual genes in wild type cells treated with carrier control or VPA analogues is displayed as percentage to the internal standard Ig7, calculated using the 2$^{-\Delta\DeltaCT}$-method. All data points displayed are averages with SEM, obtained from triplicate experiments, each comprising three repeats of the same sample. Paired student’s t-test was used for statistical analysis.
6.4 Effect of VGD on *D.discoideum* development

To investigate whether the effect of VGD treatment on gene expression correlated with behaviour, wild type (Ax2) and LisG cells were starved and allowed to develop on nitrocellulose filters in the presence of the VPA analogue VGD or ethanol (carrier control). Morphology of the fruiting body was examined after 24 hours by light microscopy; Figure 6.3 is representative for two repeats.

Both wild type (Ax2) and LisG cells aggregated and developed into mature wild type fruiting bodies in the presence of the carrier control ethanol. Furthermore, LisG cells also formed wild type fruiting bodies in the presence of 1 mM VGD, whereas wild type (Ax2) cells were impaired in their aggregation characterised by formation of few, small and aberrant fruiting bodies; this phenotype is similar to the *agg* phenotype of cARA/C− or acaA− null mutants (Kim *et al*., 1998; Pitt *et al*., 1992).

To assess whether VGD resistance was due to increased flux through the InsP-pathway, two independent clones for IMPase and Ino1 over-expressing wild type cells as well as GFP-only expressing wild type cells (control) were starved and allowed to develop on nitrocellulose filters in the presence of VGD or ethanol (carrier control). Morphology of the fruiting body was examined after 24 hours by light microscopy; Figure 6.4 is representative for two repeats.

GFP-only expressing wild type cells (Ax2:GFP) behaved similar to wild type (Ax2) cells, as they developed in the presence of ethanol but not in the presence of VGD. Over-expression of IMPase (Ax2:IMPase-GFP) or Ino1 (Ax2:ino1-GFP) in wild type cells suppressed the effect of VGD on development, as both cell types developed into mature fruiting bodies in the presence of VGD.
Figure 6.3 Effect of VGD on D.discoideum development. Wild type (Ax2) and LisG cells were developed on nitrocellulose filters in the presence of 1 mM ethanol (EtOH; carrier control) or 1 mM VGD for 30 hours. LisG cells developed into mature fruiting bodies in the presence of VGD, whereas wild type cells are impaired. Scale bar represents 0.5 mm.
Figure 6.4 Effect of VGD on Ax2:IMPase and Ax2:Ino1. Ax2:GFP (control), Ax2:IMPase-GFP and Ax2:Ino1-GFP cells were developed on nitrocellulose filters in the presence of 1 mM ethanol (EtOH, carrier control) or 1 mM VGD for 25 hours. Over-expression of IMPase or Ino1 suppresses the developmental defect of VGD. Scale bar represents 0.5 mm.
6.5 Discussion

Using the RT-PCR assay, I found that some of the mood-stabilising drugs tested during the present study indeed affect gene expression, as suggested by the therapeutic time course of mood-stabilising drugs in general (Harwood, 2005): (1) lithium increased the expression of enzymes involved in inositol phosphate signalling, with the exception of IMPase expression; (2) VPA and VPM increased expression of Ino1; (3) VGD decreased expression of IPP1, IMPase and dpoA. However, the changes in gene expression are complex and the expression patterns generated by the individual drugs were distinct, making it difficult to draw a definite conclusion from this study about how the drugs affect gene expression in general.

As part of this study, I also demonstrated that the newly developed VPA-analogue VGD, similar to its parent compound VPA, impaired aggregation of wild type D.discoideum cells. Conversely, I found that the lithium resistant mutant LisG and wild type cells over-expressing ino1 or IMPase were also resistant to VGD, when development of these cells was analysed on filters in the presence of VGD, suggesting a similarity of the effects of lithium and VGD.

6.5.1 Lithium affects gene expression in D.discoideum

For the first time, a transcriptional co-regulation of Ino1, IPP1 and the 5'-phosphatases was observed in response to lithium treatment, using the RT-PCR approach, demonstrating that the mood-stabilising drug lithium indeed affects gene expression. The finding of increased Ino1 expression due to lithium treatment is in agreement with studies in yeast (Vaden et al., 2001), mice hippocampus (Shamir et al., 2003) and D.discoideum (Williams et al., 2002). In yeast, Ino1 expression is increased in response to limiting inositol conditions; thus, as Ino1 expression was increased in response to treatment with lithium, the authors proposed that the mood-stabilising drug lithium depletes inositol (Vaden et al., 2001). In D.discoideum, however, an increase in Ino1 expression has also been shown in the mutant LisG and as a result of loss of PO, as
described in the previous chapters of this work; furthermore, 10 mM lithium decreased the intracellular inositol pool in *D. discoideum* by only 20% (Van Dijken *et al.*, 1996). Taking together, these findings suggest that lithium does not deplete inositol to the point of becoming limiting in the cell, thus lithium-induced inositol depletion cannot be the underlying reason for the observed changes in gene expression in *D. discoideum*. In addition, a double gene disruption of both IMPase homologues in yeast did not lead to inositol auxotrophy (Lopez *et al.*, 1999), suggesting that inositol depletion as such may not be the main effect of lithium. The authors suggested that non-specific phosphatases might compensate for the loss of IMPase. Interestingly, the present study found an increase in expression of *IPP1* and the 5'-phosphatases as a result of treatment with lithium; IPP1 and the 5'-phosphatases play a role in the breakdown of Ins(1,4,5)P3 in the recycling pathway (Manahan *et al.*, 2004). It would be difficult to overcome the uncompetitive inhibition of lithium by increasing the expression of *IPP1* and the 5'-phosphatases, thereby increasing the hydrolysis of Ins(1,4,5)P3, unless IPP1 or the 5'-phosphatases are able to substitute for IMPase, thus compensating for the effect of lithium. The finding of increased *IPP1* expression due to lithium treatment correlates with increased expression of this enzyme in the lithium resistant mutants LisA and LisG, further supporting a possible compensatory role for this enzyme in lithium resistance.

Another finding of the present study was that lithium did not change expression of IMPase, suggesting that the levels of inositol in the cell itself do not regulate IMPase expression in *D. discoideum*. Although regulation of *Ino1* gene expression has been studied in great detail, no information is available on the regulation of *IMPase* gene expression. Mice and humans possess two IMPase enzymes encoded by the gene pair *IMPA1* and *IMPA2*; a study by Shamir *et al.* found increased *IMPA1* expression and no change in *IMPA2* expression in the mice hippocampus treated with lithium (Shamir *et al.*, 2003). Both of these genes, *IMPA1* and *IMPA2*, are associated with a susceptibility to bipolar disorder. The *D. discoideum* IMPase is more closely related to the mouse *IMPA1* gene, as described in chapter 3.4.1; as expression of the mouse *IMPA1* was increased due to lithium treatment, and no change in gene expression was observed for *D. discoideum IMPase*, this suggests a distinct function for the
IMPase homologue of *D.discoideum*. However, the mechanism of IMPase regulation in general remains to be elucidated.

### 6.5.2 Some VPA analogues also affect gene expression in *D.discoideum*

Treatment of *D.discoideum* cells with VPA led to a mild increase in expression of *lno1*; this finding is in agreement with earlier studies from yeast and *D.discoideum* (Vaden et al., 2001; Williams et al., 2002). In yeast, increased *lno1* expression has been shown to be due to depletion of the intracellular inositol pool (Greenberg and Lopes, 1996), suggesting that VPA depletes inositol levels. The inositol depletion effect of VPA was confirmed in rat dorsal root ganglia (DRG) explants: treatment of DRG explants with VPA led to increased growth cone spreading; this effect could be reversed by supplementation with inositol (Williams et al., 2002). Taken together, these findings suggest that inositol concentration may regulate gene expression of *lno1* in *D.discoideum*. In addition, for the first time, a mild increase in *lno1* expression was found when treating *D.discoideum* cells with the VPA-analogue VPM, suggesting that VPM also depletes the inositol levels in the cell. However, as VPA has been shown to inhibit histone deacetylase-1 (HDAC-1) (Gurvich et al., 2004; Phiel et al., 2001), changes in gene expression may also be due to modulation of chromatin structure, and not due to changing inositol concentrations.

In contrast to lithium treatment, however, neither treatment with VPA nor treatment with VPM led to changes in gene expression of IMPase, IPP1 and the 5'-phosphatases. This difference may be attributed to the distinct mechanism of action for the individual drugs: lithium uncompetitively inhibits the recycling enzymes IMPase and IPP1 of the InsP-pathway (Hallcher and Sherman, 1980; Inhorn and Majerus, 1987), whereas VPA inhibits histone deacetylase-1 (HDAC-1) and thereby inhibits *ino1* expression and inositol de novo synthesis (Phiel et al., 2001; Shaltiel et al., 2004).
Alternatively, the difference in gene expression patterns for lithium and VPA could also be attributed to the assay design, as the concentration of 1 mM for VPA and the VPA-analogues may have not been sufficient to lead to changes in gene expression, especially as the axenic media contained undefined concentration of inositol, thereby possibly suppressing some of the effects. This hypothesis is supported by the finding that gene expression was not changed when cells were treated with the VPA-analogues PIA, ZW1 and 2M2P, although all of these analogues have been shown to deplete the intracellular inositol pool (Eickholt et al., 2005) (J. A. Shimshoni, personal communication).

As the VPA analogue VGD has been found to be 2-fold more potent than its parent compound VPA (Isoherranen et al., 2001), the concentration used may have been sufficient to observe an effect. VGD is a conjunction product between VPA and glycinamide, and its non-teratogenic property suggests that it may not inhibit HDAC-1 activity. Nonetheless, changes in gene expression were observed as a result of VGD treatment: VGD did not increase expression of Ino1, suggesting that it does not deplete the intracellular inositol pool; however, VGD decreased the expression of IMPase, IPP1 and dpoA. Like VPA, VGD also inhibits aggregation of wild type D.discoideum cells, suggesting that VGD affects InsP-signalling. Interestingly, the lithium resistant mutant LisG was also cross-resistant to VGD, as were cells over-expressing Ino1 or IMPase. So far, it is still unknown how the remaining enzymes involved in InsP-signalling are affected by over-expressing either IMPase or Ino1. However, in the LisG mutant, both IMPase and dpoA expression were decreased, similar to treating wild type cells with VGD; interestingly, expression of IPP1 was increased in the LisG mutant, which is the opposite of the changes observed in wild type cells treated with VGD. Taken together, these findings suggest that modulation of IPP1 expression may not only be important for lithium resistance, but equally important for VGD resistance. If this were true, then the LisA mutant, which has

---

4 J. A. Shimshoni1, E. C. Dalton2, A. Jenkins3, S. Eyal1, K. Ewan2, R. S. B. Williams3, B. Yagen4,5, A. J. Harwood3 and M. Bieler1,5 (1 Department of Pharmaceutics, School of Pharmacy, The Hebrew University of Jerusalem, Israel; 2 School of Biosciences, Cardiff University, UK; 3 Department of Biology, UCL, UK; 4 Department of Medicinal Chemistry and Natural Products, School of Pharmacy, The Hebrew University of Jerusalem, Israel; 5 David R. Bloom Center for Pharmacy, School of Pharmacy, The Hebrew University of Jerusalem, Israel).
been shown to have elevated *IPP1* expression, should also be resistant to VGD. The mechanism of action for VGD needs to be elucidated.

In summary, changes in gene expression as a result of treatment of *D. discoideum* cells with VPA or its analogues VPM and VGD were observed in the present study, but it is still unclear whether these changes in gene expression are independent or dependent on the inositol concentration. Nonetheless, caution in interpreting the data for the remaining drugs is advisable, as the drug concentration used may not have been sufficient to induce an effect.

6.5.3 Summary

- Lithium increased the expression of enzymes involved in InsP signalling, with exception of *IMPase*.
- VPA and VPM increased expression of *Ino1*.
- VGD decreased expression of *IPP1*, *IMPase* and *dpoA*.
- Loss of LisG and overexpression of *Ino1* or *IMPase* in wild type cells led to VGD resistance.
Chapter 7

Discussion
7.1 Aims of this work

The aim of this thesis was to investigate whether lithium sensitivity is due to changes in gene expression. To address this problem, this thesis answers the following sub-questions:

- Can changes in gene expression be observed?
- How does the lithium resistant mutant LisG relate to gene expression?
- Does an increase in expression of IMPase or Ino1 give rise to lithium or VPA resistance?
- How do bipolar drugs affect gene expression?

7.2 Can changes in gene expression be observed?

I have shown that changes in gene expression can indeed be observed in the lithium resistant mutant LisG and as a result of loss of PO, supporting the hypothesis that lithium sensitivity may correlate with changes in gene expression. However, the overall changes in gene expression observed in the LisG mutant were distinct from those observed as a result of loss of PO, although some similarities existed. In both mutants, expression of Ino1 and IPP1 was found to be increased, suggesting that an increase in expression of Ino1 or IPP1 or an increase in both may lead to lithium resistance. In contrast, an increase in the expression of IMPase and the 5'-phosphatases 5P2 and 5P3 were observed as a result of loss of PO, whereas a decrease in the expression of IMPase and no change in the expression of the 5'-phosphatases 5P2 and 5P3 were found in the lithium resistant mutant LisG, suggesting that these changes are characteristic for the individual mutants. As the two mutants are deficient for distinct proteins – LisG encodes the ATPase subunit of a chromatin-remodelling complex (yet to be confirmed) and LisA encodes a prolyl oligopeptidase (Williams et al., 1999) – the gene expression pattern for the individual mutants may also be expected to be different. In addition, results from the gene expression studies argue against the hypothesis that LisG may regulate dpoA expression, although cells that have lost LisG do not respond to PO.
These differences in gene expression changes also correlated with differences in motile behaviour, when cells were analysed in a spatial gradient of the chemoattractant cAMP (without lithium present): whereas the behaviour of the LisG mutant resembled that of wild type cells, the LisA mutant was characterised by formation of fewer lateral pseudopods, resulting in a straighter path towards the cAMP source. Localisation of the membrane bound PIns(3,4,5)P$_3$ to the site of pseudopod formation has been shown to play an important role in polarisation of the *D.discoideum* cells (Postma *et al.*, 2004a). Thus, as LisA is characterised by increased intracellular concentrations of Ins(1,4,5)P$_3$ owing to increased breakdown of higher inositol phosphates (Williams *et al.*, 1999), more substrate may be available for the generation of PIns(3,4,5)P$_3$ at the leading edge via the recycling pathway, thereby increasing activation of the actin cytoskeleton. Increased PIns(3,4,5)P$_3$ production at the leading edge may also lead to increased activation of myosin heavy chain kinase A (MHCKA), which locally disassembles myosin-II filaments, establishing cell polarity (Egelhoff *et al.*, 2005; Liang *et al.*, 2002). Simultaneously, increased PIns(3,4,5)P$_3$ production at the side and rear of the cell may lead to increased activation of the PI3K/PKB-pathway, which has been shown to activate p21-activated kinase A (PakA) involved in myosin-II filament assembly, thereby suppressing lateral pseudopod formation (Chung *et al.*, 2001). Taken together, elevated PIns(3,4,5)P$_3$ production due to increased higher inositol phosphate breakdown is likely to explain the increased polarity observed in the spatial gradient compared to wild type and LisG cells. However, as the LisG mutant is not characterised by formation of fewer lateral pseudopods, this also implies that the LisG mutant may not have increased Ins(1,4,5)P$_3$ levels, although this remains to be investigated, and that lithium resistance is not caused by an increase in total mass of the inositol phosphates. This possible explanation is in agreement with the uncompetitive mechanism of inhibition of lithium (Hallcher and Sherman, 1980): lithium only binds to the substrate-enzyme complex, and traps and prevents the release of the enzyme product. Therefore, an increase in total inositol phosphate mass will not substitute for the inactivated enzyme IMPase. Thus, an increase in inositol *de novo* synthesis, leading to increased total InsP mass, due to increased expression of *lno1* as observed in both
lithium resistant mutants will amplify the uncompetitive inhibition of IMPase by lithium. Interestingly, IMPase expression was only increased in the LisA mutant but not in the LisG mutant, suggesting that IMPase may have a less prominent role in conferring lithium resistance as implied by the inositol depletion hypothesis (Berridge et al., 1989).

The only similarity remaining between LisA and LisG is elevated expression of IPP1, the phosphatase responsible for the hydrolysis of Ins(1,4,5)P_{3} and Ins(1,4)P_{2} in the recycling pathway. Similar to IMPase, IPPase from calf brain is also uncompetitively inhibited by lithium. This poses the question what the possible reasons could be for increased IPP1 expression, observed in both lithium resistant mutants. Van Dijken et al. found that 10 mM lithium decreased the intracellular pool of inositol by only 20% (Van Dijken et al., 1996). In addition, in yeast it has been shown that a double gene disruption of the IMPase orthologues imp1 and imp2 did not lead to inositol auxotrophy (Lopez et al., 1999). Taken together, these findings suggest that non-specific InsP_{1}-phosphatases may exist and are able to substitute for the lithium sensitive IMPase. In D.discoideum, three IMPase activities were shown, with two of those being lithium resistant (Van Dijken et al., 1996); in the present study, homology searches of the full D.discoideum genome sequences with the yeast or mammalian IMPase homologues identified three genes, which may correspond to the three enzyme activities identified by van Dijken et al. (Van Dijken et al., 1996): IMPase, IPP1 and IPP2. Thus, it is possible that IPP1 or IPP2 encode the lithium resistant IMPase activities described by van Dijken et al.. Therefore, if IPP1 indeed hydrolysins Ins(1)P_{1}, an increase in IPP1 expression would overcome the uncompetitive inhibition of lithium. This also implies that, after a period of time, D.discoideum cells would overcome the inhibitory effect of lithium; this has indeed been shown in filter development assay: when cells are left for a prolonged period of time, they do eventually aggregate. However, IMPase activity assay with the recombinant IPP1 protein will be required to confirm this hypothesis.
7.3 Does an increase in the expression of IMPase or Ino1 give rise to lithium or VPA resistance?

Using filter development and non-gradient motility assays, I have shown that over-expression of IMPase or Ino1 in wild type D.discoideum cells indeed gives rise to lithium and VPA resistance, respectively, thus further supporting the hypothesis that lithium sensitivity may correlate with changes in gene expression. As lithium inhibits IMPase uncompetitively, thereby complexing the enzyme-substrate complex (Hallcher and Sherman, 1980), over-expression of IMPase resulted in increased enzyme activity and may have compensated for lithium's inhibitory effect. As IMPase expression was not elevated in the LisG mutant, this suggests that over-expression of IMPase is only one of at least two mechanisms to overcome the effect of lithium. A further mechanism exists in yeast, where over-expression of IMPase led to increased activation of the cation extrusion ATPase Ena1, thereby decreasing cytoplasmic concentration of lithium (Lopez et al., 1999); whether this mechanism is present in D.discoideum remains to be investigated.

Surprisingly, over-expression of Ino1 in D.discoideum cells not only led to VPA resistance, but also to lithium resistance. This finding suggests that, despite the uncompetitive mechanism of lithium action, increasing the total mass of InsPs in the cell is sufficient to confer lithium resistance, implying that a non-specific phosphatase, which is not inhibited by lithium, may be present in D.discoideum. This non-specific phosphatase may be able to compensate for enzymes of the inositol phosphate signalling pathway, such as IMPase and IPPase, when these are impaired by lithium and InsP1 concentrations increase. Increased expression of Ino1 and IPP1 was observed in both of the lithium resistant mutants LisA and LisG. Taken together, these findings suggest that IPP1 not only hydrolyses its primary substrates Ins(1,4,5)P3 and Ins(1,4)P2, but that IPP1 may also hydrolyse InsP1, when the concentration of InsP1 reaches a certain threshold in the cell. Therefore, the cell becomes lithium resistant by increasing Ino1 and IPP1 expression simultaneously. If this were the case, then one would expect to find increased IPP1 expression in cells over-expressing Ino1; this
needs to be confirmed by RT-PCR analysis. In addition, it is essential to show the \( \text{InsP}_1 \)-hydrolytic activity of the recombinant IPP1 protein.

Furthermore, using fluorescence microscopy the subcellular localisation of Ino1 and IMPase was determined. For the first time, it has been shown that Ino1 localises only to the cytoplasm, whereas IMPase is localised throughout the cell including the nucleus, as confirmed by DAPI co-staining. The finding of nuclear IMPase is in agreement with emerging evidence of nuclear \( \text{InsP} \)-phosphate signalling. Nuclear PLC-\( \beta \)1 has been shown to be activated via the MAPK-pathway, leading to generation of DAG in the nucleus (Martelli et al., 2000; Martelli et al., 1992); DAG has been shown to recruit PKC\( \alpha \), which inactivates PLC-\( \beta \)1 (Xu et al., 2001a; Xu et al., 2001b). In the cytoplasm, activation of PLC gives rise to two products, \( \text{Ins}(1,4,5)P_3 \) and DAG. Therefore, the finding from the present study that IMPase is located in the nucleus, strongly supports that a nuclear recycling pathway exists, similar to the one in the cytoplasm. In addition, exclusive localisation of Ino1 to the cytoplasm suggests that inositol \textit{de novo} synthesis occurs only in the cytoplasm.

### 7.4 How do bipolar drugs affect gene expression?

By RT-PCR analysis, I have shown that bipolar drugs such as lithium, VPA and its two analogues VPM and VGD do indeed affect gene expression in \textit{D. discoideum}, as has been suggested by the therapeutic time course of mood-stabilisers in general (Harwood, 2005), although the overall observed gene expression changes were distinct for the individual drug treatments. Nonetheless, these findings suggest that some, but not all of the effects of the drugs can be explained by changes in gene expression, thereby further strengthening the hypothesis that lithium sensitivity may be correlated with changes in gene expression.

In agreement with their observed inositol-depletion effect (Vaden et al., 2001; Williams et al., 2002), both, lithium and VPA increased the expression of Ino1 in \textit{D. discoideum}. As VPM also increased the expression of Ino1, this finding
suggests that VPM equally depletes the intracellular pool of inositol. However, it
cannot be assumed that inositol depletion is the underlying cause for the
observed gene expression changes: the VPA analogue VGD did not increase
the expression of \textit{Ino1}, suggesting that VGD does not deplete inositol; VGD,
however, did change the expression of other genes, suggesting that changes in
gene expression are not solely due to inositol depletion, but are due to an as
yet unidentified mechanism. As VPA has been shown to inhibit HDAC-1 activity
(Gurvich \textit{et al.}, 2004; Phiel \textit{et al.}, 2001), changes in gene expression may be
due to the modulation of chromatin structure. However, as VGD has lost its free
carboxyl-group, which has been suggested to be responsible for inhibiting
HDAC-1 activity (Isoherranen \textit{et al.}, 2001), the observed gene expression
changes as a result of VGD treatment are possibly due to an as yet unidentified
mechanism.

The present work also showed that lithium increased the expression of \textit{IPP1}
and the 5'-phosphatases \textit{5P2}, \textit{5P3} and \textit{5P4}, but not the expression of \textit{IMPase}.
An increase in \textit{IPP1} expression as a result of lithium treatment correlates with
the observed increase of \textit{IPP1} expression in the lithium resistant mutants LisA
and LisG. This finding further strengthens my hypothesis that \textit{IPP1}, by
compensating for the inhibited \textit{IMPase} protein, may play an important role in
conferring lithium resistance. However, this hypothesis remains to be
confirmed, by demonstrating lithium resistance and InsP$_1$-hydrolytic activity for
the recombinant \textit{IPP1} protein.

Similar to lithium, VGD also inhibited the aggregation of wild type \textit{D.discoideum}
cells. Interestingly, the lithium resistant mutants LisG and cells over-expressing
\textit{Ino1} or \textit{IMPase} are also cross-resistant to VGD. In contrast to lithium, however,
VGD decreased the expression of \textit{IPP1} in wild type cells. \textit{IPP1} expression was
increased in the LisG mutant, suggesting that modulation of \textit{IPP1} expression
may be equally important in conferring resistance to lithium as to VGD; if this
were the case, then the LisA mutant, which has increased \textit{IPP1} expression
should also be resistant to VGD. In addition, the expression of \textit{IPP1} in cells
over-expressing \textit{Ino1} or \textit{IMPase} is so far unknown, but RT-PCR analysis of
these cells could be used to confirm this theory.
7.5 Summary

Taking the findings from the individual chapters together, I have shown that lithium sensitivity does correlate with changes in gene expression. This is supported by the findings that gene expression changes occur in the lithium resistant mutants LisA and LisG, and, vice versa, that modulating gene expression can equally give rise to lithium resistance. Furthermore, although the mutant LisG needs to be recapitulated, LisG encodes the ATPase subunit of a chromatin-remodelling complex, strongly supporting a link between lithium sensitivity and regulation of gene expression. In addition, this hypothesis is supported by the finding that some, but not all of the effects of mood-stabilising drugs can be explained by changes in gene expression. However, it has proven difficult to establish a definite conclusion about the common mechanism of action for the mood-stabilising drugs, as the overall changes in gene expression observed for the individual drug treatments were distinct. Furthermore, from the findings presented in this work I could not conclude that a correlation between inositol-depletion, as a result of treatment with lithium or VPA, and the observed changes in gene expression exists. However, I have presented a possible mechanism for conferring lithium resistance that is shared by at least two independent lithium resistant mutants: the lithium resistant IPP1 may substitute for the loss of IMPase, when a threshold of intracellular InsP₁ is reached; therefore, by increasing the expression of Ino1 simultaneously with IPP1, InsP₁ accumulates in the cell, thereby leading to lithium resistance. This hypothesis, however, remains to be proven.
Chapter 8

Appendix
8.1 Malachite green standard curve
Phosphate release was detected using the established malachite green method which is based on the principle that malachite green at a lower pH will form a complex with phosphomolybdate with a marked shift of the absorption maximum; this also correlates with a colour change from brown (without phosphate) to green (with phosphate) (Bernal et al., 2005; Itaya and Ui, 1966). To establish the sensitivity of the assay, a standard curve for a range of sodium hydrogen phosphate concentrations was obtained. The standard curve was linear between 10 and 40 μM phosphate concentration with absorbance readings between 0.6 and 1 (Figure 8.1).
Figure 8.1 Malachite green standard curve measuring phosphate release. 10, 20, 30 and 40 μM of Na₂HPO₄ buffer were added to malachite green and incubated at room temperature for 10 min. Phosphate release was detected by measuring absorbance at 620 nm using a spectrophotometer.
8.2 Optimisation of RT-PCR

Primers were designed to the coding region of the genes of interest to yield a PCR product between 300 and 600 bp. Main properties of the primers are given in Table 8.1.

Table 8.1 RT-PCR primers used to generate DNA standards.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Forward primer/ Reverse primer</th>
<th>Product size [bp]</th>
<th>Tm* [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ino1</td>
<td>5'-GACACCGTCGCCTATGTTGTTGCCG-3' 439</td>
<td>83.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-GCAATCATATCAGACAAGATTTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMPase</td>
<td>5'-GTTATGACCAAGCTGGAATTTG-3'</td>
<td>468</td>
<td>79.2</td>
</tr>
<tr>
<td></td>
<td>5'-GCTGTACCTGAATCTTTAATGCTGAAC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPP1</td>
<td>5'-AGAGGAAGCAATGGAATACCAAC-3'</td>
<td>423</td>
<td>82.4</td>
</tr>
<tr>
<td></td>
<td>5'-TTGACATTTTACATTGAAAGAGGGC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPP2</td>
<td>5'-GATCAAGAGGAAACATTAAGGGTTC-3'</td>
<td>325</td>
<td>79.0</td>
</tr>
<tr>
<td></td>
<td>5'-CCTTTTGAATCTAAATTGTGTGTTT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5P2</td>
<td>5'-GGTTCATACTAGTGTTGGGATGG-3'</td>
<td>388</td>
<td>80.6</td>
</tr>
<tr>
<td></td>
<td>5'-GGAAGCAGCTGTGTTTGGAAGGG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5P3</td>
<td>5'-ACGATTGCTTTTCCAAATTACCAAGG-3'</td>
<td>384</td>
<td>79.4</td>
</tr>
<tr>
<td></td>
<td>5'-TGCAATGGAAAGCTCGGTGTGGTGGGTCG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5P4</td>
<td>5'-GTATTATCAATGATCATCAATCACC-3'</td>
<td>418</td>
<td>78.6</td>
</tr>
<tr>
<td></td>
<td>5'-CGCTGTGTTCTCAAATGACAAGCTTTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DpoA</td>
<td>5'-GGCAGGTCTGATAAGGTAAGATTTG-3'</td>
<td>446</td>
<td>81.0</td>
</tr>
<tr>
<td></td>
<td>5'-ACATAGATTAGCTCAACATTGCAG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ig7</td>
<td>5'-GTACTTAAACCGCATGGAATTTTAC-3'</td>
<td>305</td>
<td>83.2</td>
</tr>
<tr>
<td></td>
<td>5'-CGCTACCCCTTAGAACGTCATTAGTTA-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Tm denotes the melting temperature of the amplified PCR product.

The first step was to generate plasmid standard curves. For this, the PCR product was cloned into a TOPO vector for each gene used in the RT-PCR analysis. Subsequently, serial dilutions of each plasmid were made from 200 pg to 0.0002 pg and used as template for optimisation of the RT-PCR conditions. All RT-PCR reactions were set up in triplicates.
As an example, a representative data file for a RT-PCR run for the Ig7 gene is shown in Figure 8.2. The top panel shows the kinetics of a typical PCR reaction, by plotting fluorescence against cycle number. As the PCR product is amplified, fluorescence increases due to binding of the dye SybrGreen to double stranded DNA. The Cycle threshold (Ct) is defined as the cycle when sample fluorescence exceeds background fluorescence and the reaction kinetics go into the exponential phase.

As the dye SybrGreen binds non-specifically to double-stranded DNA, it was important to test whether the fluorescence was due to amplification of the desired product or due to primer dimers. Melting curves obtained at the end of each RT-PCR run plot the change in fluorescence against temperature. The amplified product should have a single peak according to its nucleotide composition. Primer dimers usually give an extended peak approximately up to 15°C below that of the amplified product. The primers for Ig7 were specific to the gene and did not form dimers (Figure 8.2b). The primers for the remaining genes were optimised in the same way, so that they could be used with the same PCR protocol (Figure 8.3 to Figure 8.11).
Figure 8.2 RT-PCR output for Ig7. (A) Amplification plot showing the fluorescence plotted against the cycle number for the standard dilution series for Ig7. (B) Melting curve showing fluorescence plotted against temperature were acquired as described in methods. These data are representative of multiple experiments.
Figure 8.3 RT-PCR output for Ino1. (A) Amplification plot showing the fluorescence plotted against the cycle number for the standard dilution series for Ino1. (B) Melting curve showing fluorescence plotted against temperature were acquired as described in methods. These data are representative of multiple experiments.
Figure 8.4 RT-PCR output for IMPase. (A) Amplification plot showing the fluorescence plotted against the cycle number for the standard dilution series for IMPase. (B) Melting curve showing fluorescence plotted against temperature were acquired as described in methods. These data are representative of multiple experiments.
Figure 8.5 RT-PCR output for IPP1. (A) Amplification plot showing the fluorescence plotted against the cycle number for the standard dilution series for IPP1. (B) Melting curve showing fluorescence plotted against temperature were acquired as described in methods. These data are representative of multiple experiments.
Figure 8.6 RT-PCR output for IPP2. (A) Amplification plot showing the fluorescence plotted against the cycle number for the standard dilution series for IPP2. (B) Melting curve showing fluorescence plotted against temperature were acquired as described in methods. These data are representative of multiple experiments.
Figure 8.7 RT-PCR output for 5P2. (A) Amplification plot showing the fluorescence plotted against the cycle number for the standard dilution series for 5P2. (B) Melting curve showing fluorescence plotted against temperature were acquired as described in methods. These data are representative of multiple experiments.
Figure 8.8 RT-PCR output for 5P3. (A) Amplification plot showing the fluorescence plotted against the cycle number for the standard dilution series for 5P3. (B) Melting curve showing fluorescence plotted against temperature were acquired as described in methods. These data are representative of multiple experiments.
Figure 8.9 RT-PCR output for 5P4. (A) Amplification plot showing the fluorescence plotted against the cycle number for the standard dilution series for 5P4. (B) Melting curve showing fluorescence plotted against temperature were acquired as described in methods. These data are representative of multiple experiments.
Figure 8.10 RT-PCR output for dpoA. (A) Amplification plot showing the fluorescence plotted against the cycle number for the standard dilution series for dpoA. (B) Melting curve showing fluorescence plotted against temperature were acquired as described in methods. These data are representative of multiple experiments.
Figure 8.11 RT-PCR output for LisG. (A) Amplification plot showing the fluorescence plotted against the cycle number for the standard dilution series for LisG. (B) Melting curve showing fluorescence plotted against temperature were acquired as described in methods. These data are representative of multiple experiments.
The calibration curves for the plasmid standards are shown in Figure 3.4 to Figure 3.9 and Figure 4.2, where mean C\textsubscript{T}-values of plasmid dilutions for each gene were plotted against log copy numbers resulting in a linear regression with a good R\textsuperscript{2} value. Table 8.2 shows the PCR efficiencies deducted from the standard curves via the equation $\varepsilon=10^{-1/m}$. It is a requirement for the 2-\textsuperscript{ΔΔCT} method that the amplification of the target and the internal standard (Ig7) are approximately equal (Livak and Schmittgen, 2001). As this was the case for all genes analysed in this study, the use of the 2-\textsuperscript{ΔΔCT} method has been validated for analysis purposes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>RT-PCR efficiency $\varepsilon$</th>
<th>RT-PCR efficiency [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ino1</td>
<td>2.13</td>
<td>113</td>
</tr>
<tr>
<td>IMPase</td>
<td>2.05</td>
<td>105</td>
</tr>
<tr>
<td>IPP1</td>
<td>2.04</td>
<td>104</td>
</tr>
<tr>
<td>IPP2</td>
<td>2.15</td>
<td>115</td>
</tr>
<tr>
<td>5P2</td>
<td>2.09</td>
<td>109</td>
</tr>
<tr>
<td>5P3</td>
<td>2.09</td>
<td>109</td>
</tr>
<tr>
<td>5P4</td>
<td>2.09</td>
<td>109</td>
</tr>
<tr>
<td>DpoA</td>
<td>2.19</td>
<td>119</td>
</tr>
<tr>
<td>Ig7</td>
<td>2.09</td>
<td>109</td>
</tr>
</tbody>
</table>

* RT-PCR efficiency $\varepsilon$ were calculated via $\varepsilon=10^{-1/m}$ and converted into percentage.
8.3 Amino acid sequence alignments

The full length amino acid sequence alignments for the families of IMPases, IPPases and PAPases were made using the ClustalW algorithm (Chenna et al., 2003), and can be found in the subsequent Figures 8.12 to 8.14.
Figure 8.12 Amino acid sequence alignment of IMPases. The clustalW program was used to align the following protein sequences: RnIMP (R. norvegicus, GeneBank™ P97697), HsIMP (H. sapiens, GeneBank™ P29218), MmIMP (M. musculus, GeneBank™ O55023), DdIMP (D. discoideum, Dictybase DDB0204100), HsIMP2 (H. sapiens, GeneBank™ O14732), MmIPPP (M. musculus, GeneBank™ P49442). * denotes identical residues, : denotes highly conserved residues, . denotes weak conserved residues.
Figure 8.13 Amino acid sequence alignment of IPPases. The clustalW program was used to align the following protein sequences: MmIP (M. musculus, GeneBank™ P49442), HsIPP (H. sapiens, GeneBank™ P49441), BtIPP (B. taurus, GeneBank™ P21327), DdIP (D. discoideum, Dictybase DDB0167248). * denotes identical residues, : denotes highly conserved residues, . denotes weak conserved residues.
Figure 8.14 Amino acid sequence alignment of PAPases. The clustalW program was used to align the following protein sequences: AtSal1 (A. thaliana, GeneBank™ Q42546), AtSal2 (A. thaliana, GeneBank™ ), ScHal2 (S. cerevisiae, GeneBank™ P32179), SpDPNP (S. pombe, GeneBank™ O94505), DdIP2 (D. discoideum, Dictybase DDB0189923). * denotes identical residues, : denotes highly conserved residues, . denotes weak conserved residues.
8.4 References


of expressed sequence tags from the first-finger stage of development. DNA Res, 5, 335-340.


Sjoholt, G., Ebstein, R.P., Lie, R.T., Berle, J.O., Mallet, J., Deleuze, J.F., Levinson, D.F., Laurent, C., Mujahed, M., Bannoura, I., Murad, I.,


HINTS AND TIPS

An Inverse PCR Technique to Rapidly Isolate the Flanking DNA of *Dictyostelium* Insertion Mutants

*Melanie Keim, Robin S. B. Williams, and Adrian J. Harwood*

Abstract

Restriction enzyme mediated integration is a widely used and effective method for insertional mutagenesis in *Dictyostelium discoideum*. In this method, plasmid rescue is used to clone the genomic deoxyribonucleic acid (DNA) sequences that flank the insertion site. For this to be effective, it is necessary to first find a suitable restriction enzyme site within the genomic DNA. This is a time-consuming process that requires Southern blot analysis of the mutant DNA. In addition, plasmid rescue requires transformation into highly competent *Escherichia coli*. Problems can arise owing to unstable genomic sequences, damage to the plasmid DNA and exogenous plasmid contamination. We have established a simple and rapid polymerase chain reaction-based technique that works for all mutants and circumvents the need for Southern blot analysis and plasmid rescue.

Index Entries: REMI mutagenesis; plasmid rescue; inverse PCR; *Dictyostelium*.

Restriction enzyme mediated integration (REMI) has proved to be an immensely useful method to generate *Dictyostelium discoideum* mutants and allows the rapid identification of the mutated genes. In this technique, a restriction enzyme is used to facilitate random insertion of plasmid deoxyribonucleic acid (DNA) into the genome. Disrupted genes are then identified by plasmid rescue, which clones the flanking genomic DNA. This process involves digestion of mutant genomic DNA with a restriction enzyme that cuts outside the inserted plasmid, circularization with T4 DNA ligase and then transformation of the ligated fragments into *Escherichia coli*. The plasmid along with the flanking genomic DNA then gives antibiotic-, usually ampicillin, resistant colonies (*I*).

Although this process is effective and rapid in comparison to mutagenesis methods used in many other organisms, identification of each mutant requires individual analysis and can present its own unique problems. First is the identification of suitably sized genomic fragments for plasmid rescue. As the efficiency of ligation and transformation decreases with increased size, each mutant requires Southern blot analysis to find suitably sized fragments. Second is the requirement for plasmid replication and selection in *E. coli*. *D. discoideum* genomic DNA can be unstable in *E. coli* or interfere with plasmid replication. In some cases the antibiotic resistance gene or plasmid replication origin can be damaged in its passage through *D. discoideum*. Finally, as the plasmid DNA has very low abundance, highly competent *E. coli* cells are required, making this method very susceptible to contaminating exogenous plasmids.

To simplify the process and avoid the potential problems of plasmid rescue, we have developed a method to isolate part of the genomic DNA at the REMI insertion site. As used for the isolation of transposon-induced insertions (*2*), this method, illustrated in *Fig. 1*, is based on inverse polymerase chain reaction (iPCR) and uses knowledge of the structure and the sequence of the inserted plasmid. The restriction enzyme *AluI* cuts at a 4-bp recognition sequence (AGCT). This occurs very fre-

*Author to whom all correspondence and reprint requests should be addressed: Dr. Adrian J. Harwood, MCR Laboratory Molecular Cell Biology and Dept. of Biology, University College London, Gower St., London WC1E 6BT, UK. Tel: +44 207 679 7527; Fax: +44 207 679 7803. E-mail: a.harwood@ucl.ac.uk

Molecular Biotechnology © 2004 Humana Press Inc. All rights of any nature whatsoever reserved. 1073-8685/2004/26:3/221–224/$25.00
Fig. 1. Inverse PCR. (A). Genomic DNA is digested with AluI, which cuts within the blasticidin gene (Bsr), and circularized by ligation with T4 DNA ligase. The genomic sequence flanking the insertion site is then amplified using the primers BsrP1 and BsrP2. (B). Conserved plasmid sequences incorporated into the iPCR product. Sequence of primers is marked by arrows and novel genomic sequence is represented by dots.
iPCR and Dictyostelium Insertion Mutants

Sequentially in the genomes of most species and in plasmid DNA. However, owing to its strong A/T-biased genome composition, AluI cuts D. discoideum DNA less frequently to give a mean fragment length of 1050 bp. This figure was calculated by taking the average frequency of AluI sites found in 10 Kb fragments randomly selected from the D. discoideum genome project DNA database. Common to most REMI insertion plasmids is a blasticidin-resistance (Bsr) cassette, which lies close to the point of insertion. AluI digests within the Bsr coding region, but not in the intervening sequence between the insertion point and the Bsr gene. An AluI digest of REMI-mutant DNA therefore generates genomic fragments distributed around 1 Kb in size. The fragment corresponding to the disrupted gene, however, will also contain a known sequence derived from the 5' region of the Bsr gene. This fragment can be isolated using iPCR (3) with primers that anneal to the Bsr gene.

This strategy has been used to clone a number of Dictyostelium REMI mutants. Genomic DNA amounting to 20 µg was digested in a total volume of 100 µL with 50 U of AluI (New England Biolabs, UK) and 2 µL of ribonuclease (10 mg/mL) at 37°C overnight. The fully digested DNA was purified using phenol/chloroform (1:1), then using chloroform, and precipitated with ice-cold 100% ethanol. The DNA was washed in 70% ethanol and dissolved in 20 µL of Tris-ethylenediaminetetraacetic acid (TBE) buffer, pH 8.0.

For the ligation, 5 µg of DNA was combined with 40 µL of 10×T4 DNA ligase buffer in a total volume of 400 µL and divided into 2 reaction tubes: 2 µL of T4 DNA ligase (New England Biolabs, UK) was added to one reaction tube and 2 µL of ddH2O to the other, and the reactions were incubated at 16°C overnight. To inactivate T4 DNA ligase, the reaction tubes were incubated at 56°C for 10 min and precipitated with 2 µL of glycogen (10 mg/mL), 20 µL of 3M sodium acetate, pH 5.5, and 500 µL of ice-cold 100% ethanol. After incubation on ice for 30 min, the DNA was pelleted at 12,000g for 30 min at 4°C, washed with 70% ethanol, air-dried, and dissolved in 50 µL of TE, pH 8.0.

The 50µL-volume iPCR reactions were set up in thin-walled 200-µL tubes (Scientific Laboratory Supply, UK) as follows: 1 µL of ligated DNA was mixed with 1 pmol of each primer (BsrP1: TATCTAGGTATACGACTCAGCTATAGGG, BsrP2: ATAGCGCGTAACTGTCGGCAG) or 1 primer plus ddH2O for controls, 200 µM of each deoxyadenosine 5'-triphosphate, deoxyctydine 5'-triphosphate, deoxycytidine 5'-triphosphate, and deoxyguanosine 5'-triphosphate (Pharmacia Biotech, UK), 1X Pfu buffer (Stratagene, UK), 2.5 U of Taq polymerase (Stratagene, UK), and 2.5 U of recombinant Pfu polymerase (Stratagene, UK). For amplification, the genomic DNA was subjected to 30 cycles of 95°C for 30 s, 58°C for 30 s, 68°C for 4 min, followed by a final extension step at 68°C for 10 min before storage at 4°C.

The products of the PCR reaction were checked by agarose gel electrophoresis. Figure 2 shows the results for seven mutants, LisF to LisL, visualized on a 1% Tris-borate-EDTA agarose gel. Amplified fragments ranged from 300 bp and 3 Kb in size (Table 1). An incomplete digest with AluI, possibly generating more than one fragment, should result in longer fragments, where smaller fragments are insufficient to find sequence homologies. Multiple fragments could also be generated by double insertion of the blasticidin–REMI cassette into the genome. Furthermore, the efficiency of the amplification for each fragment is also dependent on the length of the fragment. For Fig. 2 the PCR conditions were set to amplify fragments of approx 1 Kb in size. For one of the clones, LisK, a 3-Kb fragment could be amplified at decreased abundance. It is likely that through the use of different PCR conditions, such as longer extension time, longer fragments can also be detected more strongly.

Following confirmation of a PCR product by gel electrophoresis, the remaining PCR product was purified using a S400 spin column (Amer sham Pharmacia Biotech, UK). If sufficient concentration is available, the PCR products can be sequenced directly. We routinely cloned the fragments into a TOPO®-based plasmid vector.
Table 1
Fragments Obtained for the Dictyostelium Mutants via iPCR

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Size of Obtained Fragments (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LisF</td>
<td>550</td>
</tr>
<tr>
<td>LisG</td>
<td>950</td>
</tr>
<tr>
<td>LisH</td>
<td>900</td>
</tr>
<tr>
<td>LisI</td>
<td>900</td>
</tr>
<tr>
<td>LisJ</td>
<td>300</td>
</tr>
<tr>
<td>LisK</td>
<td>3000</td>
</tr>
<tr>
<td>LisL</td>
<td>900</td>
</tr>
</tbody>
</table>

Fig. 2. Fragments obtained for the mutants LisF to LisL via iPCR are visualized on a 1% TBE agarose gel (shown in negative for better contrast). Lane 1: 1-Kb marker; Lane 2: no DNA control; Lane 3–9: LisF–LisL.

(Invitrogen, UK) and then sequenced them with the sequence primers M13rev(-29) and M13uni(-21) (Invitrogen, UK). Fragments obtained by this iPCR method are generally smaller than those obtained by plasmid rescue; however, this is not a problem as only a small stretch of sequence is required to identify the complete gene sequence from the Dictyostelium genome project database. In the case of LisJ, a large genomic sequence could be identified from a 300-bp fragment.

We find this iPCR approach to be an efficient and routinely successful technique for identifying genes disrupted by REMI mutagenesis. This technique could also be adapted for different insertion vectors and for use in different organisms. Furthermore, this technique could form the basis of a high-throughput approach to identifying disrupted D. discoideum genes.

Acknowledgments

Sequence data for D. discoideum was obtained from the Genome Sequencing Centre Jena Website (http://genome.imb-jena.de/dictyostelium/). The German part of the D. discoideum Genome Project is carried out by the Institute of Biochemistry I, Cologne, and the Dept. of Genome Analysis, IMB Jena with support from the Deutsche Forschungsgemeinschaft (No 113/10-1 and 10-2). A.J.H. is a Wellcome Trust Senior Fellow.

References