The Role of a Collybistin-Kinesin Complex in Gephyrin Trafficking to Inhibitory Synapses

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Submitted for the degree of Doctor of Philosophy
The School of Pharmacy, University of London

March 2006
‘Just never give up... absolutely... just never give up’

Shane Warne
Plagiarism Statement

This thesis describes research conducted in the School of Pharmacy, University of London, between 2001 and 2005 under the supervision of Dr. B. R. Pearce and Dr. R. J. Harvey. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text written herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature

Date
Abstract

Neurons maintain the fidelity of neurotransmission by accumulating and clustering neurotransmitter receptors at sites precisely opposite presynaptic transmitter release. A long standing question is how this alignment is achieved. One route of investigation is to study the different protein complexes which contribute to the trafficking and localisation of postsynaptic receptors. Gephyrin is a major component of the submembrane scaffold that supports glycine and GABA_A receptors at inhibitory synapses. In transfected mammalian cells, gephyrin is translocated to submembranous sites by the GDP-GTP exchange factor collybistin. Three isoforms of rat collybistin have been identified, all of which have a N-terminal SH3 domain and alternative C-termini (CB1, CB2, CB3). The SH3 domain negatively regulates the ability of collybistin to translocate gephyrin to submembranous sites in transfected mammalian cells. Since the majority of native collybistin isoforms appear to harbour the SH3 domain, this suggests that protein-protein interactions or post-translational modifications at the SH3 domain may regulate collybistin activity at specific subcellular locations.

The following study was conducted to investigate the mechanisms by which collybistin facilitates gephyrin trafficking and clustering. It was previously suggested that collybistin activated Cdc42, which, in turn, regulated the dynamics of the actin cytoskeleton to facilitate the accumulation of gephyrin and glycine receptors at synapses. One objective of this study was to investigate this proposed role for collybistin. In addition, a library screen conducted to identify collybistin-interacting proteins revealed a number of proteins involved in intracellular trafficking. These included two kinesin motor proteins – KIF5C and KIF3A, which mediate the intracellular anterograde transport of newly synthesised proteins. The cytoskeleton of neuronal axons and dendrites comprise microtubules, which provide tracks upon which kinesins transport their cargo; collybistin may therefore act as a linker molecule, coupling gephyrin and associated inhibitory receptors to microtubules, via dynamic interactions with kinesins. It is proposed that kinesin, collybistin and gephyrin form an anterograde motor protein complex that delivers GABA_A and glycine receptors to inhibitory synapses.
Acknowledgements

First and foremost my sincerest thanks go to Drs. Brian Pearce and Robert Harvey for their meticulous supervision throughout this project - I really appreciate everything that you have both done for me. I would also like to thank Dr. Kirsten Harvey for her encouragement, help and guidance at the bench. Thank you also, to Prof. Alex Thomson for her continued support and advice and Prof. Sandy Florence for generously extending my studentship.

There are a number of people who have contributed to the work presented in this thesis and I would like to acknowledge the following for their generous gifts: Prof. Alan Hall (UCL, UK) for the pRK5Myc and pRK5Flag vectors and the constitutively-active and dominant-negative GTPase constructs; Drs. Philipe Fort and Anne Blangy (Centre de Recherches en Biochimie Macromoléculaire, France) for the REF52 cells and YEA constructs; and Prof. Heinrich Betz (Max Planck Institute for Brain Research, Germany) for the mAb7a antibody and pCisMyc-gephyrin construct. Thank you also to Prof. Bernhard Lüscher (Penn State University, USA) for the cortical neuron images; Dr. Harry Mellor (University of Bristol, UK) for the NIH3T3 images and Dr. Kanamarlapudi Venkateswarlu (University of Bristol, UK) for the lipid assay data.

I would like to thank Drs. Ian Duguid and David Gathercole for all their help with the confocal microscope, and Graham, Paul and Annie in the computer/multimedia unit for sorting out my numerous computer ‘issues’. A big thank you also goes to everyone in the pharmacology department for making it such a welcoming and entertaining place to work in especially Vicky and my office mates Jo, Alexis and Andy for all those cups of tea.

“There is no better way to become friends than to laugh together”- I would like to give a special thanks to Amina, Suzie, Pav, Bal, Sukh and Rob whose friendship has ensured that I leave SoP with the fondest (and funniest!) of memories dating back to that ‘evening stroll’ we had in Paris. Your support has been invaluable to me especially when my Qi got blocked! Thank you also to all my friends especially Mella, Fran, Liz, Ruth, Nicky, Charlotte, Aimee, Mike and Tim who provided a haven when escape was needed and for their patience during my self-imposed exile.

Finally, to save the best ‘til last, my most heartfelt and warmest thanks and appreciation go to Ba, Dad, Mum and Priyank, whose love, education and guidance throughout my life has made me who I am today. I thank you all for your unwavering support, encouragement and late night lifts especially in the last two years. Every step of this journey was achievable because you were all with me and this PhD is as much yours, as it is mine.
Table of Contents

Plagiarism Statement.................................................................................. I
Abstract...................................................................................................... II
Acknowledgements...................................................................................... III
Table of Contents......................................................................................... IV
List of Figures................................................................................................ VIII
List of Tables.................................................................................................. X
Abbreviations............................................................................................... XI

Chapter 1: General Introduction

1.1 Inhibitory amino acid neurotransmitter receptors.......................... 1
1.2 Gephyrin............................................................................................... 5
1.2.1 Functions of gephyrin................................................................. 7
1.2.2 Gephyrin associated proteins.................................................. 9
1.3 Glycine receptor clustering.............................................................. 12
1.4 Aims................................................................................................. 16

Chapter 2: Materials and Methods

2.1 Molecular Biology............................................................................... 18
2.1.1 Polymerase Chain Reaction.................................................... 19
2.1.2 Site-directed mutagenesis......................................................... 23
2.1.3 Agarose gel electrophoresis..................................................... 23
2.1.4 Agarose gel purification.......................................................... 24
2.1.5 Restriction enzyme digest......................................................... 25
2.1.6 Phenol:Chloroform extraction................................................ 26
2.1.7 Ethanol precipitation................................................................. 27
2.1.8 Ligation reactions................................................................. 27
2.1.9 Preparation of competent \textit{E. coli} cells................................ 28
2.1.10 Transformation of DNA into \textit{E. coli} cells.............................. 28
2.1.11 Mini-preparation of plasmid DNA........................................ 29
2.1.12 Midi/Maxi preparation of plasmid DNA.............................. 30
2.1.13 Determination of DNA yield................................................ 30
2.1.14 DNA sequencing................................................................. 31
2.2 Yeast Two-Hybrid system................................................................ 33
2.2.1 Yeast strains.............................................................................. 34
2.2.2 Yeast expression vectors......................................................... 34
2.2.3 Yeast medium and agar plates................................................. 34
2.2.4 Preparation of DNA for yeast transformations.................... 35
2.2.5 Transformation of yeast......................................................... 35
2.2.6 Integration of yeast strains..................................................... 36
2.2.7 Replica plating................................................................. 37
2.2.8 Freeze fracture \textit{LacZ} assay.................................................... 37
2.2.9 Isolation of plasmid DNA from yeast cells.......................... 38
2.2.10 Nutritional growth assays...................................................... 39
2.3 Mammalian cell culture................................................................. 39
2.3.1 Cell lines................................................................................. 39
2.3.2 Mammalian expression vectors........................................... 40
Chapter 3: Collybistin and Gephyrin

3.1 Introduction................................................................................. 45
3.1.1 Collybistin ............................................................................. 45
3.1.1.1 Structure of collybistin............................................................ 45
3.1.1.2 Expression and distribution of collybistin ......................... 48
3.1.1.3 GTPase Exchange Factors.................................................... 48
3.1.1.4 The membrane activation model ................................. 52
3.1.2 Aims....................................................................................... 52
3.2 Methods..................................................................................... 53
3.2.1 Yeast expression vectors......................................................... 53
3.2.1.1 pYTH9-CB2SH3.......................................................... 53
3.2.1.2 pYTH9-CB2SH3ΔRhoGEF and pYTH9-CB2SH3ΔPH.... 53
3.2.1.3 pYTH16-RhoGEF and pYTH16-PH.............................. 54
3.2.1.4 pGBK7-hPEM-2......................................................... 54
3.2.2 Mammalian expression vectors............................................. 55
3.2.2.1 pRK5Myc-CB2SH3.................................................. 55
3.2.2.2 pRK5Myc-CB2SH3ΔRhoGEF and pRK5Myc-CB2SH3ΔPH.. 55
3.2.2.3 pEGFP-gephyrin......................................................... 56
3.2.2.4 pDsRed-glycine receptor β subunit............................... 56
3.2.3 The phosphoinositide specificity of the PH domain of collybistin 57
3.3 Results....................................................................................... 58
3.3.1 Interactions between collybistin, hPEM-2 and gephyrin......... 58
3.3.1.1 Interactions in Yeast...................................................... 58
3.3.1.2 Interactions in HEK293 cells........................................... 60
3.3.1.3 Interactions in cortical neurons...................................... 63
3.3.2 Gephyrin and the RhoGEF domain.................................... 64
3.3.3 Collybistin and gephyrin mutants A4 and A5..................... 64
3.3.4 Gephyrin and the PH domain.............................................. 67
3.3.5 Glycine receptor β subunit, gephyrin and CB2SH3............ 69
3.4 Discussion............................................................................... 72
3.4.1 Collybistin, hPEM-2 and gephyrin interactions.................. 72
3.4.2 The SH3 domain............................................................... 73
3.4.3 The RhoGEF domain......................................................... 75
3.4.4 The PH domain............................................................... 76
3.4.5 Revision of the membrane activation model....................... 79
3.5 Conclusions............................................................................ 80

Chapter 4: Collybistin and Rho-family GTPases

4.1 Introduction............................................................................. 81
4.1.1 Rho-family GTPases........................................................... 81
4.1.2 RhoA, Rac1 and Cdc42 induced changes in actin structures.... 84
4.1.2.1 RhoA................................................................. 84
# Table of Contents

4.1.2.2 Rac1 and Cdc42 ............................................................. 87
4.1.3 Role of GTPase activity in the membrane activation model .......... 90
4.1.4 Aims ............................................................................... 92
4.2 Cellular Assays ................................................................. 93
4.2.1 Establishment of cell phenotypes ........................................ 93
4.2.1.1 Drug incubations .......................................................... 93
4.2.1.2 Transfection of DNA constructs ...................................... 93
4.2.1.3 Identification of actin structures using phalloidin ............... 94
4.2.2 Results ........................................................................... 94
4.2.3 Discussion ....................................................................... 98
4.3 Yeast Exchange Assay .......................................................... 99
4.3.1 Yeast strains .................................................................... 99
4.3.2 Yeast constructs ............................................................... 99
4.3.3 Transformations and Assay ................................................ 101
4.3.4 Results .......................................................................... 102
4.3.5 Discussion ...................................................................... 109
4.4 Cdc42 activation assay ......................................................... 111
4.4.1 Sample preparation .......................................................... 111
4.4.2 Activation assay .............................................................. 111
4.4.2.1 Positive and negative controls ........................................ 111
4.4.2.2 Cdc42 pull-down assay .................................................. 112
4.4.3 Gel electrophoresis, Western blot and Detection .................... 112
4.4.4 Results and Discussion .................................................... 113
4.5 Discussion and Conclusions ................................................ 114

## Chapter 5: Collybistin Interacting Proteins

5.1 Introduction ...................................................................... 118
5.1.1 Aims ............................................................................ 119
5.2 Methods .......................................................................... 120
5.2.1 Library Transformations .................................................. 121
5.2.2 Library screen DNA mix .................................................. 121
5.2.3 Library screen agar plates ............................................... 121
5.2.4 Colony selection ............................................................ 122
5.2.5 Analysis of 'chosen' hits .................................................. 122
5.3 Results ............................................................................. 123
5.3.1 NGEF ........................................................................... 125
5.3.1.1 LacZ assays in Y190 ....................................................... 125
5.3.1.2 Nutritional growth assays in AH109. .............................. 125
5.3.2 GATE-16 ....................................................................... 127
5.3.2.1 LacZ assays in Y190 ....................................................... 127
5.3.2.2 Nutritional growth assays in AH109. .............................. 127
5.3.3 KIF5C ........................................................................... 129
5.3.3.1 LacZ assays in Y190 ....................................................... 129
5.3.3.2 Nutritional growth assays in AH109. .............................. 129
5.3.4 KIF3A ........................................................................... 132
5.4 Discussion ....................................................................... 133
5.4.1 NGEF and collybistin ...................................................... 133
5.4.2 GATE-16 and collybistin ................................................ 135
5.4.3 KIF5C and collybistin ..................................................... 138
Chapter 6: Collybistin and Kinesins

6.1 Introduction
6.1.1 Microtubules and molecular motors
6.1.2 The kinesin superfamily
6.1.3 The general structure of kinesins
6.1.4 The motor domain and kinesin processivity
6.1.5 The tail domain and directional transportation of cargo
6.1.5.1 Kinesin light chains
6.1.5.2 Cargo recognition and directional transport
6.1.6 Kinesin-1
6.1.6.1 Kinesin-1 knockout mice
6.1.7 Aims

6.2 Methods
6.2.1 Yeast expression constructs
6.2.1.1 pACT2-KIF5A
6.2.1.2 pACT2-KIF5B
6.2.2 Mammalian expression constructs
6.2.2.1 pEGFP-KIF5C-wt
6.2.2.2 pEGFP-KIF5C-hdl
6.2.2.3 pRK5Flag-CB2SH3 and pRK5Flag-CB2SH3+
6.2.2.4 pCisMyc-gephyrin

6.3 Results
6.3.1 Interactions between collybistin and other kinesin heavy chains
6.3.2 Expression of transfected KIF5C in mammalian cells
6.3.3 KIF5C and gephyrin
6.3.4 KIF5C and collybistin
6.3.5 KIF5C, collybistin and gephyrin
6.4 Discussion
6.4.1 Kinesin-collybistin-gephyrin complex
6.4.2 Kinesin and receptor trafficking
6.5 Conclusions

Chapter 7: General Discussion

7.1 Membrane activation model revisited
7.2 Kinesin-collybistin-gephyrin as a trafficking complex
7.3 Could collybistin play other roles in glycine receptor clustering?
7.4 A new model

Appendix 1:

References
List of Figures

Chapter 1 General Introduction

1.1 The glycine receptor ............................................................. 4
1.2 Gephyrin ............................................................................... 6
1.3 Membrane activation model ............................................. 13
1.4 The role of collybistin in the membrane activation model 16

Chapter 2 Materials and Methods

2.1 Cloning flowchart ............................................................... 18
2.2 PCR schematic ..................................................................... 21
2.3 DNA agarose gel ................................................................. 24
2.4 DNA sequencing ............................................................... 32
2.5 Yeast Two-Hybrid system .............................................. 33
2.6 LacZ assay ............................................................................ 37

Chapter 3 Collybistin and Gephyrin

3.1 Collybistin variants ............................................................... 46
3.2 Collybistin isoform amino acid alignments ........................ 47
3.3 GEF phylogenetic tree .......................................................... 49
3.4 pYTH9-CB2SH3 vector schematic ..................................... 53
3.5 pRK5Myc-CB2SH3 vector schematic .................................. 55
3.6 LacZ assays of collybistin constructs ................................. 59
3.7 CB2SH3 and gephyrin .......................................................... 61
3.8 CB2SH3 and gephyrin .......................................................... 62
3.9 Collybistin in cortical neurons .......................................... 63
3.10 The RhoGEF domain ............................................................ 65
3.11 Gephyrin mutants A4 and A5 and collybistin .................... 66
3.12 The PH domain .................................................................... 68
3.13 Phosphoinositide assay .................................................. 70
3.14 Glycine receptor β subunit, gephyrin and collybistin ..... 71

Chapter 4 Collybistin and Rho-family GTPases

4.1 Rho-family GTPase activation cycle .................................. 83
4.2 RhoA induced stress fibres .................................................. 86
4.3 Rac1 and Cdc42 induced actin cytoskeletal changes .......... 88
4.4 Cdc42 activation by hPEM-2 ............................................. 91
4.5 NIH 3T3 cells ..................................................................... 95
4.6 REF52 cells ....................................................................... 97
4.7 Yeast Exchange Assay ..................................................... 100
4.8 Cdc42 YEA filter assays .................................................. 108
4.9 Rho-family GTPase phylogenetic tree ............................. 116

VIII
List of Figures

Chapter 5 Collybistin Interacting Proteins

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Flowchart of Yeast Two-Hybrid library screen</td>
<td>120</td>
</tr>
<tr>
<td>5.2</td>
<td>NGEF</td>
<td>126</td>
</tr>
<tr>
<td>5.3</td>
<td>GATE-16</td>
<td>128</td>
</tr>
<tr>
<td>5.4</td>
<td>KIF5C</td>
<td>131</td>
</tr>
<tr>
<td>5.5</td>
<td>KIF3A</td>
<td>132</td>
</tr>
<tr>
<td>5.6</td>
<td>Kinesin-2 holoenzyme</td>
<td>140</td>
</tr>
</tbody>
</table>

Chapter 6 Collybistin and Kinesins

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>Kinesin protein domain alignments</td>
<td>145</td>
</tr>
<tr>
<td>6.2</td>
<td>Kinesin-1 holoenzyme</td>
<td>152</td>
</tr>
<tr>
<td>6.3</td>
<td>Schematic of wild-type and mutant KIF5C</td>
<td>157</td>
</tr>
<tr>
<td>6.4</td>
<td>KIF5A</td>
<td>160</td>
</tr>
<tr>
<td>6.5</td>
<td>KIF5B</td>
<td>161</td>
</tr>
<tr>
<td>6.6</td>
<td>Wild-type and mutant KIF5C</td>
<td>163</td>
</tr>
<tr>
<td>6.7</td>
<td>Kinesin and gephyrin</td>
<td>164</td>
</tr>
<tr>
<td>6.8</td>
<td>KIF5C and collybistin</td>
<td>165</td>
</tr>
<tr>
<td>6.9</td>
<td>KIF5C-wt, CB2SH3- and gephyrin, single section</td>
<td>167</td>
</tr>
<tr>
<td>6.10</td>
<td>KIF5C-hdl, CB2SH3- and gephyrin, single section</td>
<td>168</td>
</tr>
<tr>
<td>6.11</td>
<td>Kinesin, collybistin and gephyrin</td>
<td>169</td>
</tr>
<tr>
<td>6.12</td>
<td>Headless and mutant KIF5C in HEKs</td>
<td>171</td>
</tr>
<tr>
<td>6.13</td>
<td>Trafficking complexes</td>
<td>176</td>
</tr>
</tbody>
</table>

Chapter 7 General Discussion

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
<td>The role of collybistin in the membrane activation model</td>
<td>178</td>
</tr>
<tr>
<td>7.2</td>
<td>New role for collybistin</td>
<td>181</td>
</tr>
<tr>
<td>7.3</td>
<td>A new model</td>
<td>185</td>
</tr>
</tbody>
</table>
List of Tables

Chapter 1 General Introduction

1.1 Gephyrin interactors........................................... 10

Chapter 2 Materials and Methods

2.1 PCR reagents..................................................... 20
2.2 PCR primers..................................................... 22-23
2.3 Restriction enzymes........................................... 26
2.4 Sequencing dyes................................................ 31
2.5 Yeast expression vectors..................................... 34
2.6 Mammalian cell lines......................................... 39
2.7 Mammalian expression vectors............................. 40
2.8 Electroporator settings....................................... 42
2.9 Primary antibodies............................................ 44
2.10 Secondary antibodies........................................ 44

Chapter 3 Collybistin and Gephyrin

3.1 Yeast two-hybrid constructs................................. 54
3.2 Mammalian expression constructs........................ 56
3.3 Nutritional selection results: collybistin................ 58

Chapter 4 Collybistin and Rho-family GTPases

4.1 Summary of signalling pathways......................... 84
4.2 YEA constructs.................................................. 101
4.3 YEA results: TC10.............................................. 103
4.4 YEA results: TCL.............................................. 103
4.5 YEA results: RhoA............................................. 104
4.6 YEA results: Rac1............................................. 105
4.7 YEA results: RhoG............................................. 105
4.8 YEA results: Cdc42........................................... 107
4.9 Summary of YEA............................................... 110

Chapter 5 Collybistin Interacting Proteins

5.1 Library screen results........................................ 124
5.2 Nutritional selection results: NGEF................. 127
5.3 Nutritional selection results: GATE-16... 129
5.4 Nutritional selection results: KIF5C...... 130

Chapter 6 Collybistin and Kinesins

6.1 Kinesin superfamily........................................... 146-147
6.2 KIF5A and KIF5B............................................. 153
6.3 Constructs and antibodies.................................. 158
6.4 Motor proteins and scaffold proteins.......... 173
# Abbreviations

5-HT - 5-hydroxytryptamine  
Ach - Acetylcholine  
AD - Activation Domain  
ADP - Adenosine Diphosphate  
AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid  
ATP - Adenosine Triphosphate  
BSA - Bovine Serum Albumin  
CAMKII - Calcium/calmodulin dependent Kinase II  
CBD - Cargo Binding Domain  
Cdc42 - Cell division cycle 42  
CNS - Central Nervous System  
DAG - Diacylglycerol  
DB - DNA Binding Domain  
ddNTPs - Dideoxy Nucleotide Triphosphates  
DH - Dbl Homolgy  
Dia - Diaphonous  
Dlc - Dynein Light Chain  
dNTPs - Deoxy Nucleotide Triphosphates  
E.coli - Escherichia coli  
EGFP - Enhanced Green Fluorescent Protein  
f-actin - Filamentous actin  
FCS - Foetal Calf Serum  
FYVE - Fab1p/YOTP/Vac1p/EEA1  
GABA - γ-aminobutyric acid  
GABARAP - GABA<sub>A</sub> Receptor Associated Protein  
g-actin - Globular actin  
GAP - GTPase Activating Protein  
GATE-16 - Golgi-associated ATPase Enhancer of 16KDa  
GDI - Guanosine Dissociation Inhibitor  
GDP - Guanosine Diphosphate  
GEF - Guanosine Exchange Factor  
GOS-28 - Golgi specific v-SNARE  
GPHN - Gene encoding gephyrin  
GRIP - Glutamate Receptor Interacting Protein  
GTP - Guanosine Triphosphate  
HEK293 - Human Embryonic Kidney 293 cells  
HMM - Hidden Markov Models  
hPEM-2 - Human homologue of Posterior End Mark 2  
IP<sub>3</sub> - Inositol triphosphate  
JIP - JNK Interacting Proteins  
JNK - c-jun N-terminal Kinase  
KAP3 - Kinesin Associated Protein  
KCC2 - K+/Cl- co-transporter  
KIF - Kinesin Family  
KLC - Kinesin Light Chain  
LC3 - Light Chain 3
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGIC</td>
<td>Ligand Gated Ion Channels</td>
</tr>
<tr>
<td>LIMK</td>
<td>Lin-11, Isl-3, Mec-3 domain containing kinase 1</td>
</tr>
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<td>LiOAc</td>
<td>Lithium Acetate</td>
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<td>LPA</td>
<td>Lysophosphatic acid</td>
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<td>MAP</td>
<td>Microtubule Associated Protein</td>
</tr>
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<td>MNTB</td>
<td>Medial Nucleus of the Trapezoid Body</td>
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<tr>
<td>MLCK</td>
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<td>MoCo</td>
<td>Molybdenum Cofactor</td>
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<td>MTGC</td>
<td>Microtubule Organising Centre</td>
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<td>Neuronal Guanosine Exchange Factor</td>
</tr>
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<td>NIH 3T3</td>
<td>National Institute of Health 3T3 mouse fibroblast cells</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethyl-maleimide-sensitive factor</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neuronally enriched WASP</td>
</tr>
<tr>
<td>PAK</td>
<td>P21 activated kinase</td>
</tr>
<tr>
<td>PBD</td>
<td>p21 GTPase Binding Domain</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95/Discs large/ZO1</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>Pfam</td>
<td>Protein Family</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI3,4,5P3</td>
<td>Phosphatidylinositol-3,4,5-triphosphate</td>
</tr>
<tr>
<td>PI3,4P2</td>
<td>Phosphatidylinositol-3,4-bisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-Kinase</td>
</tr>
<tr>
<td>PI3P</td>
<td>Phosphatidylinositol-3-phosphate</td>
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<td>Phosphatidylinositol-4,5-bisphosphate</td>
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<td>Rapamycin and FKBp12 target protein</td>
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<td>REF52</td>
<td>Rat Embryonic Fibroblasts 52</td>
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<td>RhoA</td>
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<tr>
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<td>Rho GTPase Exchange factor domain</td>
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<td>Ribonuclease A</td>
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Chapter 1: General Introduction
Chapter 1: General Introduction

1. Introduction

1.1 Inhibitory amino acid neurotransmitter receptors

An optimal excitatory and inhibitory balance is the basis for proper brain function. Inhibitory neurotransmission within the CNS is predominantly mediated by two amino acids – γ-aminobutyric acid (GABA) and glycine (Werman et al., 1967; Roberts, 1986; Aprison, 1990). GABA is the major inhibitory neurotransmitter found in the brain and glycine is mainly concentrated in the spinal cord and brainstem, however, both are found throughout the CNS. Glycine has been implicated in many motor and sensory pathways. In the spinal cord and brainstem, inhibitory interneurons containing glycine are involved in the control of motor rhythm generation that underlie locomotor behaviour and also have an important role in the co-ordination of spinal reflexes (Baldissera et al., 1981; Grillner, 1981; Grillner et al., 1998). In the brain, glycine is implicated in inhibitory transmission within the striatum, substantia nigra, hippocampus and the hypothalamus (reviewed in Legendre, 2001; Lynch, 2004).

In addition to their inhibitory function, GABA and glycine also have an excitatory role in the developing CNS. Immature neurons contain high intracellular concentrations of Cl⁻, and in these neurons glycine can induce increases in Cl⁻ conductance leading to Cl⁻ efflux which causes membrane depolarisation and eventually neurotransmitter release (Boehm et al., 1997). This is thought to be important for synaptogenesis as increases in intracellular Ca²⁺ concentrations triggered by glycine are crucial for the formation of many specialisations including glycinergic synapses (Kneussel and Betz, 2000). The switch to the mature neuron phenotype is believed to be due to the expression of the K⁺/Cl⁻ co-transporter – KCC2. This transporter orchestrates the lowering of internal Cl⁻, thus shifting the Cl⁻ equilibrium potential of the cell, converting the actions of glycine receptor activation from excitatory to inhibitory (Hubner et al., 2001; Stein and Nicoll, 2003). Glycine is also an essential co-agonist at glutamatergic synapses containing N-methyl-D-aspartate (NMDA) receptors. Glycine binds to a site distinct to that occupied by glutamate and is required for efficient channel gating (Johnson and Ascher, 1987).

GABA and glycine mediate their effects through receptors, GABA can activate three types of receptors – ionotropic GABAₐ and GABAₖ and metabotropic GABAₐ. GABAₐ and glycine receptors along with the nicotinic acetylcholine (nAChR) and the 5-hydroxytryptamine type 3 receptor (5-HT₃) belong to the cys-loop family of ligand
Chapter 1: General Introduction

gated ion channels, so called because their N-termini contain two cysteine residues, which are separated by 13 amino acid residues and are joined by a disulphide bridge. These receptors mediate rapid synaptic transmission throughout the CNS and are integral to the efficient and fast communication between neuronal cells. Glycine and GABA_A receptors are anion permeable and mediate inhibitory transmission, whilst excitatory transmission is mediated by nAChR, and 5-HT_3 receptors along with the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), NMDA and kainate glutamate receptors (Ortelis and Lunt, 1995). GABA_A receptors display extensive structural heterogeneity, with 19 different subunit genes identified to date, α1-6, β1-3, γ1-3, δ, ρ1-3, ε, π, and θ. The accepted consensus for the native receptor stoichiometry is 2α, 2β and 1γ or δ. In the brain, GABA_A receptors are thought to contain the γ2 subunit in combination with any of the α and β subunit variants (reviewed in Lüscher and Kellar, 2004).

The postsynaptic glycine receptor was the first receptor protein to be isolated from the mammalian brain (Pfeiffer et al., 1982; Graham et al., 1985), and found to consist of a 48 kDa α-subunit and a 58 kDa β-subunit (Langosch et al., 1988). There are four known isoforms of the α subunit (α1-4) which have different temporal and spatial distributions. For example, the α2-subunit gene is expressed throughout the late embryonic stages, with a developmental switch to α1 occurring during the early postnatal stages (Becker et al., 1988; Malosio et al., 1991a; Kuhse et al., 1991; Piechotta et al., 2001). Both these receptor subunits have a widespread distribution throughout the CNS, whereas the α3 subunit is distinctly expressed in the superficial layers of the spinal cord dorsal horn (Harvey et al., 2004a). The α4 subunit has only been detected in the mouse, chick and zebrafish where its expression has been localised to the embryonic sympathetic nervous system, spinal cord and male genital ridge (Matzenbach et al., 1994, Harvey et al., 2000, Devignot et al., 2003). In contrast to the α subunits, only one β subunit gene has been identified, which is expressed at all developmental stages throughout the CNS (Malosio et al., 1991b; Fujita et al., 1991). Fig 1.1 is a schematic representation of a glycine receptor subunit which consists of an extracellular N-terminus thought to harbour the ligand binding sites, four transmembrane domains (TM1-4), of which TM2 is thought to contribute to the ion channel pore, a large intracellular loop between TM3 and TM4 which contains a number of putative protein binding and modulatory sites, and an extracellular C-
terminus (Ortells and Lunt, 1995). The intracellular loop of the β subunit harbours the gephyrin binding site.

Fig. 1.1 also shows that the glycine receptor has a pentameric quaternary structure, consisting of two α subunits and three β subunits, although, functional receptors can be expressed consisting of five α subunits (Takahashi et al., 1992; Grudzinska et al., 2005). In heterologous expression systems, the α subunits can form functional homomeric receptors (Kuhse et al., 1995; Laube et al., 2002), which in vivo are found at extrasynaptic sites (Takahashi et al., 1992). The β subunit can not form functional homomeric receptors, however recent data have revealed that the β subunit determines the ligand binding properties of synaptic glycine receptors (Grudzinska et al., 2005), in addition to this, the β subunit is also required for binding to gephyrin and anchoring glycine receptors at postsynaptic sites.

Glycine receptors have also been localised to nonsynaptic sites on some neurons. For example, hippocampal CA3 neurons have nonsynaptic glycine receptors which are believed to be held in a tonically active state by the local release of taurine or β-alanine (Mori et al., 2002). Developing cortical neurons also have nonsynaptic glycine receptors which may be activated by taurine released from glia, and be important for development (Flint et al., 1998).

Functional presynaptic glycine receptors have been identified at calyceal synapses in the medial nucleus of the trapezoid body (MNTB), a part of the auditory pathway (Turecek and Trussell, 2001). Activation of these presynaptic receptors triggers a weakly depolarising Cl⁻ current in the nerve terminal, which in turn causes sufficient activation of Ca²⁺ channels to facilitate glutamate release. Calyces do not receive glycinergic axo-axonic synapses, but spillover from the activation of glycinergic termini in the MNTB activates the presynaptic receptors (Turecek and Trussell, 2001). Glycine also acts at presynaptic auto-receptors on rat spinal sacral dorsal commissural nucleus neurons, causing depolarisation of the glycinergic nerve terminal, and the subsequent activation of voltage dependent Na⁺ and Ca²⁺ channels facilitates glycine release (Jeong et al., 2003).
Fig. 1.1: A schematic representation of a glycine receptor subunit and the pentameric post synaptic glycine receptor - There are four known isoforms of the α subunit and one of the β subunit. Each subunit is composed of four transmembrane domains (purple and green), a large intracellular loop between TM3 and TM4 and extracellular N- and C-termini. TM2 is thought to contribute the ion channel pore. The large intracellular loop harbours a number of protein interaction and modulatory sites and in the case of the β subunit the gephyrin binding site as well (adapted from Lynch, 2004). The pentameric glycine receptor complex is thought to be composed of two α and three β subunits (Grudzinska et al., 2005).
1.2 Gephyrin

Postsynaptic glycine receptors are aggregated in clusters at inhibitory synapses and the formation and maintenance of these clusters is dependent on a submembrane protein scaffold, of which the main constituent is a 93 kDa protein, gephyrin. Gephyrin was co-purified with the glycine receptor β subunit and polymerised tubulin (Prior et al., 1992; Kirsch et al., 1993a) and found to be involved in the postsynaptic clustering of glycine receptors, and some GABA_A receptors (Meyer et al., 1995; Essrich et al., 1998). This isoform of gephyrin, P1, is 736 amino acids long and divided into two recognised Pfam domains (Prior et al., 1992). The N-terminal region of gephyrin is homologous to the _E. coli_ protein MogA and the C-terminal is homologous to the _E. coli_ protein MoeA (Fig. 1.2B; Prior et al., 1992). Both of these proteins are involved in molybdenum cofactor synthesis, implying that gephyrin may be a multifunctional protein conserved through evolution (Feng et al., 1998). The two domains of gephyrin are separated by a 170 amino acid proline rich linker region which contains sequences that are homologous to microtubule associated proteins such as MAP, MAP2 and Tau. This region is thought to interact with tubulin (Ramming et al., 2000), however, the precise nature of the interaction between gephyrin and tubulin remains unclear. Crystal structure analysis of _E. coli_ MogA and gephyrin indicates that the MogA homology domain at the N-terminus trimerises (Fig. 1.2C; Sola et al., 2001). Similar analysis of _E. coli_ MoeA and gephyrin suggests that the MoeA homology domain dimerises through its C-terminal (Sola et al., 2004). This may produce a hexagonal lattice shape which could be the way in which gephyrin provides support to postsynaptic receptors (Fig 1.2D).

In addition to the P1 isoform of gephyrin a number of other isoforms have also been identified which are created by alternative splicing of the _GPHN_ gene. These splice variants were created by the insertion or absence of short amino acid sequences termed cassettes and the majority differ at the N-terminal region of gephyrin. Six different cassettes were identified in rat contributing to 13 alternative spliced variants. The combination of cassettes present in each isoform configures different binding properties upon gephyrin and some of these isoforms do not bind to glycine receptor β subunits (Meier et al., 2000a). An additional 4 cassettes have been identified in humans, termed C4A-D, and appear in various combinations thus extending the number of gephyrin isoforms detected in humans (Rees et al., 2003). The different isoforms of gephyrin may be the key to its diverse functional properties.
Fig. 1.2: Gephyrin - The amino acid sequence of gephyrin isoform P1, the *E.coli* homology domains MogA and MoeA in dark and light green, respectively, have been conserved through evolution and are involved with Moco synthesis (A). A schematic model of gephyrin indicating the MogA and MoeA domains, joined by the central linker region, which is believed to harbour the tubulin binding site (B). A ribbon representation of the trimeric structure of the MogA domain (C; taken from Sola *et al.*, 2001). A schematic representation of the hexagonal lattice predicted from crystal structure analysis of gephyrin, which indicates that the N-terminus trimerises and the C-terminus dimerises. This lattice structure may form the basis of the subsynaptic scaffold that supports glycine receptors at inhibitory synapses (D; Sola *et al.*, 2001, 2004). The expression of recombinant gephyrin in HEK293 cells produces characteristic cytoplasmic aggregates indicated by arrows (E).
1.2.1 Functions of gephyrin

**Molybdoenzyme activity**

Molecular cloning of gephyrin revealed that it is very similar to three *E. coli* proteins – MoeA, MoaB and Mog, the *D. melanogaster* protein “cinnamon” and the *A. thaliana* protein cnx1; all of which are involved in the synthesis of molybdenum containing cofactor essential for the activity of molybdoenzymes. The molybdoenzyme cofactor (Moco) consists of a unique and conserved pterin derivative called molybdopterin. Gephyrin is required for the insertion of molybdenum during Moco assembly. Moco deficiency results in the loss of activity of the molybdoenzymes such as sulphite oxidase, xanthine dehydrogenase and aldehyde oxidase. In humans, the loss of these metabolic enzymes manifests as progressive neurological loss resulting in death in early childhood (Reiss and Johnson, 2003). Gephyrin knockout mice displayed neurological phenotypes such as hypertonicity and myoclonus consistent with those seen in humans with autosomal recessive Moco deficiency, and with gephyrins role in glycine receptor clustering (Feng *et al.*, 1998).

**Glycine receptor clustering**

There is much evidence to show that gephyrin is crucial for glycine receptor clustering (Kirsch *et al.*, 1996). Gephyrin accumulates at developing postsynaptic sites prior to glycine receptors (Kirsch *et al.*, 1993a; Bechade *et al.*, 1996) and loss of gephyrin expression via antisense oligonucleotide depletion in primary neurons (Kirsch *et al.*, 1993b) and gephyrin gene knockout in mice (Feng *et al.*, 1998) both result in a loss of synaptic clustering of glycine receptors. However, in the hindbrain of zebrafish larva, glycine receptors composed of only α subunits can be synaptically activated, suggesting that gephyrin mediated glycine receptor clustering may not be the only way in which these receptors are clustered at postsynaptic sites (Legendre, 1997). It has also been shown that homomeric glycine receptors can aggregate at the cell surface without gephyrin, however, these receptors may be partially associated with gephyrin and their accumulation at synapses might depend on interactions between newly formed receptors and endogenous postsynaptic glycine receptor complexes (Meier *et al.*, 2000b). This suggests that clustering of glycine receptors might depend on mechanisms that are different from those involved in their accumulation. Gephyrin is crucial for the clustering and anchoring of postsynaptic glycine receptors.
Chapter 1: General Introduction

The gephyrin binding site on the glycine receptor β subunit has been localised to amino acids 378-426 of the large intracellular loop that joins transmembrane domains three and four (Meyer et al., 1995; Kneussel et al., 1999). Rees et al. (2003) showed that an intact MoeA domain is essential for glycine receptor interactions. It has been proposed that the glycine receptor β subunit interaction site on gephyrin is located over two loops within the MoeA domain, and the binding affinity for glycine receptor β subunit changes upon the dimerisation of this region. Crystal structure analysis showed that the loops which consist of amino acids 713-721, are imperative for glycine receptor β subunit binding (Sola et al., 2004).

Recently, a model has been proposed to explain the manner in which the gephyrin scaffold is formed at postsynaptic sites and how it may regulate the dynamics of glycine receptor clustering (Sola et al., 2004). Based upon crystal structure analysis and in vivo data, the authors propose that gephyrin exists within the cytosol as trimers bound together through their MogA domains. In this formation, the MoeA domains are free to bind to membrane bound glycine receptor β subunits that are either anchored in transport vesicles or embedded within the somatodendritic membrane. A yet unknown signal can then trigger a conformational change in gephyrin which allows the MoeA domain to dimerise, thus producing the hexagonal lattice structure of the gephyrin scaffold. In this conformation two glycine receptor β subunit binding sites are formed, one of high affinity and the other of low affinity. Thus, there is the possibility that either two glycine receptor β subunits from one receptor complex or one glycine receptor β subunit from two different receptor complexes bind to the subsynaptic gephyrin (Sola et al., 2004). However this model does not consider the recent findings that the subunit stoichiometry of the receptor complex is 2α and 3β (Grudzinska et al., 2005).

The subsynaptic scaffold provides physical constraints which are required to maintain the integrity of the synapse. Receptors which are not bound to a rigid structure are free to diffuse over large areas within the plasma membrane at rates above 0.1 μm s⁻¹ (Meier et al., 2001). It has been shown that at the neuromuscular junction, AChR freely diffuse at extrasynaptic sites, and are progressively captured by rapsyn (reviewed in Willmann and Fuhrer, 2002). There is now evidence to suggest that a similar phenomenon occurs with glycine receptors at postsynaptic sites during synaptogenesis. Experiments using
quantum dot technology to track single receptors revealed that three pools of receptors appear to exist within differentiating neurons. The first are mobile extrasynaptic receptors that are not associated with gephyrin. The second set are less mobile perisynaptic receptors which may associate with gephyrin. The third set of receptors diffuse slowly and are anchored by gephyrin (Dahan et al., 2003). Rapid dynamic changes are seen between these receptor pools and confirm data from other studies suggesting that clustering of glycine receptors is governed by a diffusion-trap mechanism during synaptogenesis (Kirsch and Betz 1998; Levi et al., 1998, Meier et al., 2001). The diffusion trap mechanism is best studied at the neuromuscular junction during synaptogenesis where AChR are highly mobile at extrasynaptic sites, and progressively become ‘trapped’ by the 43 kDa anchoring protein rapsyn at synaptic sites (Froehner, 1993). In the case of glycine receptors it is thought that mobile receptors become ‘trapped’ by gephyrin.

In order for glycine receptors to be anchored at their postsynaptic sites, gephyrin needs to bind to the cytoskeleton. Gephyrin binds to polymerised microtubules, but for this to be maintained an ATP-dependent auto-phosphorylation process is required (Langosch et al., 1992). If this process is disrupted using demecolcine (an alkaloid that blocks microtubule assembly), postsynaptic glycine receptor clusters are dispersed throughout the cell and the number of gephyrin clusters is reduced (Kirsch and Betz, 1995). However, this is only evident in immature neurons which suggests that microtubules are essential for the delivery of glycine receptors but not necessarily the maintenance of glycine-cergic synapses (Van Zundert et al., 2004). Recently, it has been suggested that glycine receptors associate with gephyrin on their way to the cell surface, and that this association increases their accumulation at the cell surface (Hanus et al., 2004). It has also been suggested that glycine receptors, gephyrin and dynein associate with each other in retrograde transport away from synapses (Maas et al., 2006).

1.2.2 Gephyrin associated proteins

In addition to the glycine receptor β subunit, gephyrin also interacts with a number of other proteins, some of which may participate in the formation and maintenance of the postsynaptic scaffold at inhibitory synapses. These proteins are summarised in Table 1.1 and some which may be involved in the postsynaptic scaffold will be described in the following section.
Chapter 1: General Introduction

### Table 1.1: A summary of the proteins that are known to interact with gephyrin.

<table>
<thead>
<tr>
<th>Interactor</th>
<th>Possible Function of Gephyrin</th>
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<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor subunits</td>
<td>Anchoring at postsynaptic sites</td>
</tr>
<tr>
<td>Collybistin</td>
<td>Intracellular trafficking</td>
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<tr>
<td>GABARAP</td>
<td>Interactions with GABA&lt;sub&gt;A&lt;/sub&gt; receptors</td>
</tr>
<tr>
<td>Dynein light chains 1 and 2</td>
<td>Intracellular trafficking</td>
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<tr>
<td>Profilin I and IIa,</td>
<td>Linking to microfilament system</td>
</tr>
<tr>
<td>Mena/VASP</td>
<td>Linking to microfilament system</td>
</tr>
<tr>
<td>RAFT1</td>
<td>mRNA translation at dendritic sites</td>
</tr>
<tr>
<td>Polymerised Tubulin</td>
<td>Interaction with the cytoskeleton</td>
</tr>
<tr>
<td>F-actin</td>
<td>Interaction with the cytoskeleton</td>
</tr>
<tr>
<td>Agrin</td>
<td>Association in retinal cells</td>
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</table>

A gephyrin interactor that is heavily implicated in the trafficking of GABA<sub>A</sub> receptors is the 13.9 kDa protein GABA<sub>A</sub> receptor associated protein (GABARAP; Kneussel et al., 2000). GABARAP binds to the intracellular loop of GABA<sub>A</sub> receptor γ2 subunit (Wang et al., 1999; Nymann-Anderson et al., 2002) and can induce the clustering of γ2 subunit containing receptors in heterologous cells (Chen et al., 2000). GABARAP also binds to tubulin and thus may mediate the interaction of GABA<sub>A</sub> receptors to the cytoskeleton, analogous to the function carried out by gephyrin for glycine receptors. GABARAP dimerises when in solution suggesting that it may contribute to the subsynaptic scaffold, or act as linker between gephyrin and GABA<sub>A</sub> receptors (Nymann-Anderson et al., 2002; Coyle and Nikolov, 2003). However, GABARAP distribution does not appear to support this assertion, as data indicate that GABARAP immunoreactivity is found in the Golgi compartment. Thus, colocalisation between gephyrin and GABARAP does not occur at synaptic sites (Kneussel et al., 2000; Kittler et al., 2001). There is now much data to indicate that GABARAP plays a greater role in the intracellular trafficking of GABA<sub>A</sub> receptors than in their synaptic clustering (reviewed in Lüscher and Keller, 2004).

Gephyrin also interacts with other proteins that are involved in intracellular trafficking - dynein light chain 1 (Dlc1) and dynein light chain 2 (Dlc2; Fuhrmann et al., 2002). These light chains form part of the multi-subunit dynein complex, involved in intracellular, retrograde protein transport. Dynein transports its cargo towards the minus ends of microtubules and its interaction with gephyrin suggests that it may be
involved in the trafficking of gephyrin within neurons either retrogradely along the axon and distal dendrites, or in either direction within the proximal dendrites. Disruption of the Dlc1 binding site on gephyrin did not affect the subcellular distribution of postsynaptic gephyrin clusters suggesting that this complex is involved in retrograde transport of gephyrin (Fuhrmann et al., 2002). A recent study provided evidence for the interaction between dynein light chains, gephyrin and glycine receptors, and suggests that this transport complex contributes to the dynamic and activity-dependent rearrangement of postsynaptic glycine receptors (Maas et al., 2006). Dlc1 and Dlc2 can also exist as a subunit of myosin Va. Myosin Va is involved in actin based vesicle transport. It can also bind to microtubules, and may act as a mechanical linker between microfilaments and microtubules. Myosin Va also interacts with kinesin and is found at the cell surface, therefore, it may have a role in transferring cargo from microtubule to microfilament tracks (Mallik and Gross, 2004).

Profilin is a gephyrin interactor that is associated with the cytoskeleton. It binds to actin monomers and stimulates ADP-ATP exchange that promotes the incorporation of actin monomers into actin filaments (Finkel et al., 1994). Two isoforms of profilin exist, profilin I, which is ubiquitously expressed, and IIa, which is restricted to the brain and skeletal muscle (Witke et al., 1998). Interactions between gephyrin and profilin are thought to regulate the density of glycine receptor aggregates (Kneussel and Betz, 2000; Giesemann et al., 2003). The Mammalian enabled/vasodilator stimulated phosphoprotein (Mena/VASP) complex is involved with regulating the geometry of the actin cytoskeleton. It is involved with many actin-dependent processes including axon and dendrite guidance (Menzies et al., 2004). The C-terminal MoeA region of gephyrin self-interacts, and also forms a complex with neuronal Mena and profilin. These colocalise at spinal synaptic sites and may contribute to the link between receptors and the cytoskeleton. This complex may also regulate the receptor density of inhibitory synapses (Giesemann et al., 2004).

Rapamycin and FKBP12 target protein (RAFT1) is an important target for the immunosuppressant rapamycin, and is also an important regulator of the cell cycle. RAFT1 was found to interact with gephyrin (Sabatini et al., 1999). RAFT1 also participates in signaling pathways that control mRNA translation (Sabatini et al., 1997). High levels of glycine receptor α subunit mRNA have been found located subsynaptically in spinal cord cell dendrites (Racca et al., 1997). Therefore, the presence
of RAFT1, a regulator of translational machinery at inhibitory synapses, suggests that it may regulate dendritic mRNA translation at synapses (Triller et al., 1990; Sabatini et al., 1999; Kneussel and Betz, 2000).

1.3 Glycine receptor clustering

A prerequisite for fast synaptic transmission between neurons is the placement of postsynaptic receptors opposite their corresponding presynaptic terminals. Glycine receptors are found in dense clusters at postsynaptic specialisations opposite axon terminals releasing glycine (Triller et al., 1985). There is a wealth of evidence to suggest that the localisation of these receptors is orchestrated by a number of receptor associated proteins. Without the support of the subsynaptic scaffold, the receptors would diffuse over large areas within the membrane, therefore, these physical constraints are required to maintain the integrity of the synapse. The selective clustering of glycine receptors at inhibitory sites appears to be a complex procedure consisting of many different mechanisms which regulate synthesis, trafficking and localisation of receptors and gephyrin. In 2000, Kneussel and Betz proposed a model for the molecular mechanisms by which submembranous gephyrin aggregates, and associated glycine receptors are specifically localised at inhibitory postsynaptic sites.

The “membrane activation model” proposes that gephyrin accumulation and subsequent glycine receptor clustering is initiated by receptor driven activation of phosphatidylinositol-3-kinase (PI3K) and Ca^{2+} influx (Fig. 1.3). There are many isoforms of PI3K, which are either activated by receptor tyrosine kinases (RTK) or G-protein coupled receptors (Maier et al., 1999). Activated PI3K phosphorylates phosphatidylinositol 4,5 bisphosphate (PI_{4,5}P_{2}) to phosphatidylinositol 3,4,5 trisphosphate (PI_{3,4,5}P_{3}). This model suggests that PI3K is activated by an as yet unknown presynaptically released factor which may act on RTKs of the epidermal growth factor/platelet derived growth factor/insulin superfamily. This is analogous to agrin in muscle (Hoch, 1999). RTKs are also known to activate the small G-protein cell division cycle 42 (Cdc42), which is a monomeric GTPase that plays a vital role in regulating intracellular processes such as the rearrangement of the actin cytoskeleton. The authors suggested that the accumulation of PI3,4,5P_{3} recruits collybistin - a guanosine exchange factor (GEF), and profilin to the activated membrane domain, which in turn cause cytoskeletal rearrangements and the apposition of gephyrin to the plasma membrane. Fig. 1.3 is a schematic representation of the proteins and signalling pathways implicated in the membrane activation model.
Fig. 1.3: The membrane activation model - This model proposes that glycine receptor clustering at the postsynaptic membrane is mediated by an activity-dependent and trophic-factor controlled signalling mechanism. According to this model, Ca²⁺ influx and local PI3K activation results in the assembly of a submembranous gephyrin scaffold at developing postsynaptic sites. PI3K activation results in the accumulation of PIP₃, which recruits the GDP-GTP exchange factor (GEF) collybistin and profilin to the membrane. Collybistin is predicted to activate Cdc42, which regulates the rearrangement of the actin cytoskeleton. In addition, profilin encourages the formation of filamentous-actin (f-actin) and is thought to regulate the density of the glycine receptor aggregates. Gephyrin accumulation may also stimulate the kinase activity of RAFT1, which regulates the subsynaptic translational machinery via S6 kinase and the elongation factor eIF-4F. Cdc42-GTP may also stimulate pp70s6 kinase activity. Cdc42 can also activate microtubule associated protein kinases (MAPks) and p21 activated kinases (PAKs), which regulate gene expression and may also contribute to the differentiation of postsynaptic membranes during synaptogenesis (adapted from Kneussel and Betz, 2000).
Chapter 1: General Introduction

Gephyrin accumulation and postsynaptic clustering of glycine receptors was prevented by strychnine treatment, which blocks glycine receptor activation, and by L-type Ca$^{2+}$ channel blockers or tetrodotoxin, which blocks sodium channels thus preventing action potentials (Kirsch and Betz, 1998; Levi et al., 1998). In addition, glycine had a depolarising effect on developing neurons which caused Ca$^{2+}$ influx, a trigger for neurotransmitter release (Boehm et al., 1997). Taken together, this evidence indicates that glycine receptor activation and increased intracellular Ca$^{2+}$ levels may be required for the accumulation and clustering of gephyrin and glycine receptors at postsynaptic specialisations. However, it has been shown that GABA$_A$ receptor activity was not required for receptor clustering (Craig and Banker, 1994), suggesting that receptor activation may be involved in discriminating between functional and non-functional synapses, instead of being the primary signal for receptor clustering (Kirsch and Betz, 1998).

In 2000, Kins et al. identified collybistin as a diffuse B cell lymphoma-like (dbl-like) GEF that interacted with gephyrin. They isolated two isoforms of collybistin (I and II), both of which harboured the dbl-homology (DH, also known as RhoGEF) and pleckstrin homology (PH) tandem domain typical of the dbl-like family of proteins. In addition, collybistin I had a N-terminal Src-homology 3 (SH3) domain and a coiled-coil C-terminal tail. Based upon sequence homology they suggested that collybistin was a GEF for the Ras homologous A (RhoA)-family of GTPases, which also includes Ras-related C3 botulinum substrate 1 (Rac1) and Cdc42. They also noted that the SH3 domain of collybistin I displayed high homology to the SH3 domains of cytoskeletal proteins such as myosin and spectrin. Upon co-expression of collybistin and gephyrin in human embryonic kidney 293 (HEK293) cells, collybistin I targeted to cytoplasmic regions rich in gephyrin whereas collybistin II caused the relocalisation of cytoplasmic gephyrin to submembranous microaggregates which resembled gephyrin microaggregates observed during glycine receptor clustering.

For those reasons, Kins et al. (2000) speculated that the molecular mechanisms underlying the redistribution of gephyrin from intracellular aggregates to submembranous microaggregates involved the activation of one or more Rho-family GTPases. These GTPases are implicated in the regulation of many cellular processes, but most prominently in the regulation of the actin cytoskeleton (Hall, 1998). Rho-family GTPases have also been implicated in signaling pathways which activate
phospholipase C (PLC), which cleaves PI$_{4,5}^2$ to liberate inositol 1,4,5 trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ can trigger the release of Ca$^{2+}$ from intracellular stores, whilst DAG activates protein kinase C (PKC), which has many targets implicated in a variety of cellular activities. Additionally, PH domains, like the one present in collybistin, are thought to bind to PI$_{4,5}^2$ in order to mediate correct subcellular localisation of proteins. The presumed binding of collybistin to PI$_{4,5}^2$ implies that this phospholipid and its derivatives may be in close proximity to gephyrin. The increase in intracellular Ca$^{2+}$ by IP$_3$ derived from PI$_{4,5}^2$ may contribute to the microdomains of elevated Ca$^{2+}$ that are implicated in the generation of gephyrin clusters in differentiating spinal neurons (Kirsch and Betz, 1998). Kins et al. (2000) concluded that collybistin initiated actin rearrangements, which caused the accumulation of gephyrin at presumptive postsynaptic sites, which in turn facilitated glycine receptor clustering.

In 1999, Reid et al. identified and characterised the human homologue of collybistin - Human homologue of Posterior End Mark 2 (hPEM-2) as a Cdc42-specific GEF. hPEM-2 has tandem RhoGEF-PH domains identical to collybistin, a N-terminal SH3 domain like collybistin I and a C-terminal which is different to that of the collybistin isoforms identified by Kins et al. (2000). The findings of Kins et al. (2000), taken with the data presented by Reid et al. (1999), were encompassed into the membrane activation model, which suggests that collybistin is involved in the activation of Cdc42, and that Cdc42 influences glycine receptor clustering at many levels. In parallel to this, Cdc42 may also exert translational and transcriptional control via its downstream targets (Cerione, 2004). The gephyrin interactor RAFT1 is also involved in translational signalling pathways. The targets of raised cytosolic Ca$^{2+}$ in the membrane activation model are unknown, but may include proteins that influence microfilament and microtubule dynamics (Chou and Blenis, 1996; Fanger, 1999; Daniels and Bokoch, 1999).
1.4 Aims

The overall aim of this study was to establish the role(s) played by collybistin in the clustering of gephyrin at postsynaptic membranes. Although the membrane activation model addresses what may be happening to gephyrin and glycine receptors at developing synapses, it does not fully account for the trafficking and clustering of inhibitory neurotransmitter receptors. Also, there are many aspects of this model which remain unclear, particularly the specific role(s) of collybistin, and its contribution to the clustering of gephyrin at postsynaptic sites. Kneussel and Betz (2000) suggested that collybistin was recruited by PI4,5P2 or PI3,4,5P3, thus restricting collybistin function to the postsynaptic membrane, where it catalysed GDP-GTP exchange activity on Cdc42, which in turn regulated the local actin cytoskeleton thus facilitating the accumulation of gephyrin. However, there is very little evidence to directly link collybistin to Cdc42 or PI4,5P2 and PI3,4,5P3. Kins et al. (2000) identified two collybistin isoforms which gave different phenotypes when co-expressed with gephyrin in HEK293 cells. The isoform lacking the SH3 domain translocated gephyrin from intracellular to submembranous sites, which implies that the interaction between collybistin and gephyrin may occur within neurons at sites other than the synapse. Fig 1.4 is a schematic of the contribution made by collybistin to gephyrin clustering, as suggested in the membrane activation model, and the focus of this study.

![Diagram](image_url)

**Fig. 1.4:** A schematic representation of the role played by collybistin in the membrane activation model.
Chapter 1: General Introduction

The aims of this study were to:

- Investigate how the SH3, RhoGEF and PH domains of collybistin interact with gephyrin and contribute to the overall function of collybistin in the translocation of gephyrin as demonstrated by Kins et al. (2000), and establish an explanation for the functional differences observed between the two collybistin isoforms. This will be presented in Chapter 3.

- Establish whether collybistin activates Cdc42, as suggested in the membrane activation model. Kins et al. (2000) stated they were unable to show this but, as it is an important part of the model, it should be verified. This will be presented in Chapter 4.

- Identify any other proteins that may interact with collybistin in order to find out more about its function. It is increasingly evident that protein-protein interactions form the backbone of most cellular processes, and it was hypothesised that collybistin could have other interacting partners which may aid it in clustering glycine receptors. A cDNA library screen using the yeast two-hybrid system was carried out to identify any potential interactors and the results of this work will be presented in Chapters 5 and 6.
Chapter 2: Materials and Methods
2. Materials and Methods

The following chapter includes all the general methods that were used throughout the project including techniques for molecular biology procedures, yeast two-hybrid, cell culture and immunocytochemistry.

2.1 Molecular Biology

![Flowchart showing the techniques used throughout this study to clone cDNA into an expression vector.](image-url)

**Fig. 2.1:** Flowchart showing the techniques used throughout this study to clone cDNA into an expression vector.
2.1.1 Polymerase chain reaction (PCR)

The polymerase chain reaction is an *in vitro* technique employed to amplify a fragment of DNA between two known sequences from either genomic DNA or cDNA. For successful amplification, two synthetic oligonucleotide primers, which are complimentary to two regions that flank the target DNA (one for each strand) are required. These primers are added to a small quantity of target DNA and, in the presence of a heat-stable DNA polymerase, a buffer and an excess of deoxynucleotides (dNTPs), are subjected to a series of temperature cycles. Typically there are 30-40 cycles in which the target DNA is repeatedly denatured, annealed to the primers and a daughter strand is extended from the primers. The daughter strands can act as a template, thus the DNA is amplified exponentially. The amplified DNA can then be purified and cloned into a vector. Fig. 2.2 shows a schematic representation of PCR.

All reactions were set up as shown in Table 2.1 and carried out in a Thermo Hybaid PX2 thermal cycler machine. Two different DNA polymerase enzymes were used for PCR reactions depending on the size of the DNA fragment being amplified and whether high or low fidelity was required.

The first enzyme used was *Pfu* DNA polymerase (Stratagene), which is a proof reading enzyme isolated from *Pyrococcus furiosus*. It has 3'-5' exonuclease activity, which enhances the fidelity of the DNA synthesis as it will excise incorrectly or mismatched 3'-terminal nucleotides from the primer/template and incorporate the correct nucleotide, thus giving it a low error rate (1.3×10⁻⁵, the error rate equals mutation frequency per base pair per duplication). The 10× reaction buffer used with this enzyme contains 200 mM Tris-HCl; 20 mM MgSO₄; 100 mM KCl; 100 mM (NH₄)₂SO₄; 1% (v/v) Triton X-100 and 1 mg/ml nuclease-free BSA; pH 8.8.

The second enzyme used was the Advantage 2 system (BD Biosciences) *Taq* polymerase isolated from *Thermus aquaticus*. This enzyme is ideal for amplifying large fragments of DNA, as it can amplify up to 18 kb, although it has a higher error rate than *Pfu* DNA polymerase (error rate: 25 errors in 10,000 bp amplified). The 10× reaction buffer used with this enzyme contains 400 mM Tricine-KOH, pH 8.7; 150 mM KOAc; 35 mM Mg(OAc)₂; 37.5 μg/ml BSA; 0.05% Tween 20 and 0.05% Nonidet-P40.
Chapter 2: Materials and Methods

The PCR primers were designed ‘in house’ and synthesised by Sigma Genosys. For cloning, the primers were designed so that the resulting PCR product would incorporate a restriction enzyme site at the 5’ and 3’ end of the target sequence. These sites were then used to clone the target sequence cDNA into an expression vector. For each of the target sequences, two primers were designed of different sequences that would anneal to complimentary strands on the target cDNA. Primers were 15-20 bp long, with the restriction enzyme site incorporated at the 5’ end of each primer. Fig. 2.2 shows a schematic representation of primer design. For site-directed mutagenesis primers were designed so that the resulting PCR product contained the required mutation. All the PCR primers were reconstituted in buffer EB (10 mM Tris-HCl, pH 8.5) to stock concentrations of 100 pmol/μl, and stored at -20°C.

Some PCR reactions required the addition of GC melt (5x stock concentration composed of 25% DMSO and 5 M GC melt, BD Biosciences). GC melt enables the amplification of GC rich sequences to occur. GC rich sequences have a strong secondary structure which resists denaturation and prevents the primer from annealing. This results in poor amplification of the template. GC melt contains DMSO, as well as a proprietary reagent which weakens the G-C pairs (BD Biosciences).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock concentration.</th>
<th>Volume used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme buffer</td>
<td>10x</td>
<td>5μl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10 mM of each dNTP</td>
<td>1μl</td>
</tr>
<tr>
<td>Primer 1</td>
<td>10 pmol/μl</td>
<td>1μl</td>
</tr>
<tr>
<td>Primer 2</td>
<td>10 pmol/μl</td>
<td>1μl</td>
</tr>
<tr>
<td>Template</td>
<td>5 ng/μl or 50 ng/μl</td>
<td>1μl</td>
</tr>
<tr>
<td>Enzyme</td>
<td>2.5 U/μl</td>
<td>1μl</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>—</td>
<td>40μl</td>
</tr>
<tr>
<td>Total</td>
<td>—</td>
<td>50μl</td>
</tr>
</tbody>
</table>

Table 2.1: PCR reagents – these are the reagents and volumes used in the standard PCR reactions carried out throughout this study. Volumes were adjusted accordingly if GC melt was included in the PCR.
A Primer Design

<table>
<thead>
<tr>
<th>Target sequence</th>
<th>Primer 1</th>
<th>Primer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'</td>
<td>3'</td>
<td>5'</td>
</tr>
</tbody>
</table>

Primer were designed so that a restriction enzyme site could be incorporated into the amplified sequence, which could then be used for cloning. In general primers were 15-20bp long, with the site (underlined) at the 5' end. See example of rCB1.

rCB1 5' GTGGGATCCATGCAGTGGATTAGAGGCAGGA 3'

B

<table>
<thead>
<tr>
<th>Denaturation at 94°C</th>
<th>( 5' \rightarrow 3' )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealing at 60-65°C</td>
<td>( 5' \rightarrow 3' )</td>
</tr>
<tr>
<td>Extension at 68-72°C</td>
<td>( 5' \rightarrow 3' )</td>
</tr>
</tbody>
</table>

C

Fig. 2.2: Schematic representation of PCR - PCR is an in vitro cyclic procedure by which a target region of DNA can be amplified with the aid of two target specific primers that are complimentary to the two ends of the DNA (A). Each cycle consists of a denaturation step, an annealing step, in which the primers anneal to their respective ends, and an extension step in which the primers are extended by DNA polymerase (B). The first cycle produces two daughter strands and these strands act as templates in each subsequent cycle (C).
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>rCB1 (BamHI)</td>
<td>5'-GTGGGATCCATGCAGTGGATTAGAGCGGA-3'</td>
</tr>
<tr>
<td>rCB2 (EcoRI)</td>
<td>5'-TTCCGAAATTCCCATCCTCTGCTGTACCCCA-3'</td>
</tr>
<tr>
<td>rCB3 (BamHI)</td>
<td>5'-GGAGGATCCAAATACGTTGCCGAGCTTA-3'</td>
</tr>
<tr>
<td>rCB4 (EcoRI)</td>
<td>5'-TTAGAATTCTCTGCTCTTCTATAGGTATTA-3'</td>
</tr>
<tr>
<td>hCB1</td>
<td>5'-CATGGCCTCGTGATATGATAG-3'</td>
</tr>
<tr>
<td>hCB2</td>
<td>5'-TCCAGGTTGTCGCTGACATCG-3'</td>
</tr>
<tr>
<td>hCB3</td>
<td>5'-CTGCAATGACTGTGAGAAAAGTCC-3'</td>
</tr>
<tr>
<td>hCB4</td>
<td>5'-ATTATCTGCTCCCTGTAGGTATC-3'</td>
</tr>
<tr>
<td>rGeph1 (BclI)</td>
<td>5'-CGCTGATCAACATGGCGACCGAGGGA-3'</td>
</tr>
<tr>
<td>rGeph2 (XhoI)</td>
<td>5'-TGGCTCGAGTCATAGCCGTCCGATGA-3'</td>
</tr>
<tr>
<td>hβGlyR1 (EcoRI)</td>
<td>5'-GCTGAATTCCGACCATGCGGAGGGGA-3'</td>
</tr>
<tr>
<td>hβGlyR2 (BamHI)</td>
<td>5'-ACCGGATCCCTTGCAATAAGATCAATTCGC-3'</td>
</tr>
<tr>
<td>rPH1 (Sall)</td>
<td>5'-GAGGTGCACTGATCTACACT-3'</td>
</tr>
<tr>
<td>rPH2 (EcoRI)</td>
<td>5'-TTCCGAATTCTCTTCTCCTCTCTCTCTGA-3'</td>
</tr>
<tr>
<td>rRhoGEF1 (Sall)</td>
<td>5'-CGCGCTCGAAGTACAGTCAATGAGATC-3'</td>
</tr>
<tr>
<td>rRhoGEF2 (EcoRI)</td>
<td>5'-ACGGAATTCTCTCTATTGATCTGCTGA-3'</td>
</tr>
<tr>
<td>hPEM-2 1 (NdeI)</td>
<td>5'-GTGCATATGAGCTGACGTTG-3'</td>
</tr>
<tr>
<td>hPEM-2 2 (EcoRI)</td>
<td>5'-TTAGAATTCTCTCCTCCCTGTAGGTATC-3'</td>
</tr>
<tr>
<td>hPEM-2 3 (XhoI)</td>
<td>5'-CTCCTCGAGGCCACCACCTGACGTTGCTGATC-3'</td>
</tr>
<tr>
<td>hPEM-2 4 (BamHI)</td>
<td>5'-AGGGATCTCTTCTTTGAAAGGGGCTAAC-3'</td>
</tr>
<tr>
<td>hKIF5A 1 (BamHI)</td>
<td>5'-ACAGGATGCTGACAGTCAATGC-3'</td>
</tr>
<tr>
<td>hKIF5A 2 (XhoI)</td>
<td>5'-TGTCCTCGAGTTAGTGCTGCTG-3'</td>
</tr>
<tr>
<td>hKIF5B 1 (BamHI)</td>
<td>5'-ACAGGATCCCTGATCGTATAATCG-3'</td>
</tr>
<tr>
<td>hKIF5B 2 (XhoI)</td>
<td>5'-TATCTCAGGTATCAAACTGTTGTTG-3'</td>
</tr>
<tr>
<td>hKIF5C 1 (BclI)</td>
<td>5'-CCGCTGATCAATGGCGATCCAGGCCG-3'</td>
</tr>
<tr>
<td>hKIF5C 2 (BclIB)</td>
<td>5'-ACCTGATCAATACAGTCTCTGCTGA-3'</td>
</tr>
<tr>
<td>hKIF5C 3 (Sall)</td>
<td>5'-ATTGTGCTGACCTTATTTCTGCTGGAATG-3'</td>
</tr>
<tr>
<td>hKIF5C 4 (BamHI)</td>
<td>5'-ACAGGATCCCTGCTCCGGGACACGC-3'</td>
</tr>
<tr>
<td>pYTH9 reverse</td>
<td>5'-AAACGTCCACCGCGTG-3'</td>
</tr>
<tr>
<td>pGBK7 reverse</td>
<td>5'-AATCATATAAGAAATTCGC-3'</td>
</tr>
<tr>
<td>T7</td>
<td>5'-TAATACGACTCATATAGGG-3'</td>
</tr>
<tr>
<td>pACT2 M2</td>
<td>5'-TTCAGTATCTACGATTCATAG-3'</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAL4AD</td>
<td>5'-AATACCACCTACAATGGATGATGTAT-3'</td>
</tr>
<tr>
<td>GAL4BD</td>
<td>5'-TCATCGGAAGAGAGTAGTAACAAAG-3'</td>
</tr>
<tr>
<td>GFP Seq1 (C1, C2)</td>
<td>5'-CATGGTCCTGCTGGAGGTTCGTG-3'</td>
</tr>
<tr>
<td>pRK5 Seq</td>
<td>5'-TATAGAATAAACATCCAC-3'</td>
</tr>
<tr>
<td>CB Seq1</td>
<td>5'-TGTCATCAATGAGATCA-3'</td>
</tr>
<tr>
<td>CB Seq2</td>
<td>5'-CTTGCAGCAGATGATCG-3'</td>
</tr>
<tr>
<td>CB Seq3</td>
<td>5'-GATGGTCTCTGCAAGA-3'</td>
</tr>
<tr>
<td>ΔRhoGEF 1</td>
<td>5'-ACCAGATGCGGGCCAATCGCAAGCGACGTCTGGA-3'</td>
</tr>
<tr>
<td>ΔRhoGEF 2</td>
<td>5'-TCCAGACGTCGCTTGGGATGCGACGTCTGGA-3'</td>
</tr>
<tr>
<td>ΔPH 1</td>
<td>5'-TAGACAGGAGCTCGGAAATGGTACAAGAAGATGA-3'</td>
</tr>
<tr>
<td>ΔPH 2</td>
<td>5'-TCATCTTTCTAGAACCATTCCCAGCTCTGCTCTA-3'</td>
</tr>
</tbody>
</table>

**Table 2.2: PCR Primers**

2.1.2 Site-directed mutagenesis

Site-directed mutagenesis was performed using the Quickchange site-directed mutagenesis kit (Stratagene). This allowed the *in vitro* mutagenesis of plasmids, using two synthetic primers which were designed to contain the desired mutation and were complimentary to opposite strands of the template plasmid. The primers were extended for 21 cycles at 94°C for 1 min, 50°C for 1 min and 68°C for 1 min by *Pfu* polymerase, and the incorporation of oligonucleotides generated mutation-containing plasmids with staggered nicks. The PCR product was then incubated for 1 h with *DpnI*, which is an endonuclease specific for methylated and hemimethylated DNA, to digest away the parental DNA template thus leaving the mutated DNA. The mutated plasmid was subsequently transformed into competent *E. coli* cells which repaired the nicks in the vector to give the desired mutated plasmid.

2.1.3 Agarose gel electrophoresis

Agarose gels were used to separate and visualise nucleic acids for preparative and analytical purposes. Agarose is a linear polymer extracted from seaweed, and when it solidifies it forms a pore containing matrix, the size of the pore is dependent on the concentration of the agarose (w/v) and determines the range of DNA size which can be separated.
Chapter 2: Materials and Methods

The gel was formed by dissolving powdered agarose (Ultra-pure grade, Invitrogen) in 100 ml 1× TAE buffer (1× Tris-Acetate-EDTA: 40 mM Tris-base; 20 mM Acetic acid; 1 mM EDTA; pH 8.0) to the desired concentration 0.5-2% (w/v) - by heating it in a microwave until it reached boiling point. The gel was cooled by immersing the flask into cold water, 1 µl ethidium bromide (10 mg/ml, Promega) was thoroughly mixed into the cooled gel, which was subsequently poured into a gel casting plate. An appropriate well forming comb was carefully inserted into the molten gel, ensuring that all the air bubbles were eased out. Once the gel had set, it was immersed into an electrophoresis submarine-tank, with the wells positioned at the negative electrode. DNA is highly negatively charged and therefore flows towards the positive electrode. Once submerged in electrophoresis buffer (1× TAE), the comb was removed and the DNA samples were loaded. Each DNA sample was pre-mixed with 2 µl of a dense loading buffer, which prevented the samples escaping from the wells (0.2% (w/v) bromophenol blue, 40% (w/v) sucrose in water). To deduce the size of the DNA fragments, 5 µl 1 kb DNA ladder marker (Invitrogen) was also loaded into one of the wells. The gel was electrophoresed at 100 mV for 30 min-2 h and viewed under a UV transilluminator. Fig 2.3 is an example a gel image captured using a Polaroid camera and type 667 film.

2.1.4 Agarose gel purification

The PCR products were purified by resolving them on an agarose gel as described in 2.1.2. The fragment was excised from the gel and processed to purify the DNA away from the gel and to remove any traces of ethidium bromide and enzymes. A glass bead based gel purification system was employed to recover the DNA from the gel (Sephaglas Bandprep kit, Amersham Biosciences).
Chapter 2: Materials and Methods

The excised gel was incubated in 250 µl of gel solubiliser (Buffered solution containing NaI) at 60°C with constant agitation for 10 min. Once completely dissolved, 5 µl of thoroughly resuspended glass bead mixture (20% (w/v) sephaglas BP suspended in distilled water) was added to the gel solution. This was incubated at room temperature for 5 min, and vigorously mixed every minute. The gel solution was then centrifuged at 13,000 rpm for 2 min at room temperature to pellet the beads to which the DNA should be bound. The supernatant was decanted, ensuring the pellet was not disrupted. The pellet was washed by resuspending it in 80 µl of wash buffer (20 mM Tris-HCl, pH 8.0; 1 mM EDTA; 0.1 mM NaCl in ethanol), centrifuging it at 13,000 rpm for 1 min at room temperature and decanting the supernatant. The pellet was washed three times. The pellet was then thoroughly dried by dispersing it around the bottom of the tube and letting it stand at room temperature for 10 min. Once dried, the DNA was eluted from the glass beads by incubating the pellet in 25 µl of elution buffer (10 mM Tris-HCl pH 8.0; 0.1 mM EDTA) at room temperature for 5 min with periodic shaking. The solution was centrifuged at 13,000 rpm for 1 min and the DNA-containing supernatant was transferred to a fresh tube, ensuring that the glass beads did not contaminate the DNA solution. The recovery of fragments was checked using gel electrophoresis.

2.1.5 Restriction enzyme digest

Bacteria contain restriction endonucleases/enzymes which recognise specific DNA sequences. In the majority of cases, these enzymes cut DNA at or near specific recognition sequences. Different species and strains of bacteria contain their own unique enzymes and use them as a defence and protective mechanism. These enzymes have been exploited by molecular biologists to generate recombinant DNA molecules. There are three types of restriction endonucleases, but only the type II enzymes are used in molecular biology. These enzymes cleave at specific sites and are used to generate defined DNA sequences for cloning into vectors. There are over 600 known enzymes, and they are generally named with a three-letter abbreviation based on the species and genus of the source bacterium, followed by a Roman numeral which indicates the order of identification of that enzyme. These enzymes only cut double stranded DNA, and typically the sequence at which they cut are palindromic, with cleavage occurring in both DNA strands at the same recognition sequence site. Table 2.3 is a list of all the restriction enzymes used throughout the course of this study, and the cleavage site is indicated by *.
Chapter 2: Materials and Methods

Restriction enzyme digests were carried out at 37°C for 1 h. The digestion mix varied, but was composed of DNA, one or two different restriction enzymes, 1× buffer and distilled water. Generally 1 μg DNA was digested with a total of 1 U of restriction enzymes and volumes were adjusted accordingly. The appropriate buffer for the enzyme had to be used to ensure optimum digestion. Generally, efficient digestion was seen within 1 h of incubation at 37°C, although some enzyme combinations required slightly longer times or different temperatures if the buffers did not match, or the enzyme had slower rate of activity.

<table>
<thead>
<tr>
<th>Restriction Endonuclease (New England Biolabs)</th>
<th>Recognition Site</th>
<th>Buffer (New England Biolabs)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>BamHI</em></td>
<td>5'...G*GATCC...3'</td>
<td><em>BamHI</em></td>
</tr>
<tr>
<td></td>
<td>3'...CCTAG*G...5'</td>
<td></td>
</tr>
<tr>
<td><em>BclI</em></td>
<td>5'...T*GATCA...3'</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3'...ACTAG*T...5'</td>
<td></td>
</tr>
<tr>
<td><em>BglII</em></td>
<td>5'...A*GATCT...3'</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3'...TCTAG*A...5'</td>
<td></td>
</tr>
<tr>
<td><em>BspEI</em></td>
<td>5'...T*CCGGA...3'</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3'...AGGCC*T...5'</td>
<td></td>
</tr>
<tr>
<td><em>EcoRI</em></td>
<td>5'...G*AATTC...3'</td>
<td><em>EcoRI</em></td>
</tr>
<tr>
<td></td>
<td>3'...CTTAA*G...5'</td>
<td></td>
</tr>
<tr>
<td><em>NcoI</em></td>
<td>5'...C*CATGG...3'</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3'...GGTAC*C...5'</td>
<td></td>
</tr>
<tr>
<td><em>NdeI</em></td>
<td>5'...CA*TATG...3'</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3'...GTAT*AC...5'</td>
<td></td>
</tr>
<tr>
<td><em>PstI</em></td>
<td>5'...C*TGCAG...3'</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3'...GACGT*C...5'</td>
<td></td>
</tr>
<tr>
<td><em>SalI</em></td>
<td>5'...G*TGCAG...3'</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3'...CAGCT*G...5'</td>
<td></td>
</tr>
<tr>
<td><em>SmaI</em></td>
<td>5'...CCC*GGG...3'</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3'...GGG*CCC...5'</td>
<td></td>
</tr>
<tr>
<td><em>XbaI</em></td>
<td>5'...T*CTAGA...3'</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3'...AGATC*T...5'</td>
<td></td>
</tr>
<tr>
<td><em>XhoI</em></td>
<td>5'...C*TCGAG...3'</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3'...GAGCT*C...5'</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3: Restriction Enzymes – recognition sequences and buffers for the enzymes

2.1.6 Phenol:Chloroform extraction

Phenol:chloroform extraction was carried out to remove any traces of proteins that could have contaminated a DNA solution. The solution containing the DNA was made up to a volume of 100 μl with distilled water. 100 μl of phenol:chloroform:isoamyl alcohol solution (25:24:1 (v/v), Invitrogen) was added to this and vigorously agitated until the two phases were thoroughly mixed, this denatured any proteins that may have
contaminated the DNA. The mix was centrifuged at 14,000 rpm for 20 min, and the resulting solution separated into two phases. The upper aqueous phase contained the DNA and was ethanol precipitated. The interface containing denatured proteins and lower phase were discarded.

2.1.7 Ethanol precipitation

DNA solutions were concentrated by ethanol precipitation. 100 μl of DNA solution was added to 10 μl sodium acetate (0.3 M, pH 5.2), 250 μl 96% ethanol (Pure grade, BDH) and 1 μl glycogen (20 μg/μl, the glycogen is a visible carrier molecule) and incubated on dry ice for up to 30 min to aid precipitation. The solution was then centrifuged at 14,000 rpm for 30 min at room temperature. The supernatant was decanted, and the resulting DNA pellet was washed by adding 250 μl 80% ethanol and centrifuging at 14,000 rpm for 5 min at room temperature. The supernatant was decanted and the DNA pellet dried at room temperature for 2-5 min. It was imperative that there were no traces of ethanol remaining in the tube as these could have affected subsequent reactions. Once dry, the pellet was resuspended in EB buffer (10 mM Tris-HCl, pH 8.5, Qiagen).

2.1.8 Ligation reactions

Once the digested vector and PCR products were phenol:chloroform extracted and ethanol precipitated they were ligated. The vector was resuspended in 50 μl EB buffer to a final concentration of 50 ng/μl, and the purified PCR product in 10μl EB buffer. The ligation reaction, which consisted of 7 μl PCR product, 1 μl vector, 1 μl 10× ligation buffer (10×: 660 mM Tris-HCl; 50 mM MgCl₂; 50 mM dithiothreitol (DTT); 10 mM ATP; pH 7.5, Roche) and 1 μl T4 DNA ligase (Roche) was thoroughly mixed together in a small microcentrifuge tube and incubated at 4°C overnight. Half of the ligated reaction was digested with a restriction enzyme whose recognition site had been removed from the digested vector and was absent from the PCR product. This removed any unligated or re-ligated vector from the solution, thus preventing their transformation and the formation of false positive colonies. The other half was transformed without digestion.
2.1.9 Preparation of competent E. coli cells

Competent E. coli cells that were used for transformation were made and stored at -80°C until required. A freshly streaked Top10 E. coli colony (Genotype: F' mcrA Δ(mrr-hsdRMS-mcrBC) 80lacZΔM15 ΔlacX74 recA1 araΔ139 Δ(ara-leu)7697 galU galK rpsL (Str^R) endA1 mupG, Invitrogen) was inoculated in 2 ml LB medium (Luria Bertani medium: 20 g dissolved in 1 l distilled water and autoclaved at 121°C for 15 min, Sigma, composed of 10 g/l Tryptone, 5 g/l Yeast extract and 5 g/l NaCl) and incubated at 37°C overnight with constant agitation (250 rpm). After 12-16 h, this starting culture was added to 200 ml of freshly prepared LB medium and incubated at 37°C with constant agitation until the culture grew to an OD₆₀₀ reading of 0.94-0.95. The density of cells grown in culture was determined at OD₆₀₀. Cultures were diluted so that the OD₆₀₀ reading was <1. At this range, 0.1 OD₆₀₀ corresponds to ~10⁸ cells/ml. All the reagents used were kept on ice throughout the procedure. The growth culture was transferred to 50 ml conical tubes and centrifuged at 3,000 rpm for 3 min at 4°C. The supernatant was decanted and the cells were resuspended in 10 ml freshly prepared ice-cold CaCl₂/MgCl₂ solution (80 mM CaCl₂/50 mM MgCl₂). The cells were pooled together and incubated on ice for 10 min. They were then centrifuged at 3,000 rpm for 3 min at 4°C. The pellet was then resuspended in CaCl₂/MgCl₂ solution, incubated on ice for 10 min and centrifuged as before. The resulting cell pellet was resuspended in 5.5 ml of freshly prepared ice-cold 0.1 M CaCl₂ solution. An equal volume (6 ml) of 50% glycerol was added to the cells, mixed gently and aliquotted into pre-chilled microcentrifuge tubes. These were then immediately snap frozen in liquid nitrogen and stored at -80°C until required.

2.1.10 Transformation of DNA into E. coli cells

Competent E. coli cells were defrosted on ice. 1 ng of plasmid DNA (which were being retransformed) or 5 µl ligation mix was sufficient for efficient transformation. The DNA was added to 100 µl of defrosted competent cells, gently mixed and incubated on ice for 30 min. The cells were heat shocked at 42°C for 30 s and incubated on ice for 2 min. 300 µl of sterile LB medium was added to the cell suspension and incubated at 37°C for 1 h with constant agitation. The cell suspension was spread onto selective agar plates and incubated at 37°C overnight. Selective agar plates were prepared by dissolving 16 g powdered LB agar (Invitrogen; composed of 12 g/l agar, 10 g/l yeast extract, 5 g/l peptone 140 and 5 g/l NaCl) in 500 ml distilled water, autoclaved at 121°C for 15 min,
cooled to >50°C and supplemented with the appropriate selective antibiotic, either ampicillin (final concentration: 100 µg/ml), or kanamycin (final concentration: 50 µg/ml), and poured into plastic 90 mm diameter Petri dishes. Plasmid vectors contain a gene which confers antibiotic resistance when expressed in *E. coli* allowing successfully transformed colonies to grow. Ampicillin resistance is encoded by the plasmid gene *bla* whose expression produces a periplasmic β-lactamase protein that catalyses the conversion of ampicillin to penicillic acid. Kanamycin resistance genes encode for aminoglycoside-3'-phosphotransferases I and II, which phosphorylate kanamycin rendering it inactive.

2.1.11 Mini-preparation of plasmid DNA

QIAprep spin mini-prep kits (Qiagen) were used to isolate the plasmid DNA from transformed *E. coli* cells. One colony was inoculated in 2 ml selective LB medium and incubated at 37°C overnight with agitation (250 rpm). The cell suspension was centrifuged at 13,000 rpm for 30 s at room temperature to harvest the bacterial cells. The supernatant was decanted and the pellet was resuspended in 250 µl buffer P1 (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A; Qiagen). 250 µl buffer P2 (200 mM NaOH, 1% (w/v) SDS) was then added. This is a lysis buffer which breaks open the bacterial cell wall and denatures the proteins, DNA and hydrolyses RNA. This was gently mixed and then 350 µl buffer N3 was added (neutralisation buffer; 1 M KOAc, pH 5.0). Plasmid DNA is smaller than genomic DNA therefore it does not precipitate in P1, P2 and N3, whereas the genomic DNA aggregates into an insoluble network. This mix was centrifuged at 13,000 rpm for 10 min at room temperature. This ensured that any cell components such as the cell wall, proteins and genomic DNA were removed from the solution which contained the plasmid DNA. The supernatant was decanted into a QIAprep spin column and the column was centrifuged at 13,000 rpm for 1 min. The flow through was discarded and 750 µl of buffer PE (wash buffer; 1 M NaCl, 50 mM MOPS, pH 7.0, 15% (v/v) isopropanol) was pipetted into the column. This was centrifuged as before, the flow through was discarded and the column was centrifuged once more to ensure that all the wash buffer had been removed from the column. The washed column was transferred to a clean microcentrifuge tube and 50 µl of EB buffer was added. The column was incubated at room temperature for 1 min and centrifuged as before to elute the DNA into the microcentrifuge tube. The mini preparation of plasmid DNA was then used for sequencing and subcloning.
Chapter 2: Materials and Methods

2.1.12 Midi/Maxi-preparation of plasmid DNA

Midi- and maxi-prep kits (Qiagen) were used to prepare large quantities of plasmid DNA. A single colony was inoculated in 2 ml selective LB medium and incubated at 37°C with agitation (250 rpm) for 8-10 h. 100 μl of this starter culture was added to 100 ml (50 ml for midiprep, all midiprep volumes will be in brackets after the maxiprep volumes) selective LB medium and incubated 37°C, for 12-16 h with agitation (250 rpm). The cell suspension was centrifuged at 4,000 rpm at 4°C for 30 min to harvest the bacterial cells. The resulting cell pellet was resuspended in 10 ml (4 ml) buffer P1 ensuring that there were no cell aggregates remaining in the tube. 10 ml (4 ml) buffer P2 (lysis buffer) was added, mixed gently and the suspension was incubated at room temperature for 5 min. 10 ml (4 ml) of pre-chilled buffer P3 (neutralisation buffer; 1 M KOAc, pH 5.0) was added to this mixture and incubated on ice for 20 min. The cell suspension was centrifuged at 4,000 rpm for 30 min at 4°C. During this time the columns were equilibrated by passing 10 ml (4 ml) buffer QBT (equilibration buffer; 750 mM NaCl, 50mM MOPS, pH 7.0, 15% (v/v) isopropanol, 0.15% Triton X-100; the detergent in the buffer reduces the surface tension of the tip) through the column. The supernatant was decanted into the columns ensuring that the precipitate did not enter the column as this would block the resin and slow down the filtering process. Once the supernatant had passed through, 30 ml (10 ml) buffer QC (wash buffer; 1 M NaCl, 50 mM MOPS, pH 7.0, 15% (v/v) isopropanol) was added to the column, allowed to flow through and the steps repeated. Once the resin had been washed, the DNA was eluted into a polypropylene tube with 15 ml (5 ml) buffer QF (elution buffer; 1.25 M NaCl, 50 mM Tris-Cl, pH 8.5, 15% v/v isopropanol). 10.5 ml (3.5 ml) isopropanol was then added to the eluted solution and incubated on ice for 30 min to precipitate the DNA. The solution was centrifuged at 12,000 rpm at 4°C for 20 min. The supernatant was carefully decanted and the DNA pellet was resuspended in 500 μl distilled water. The DNA was concentrated by ethanol precipitation as described above. The resulting pellet was resuspended 50-200 μl EB buffer (depending on pellet size), by incubating at 37°C with constant agitation until the pellet had dissolved.

2.1.13 Determination of DNA yield

To determine the yield of plasmid DNA retrieved from the midi- or maxi prep, 2.5 μl DNA was diluted in 498 μl distilled water and the concentration was calculated by measuring the sample at OD\textsubscript{260} (Biophotometer, Eppendorf).
2.1.14 DNA Sequencing

Plasmid DNA constructs were sequenced to ensure that the insert was correctly cloned into the vector. The gene of interest needs to be inserted into a specific reading frame of the vector so that it is translated correctly. Sequencing also checks for any mutations or errors that may have occurred either during the PCR amplification or by the bacteria during amplification. Sequencing was also used to determine the identity of the 'hits' isolated from the yeast two-hybrid library screen (see chapter 5). To sequence plasmid DNA, a sequencing primer was required that started within the vector upstream of the insert or within the insert itself. Sequencing reactions only require one primer, therefore plasmids could be sequenced with a primer in one direction, although where possible forward and reverse primers were used.

The BigDye terminator sequencing technology (ABI, Perkin-Elmer), used throughout this project is based upon the Sanger dideoxy 'terminator' method (Sanger et al., 1977) and allows the rapid sequencing of DNA. Each dideoxy nucleoside triphosphate (ddNTP) is labelled with a high-sensitivity dye which contains a fluorescein donor dye such as 6-carboxyfluorescein linked to a dichlorodiamine acceptor dye (see Table 2.4 for each ddNTP). The excitation of each dye is that of the donor and has been optimised to absorb the excitation energy of the argon ion laser in the sequencing machine. The emission maximum of each dye is that of the dRhodamine acceptor and is detected by a real-time laser gel scanner sent to the computer for analysis.

<table>
<thead>
<tr>
<th>ddNTP</th>
<th>Acceptor dye</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>dRGG</td>
<td>Green</td>
</tr>
<tr>
<td>C</td>
<td>dROX</td>
<td>Red</td>
</tr>
<tr>
<td>G</td>
<td>dR110</td>
<td>Blue</td>
</tr>
<tr>
<td>T</td>
<td>dTAMRA</td>
<td>Black</td>
</tr>
</tbody>
</table>

Table 2.4: Acceptor dye and colour of BigDye terminators

The BigDye Terminator cycle sequence ready reaction mix (sequencing reagent, Perkin-Elmer) combines AmpliTaq DNA polymerase FS (which contains two mutations, one which results in less discrimination against ddNTPs and the other which abolishes 5'-3' nuclease activity), dye terminators, deoxynucleoside triphosphates (dNTPs), rTth pyrophosphatase, MgCl₂ and buffer. For the sequencing reactions 2.5 µl sequencing reagent, 1 µl sequencing primer, plasmid DNA (either 3 µl of a miniprep or 1 µl DNA at 0.5 µg/µl) and distilled water (to make up total volume to 10 µl) were thoroughly
mixed together in a small PCR tube. The sequencing reactions were performed under the PCR conditions – 30 cycles at 94°C for 50 s, 50°C for 50 s and 72°C for 4 min.

Once the sequencing reactions were complete they were phenol:chloroform:isoamyl alcohol extracted and ethanol precipitated as described in section 2.1.5 and 2.1.6. Once the DNA pellet was dry, it was resuspended in 20 μl template suppression reagent (Perkin-Elmer Life Sciences), and incubated at 37°C for 5 min with constant agitation. The solution was then briefly centrifuged and transferred to a 310 sequencing tube, covered with a septa and denatured at 96°C for 2 min. The sequencing samples were incubated on ice until they could be loaded onto the automated DNA sequencer (ABI Prism™310 Genetic Analyzer, Applied Biosystems, Perkin-Elmer). The fragments generated by the cycling reaction were separated by size through capillary electrophoresis and only fragments that had incorporated a ddNTP were dye labelled and detected by the laser scanner. The capillaries contained a non-polymerised gel matrix. Sequences were analysed using the Sequencher programme (GeneCode).

Fig. 2.4: Example of sequencing peaks using BigDye Terminator technology
2.2 Yeast Two-Hybrid System

Protein-protein interactions are fundamental to all cellular activities and therefore identifying them is crucial to establishing how cells function. Virtually all processes involve protein-protein interactions ranging from DNA replication to the formation of large complex structures such as the cytoskeleton.

The yeast two-hybrid system enables the identification of protein-protein interactions in vivo, and also the characterisation of known interacting couples (Fields and Song, 1989). The yeast two-hybrid system is based upon the modular properties of eukaryotic transcription factors such as GAL4. Transcription factors consist of two functional domains, the DNA binding domain (DB) which directs binding to a promoter DNA sequence, and an activation domain (AD) which activates transcription of a reporter gene. The yeast two-hybrid system exploits the fact that the DB is incapable of activating transcription unless it is physically associated with the AD. Generally the protein of interest or ‘bait’ is fused to the DB and the potential interactors or a library of cDNA (prey) clones are fused to the AD. These are then transformed into a host yeast strain which contains a reporter gene and only if there is an interaction between the two proteins will the reporter gene be activated.

![Diagram of the Yeast Two-Hybrid System](image)

**Fig 2.5: Yeast Two-Hybrid** - Schematic representation of the Yeast Two-Hybrid System.
2.2.1 Yeast Strains

Y190 has the genotype MAT\textalpha, gal4\Delta, gal80\Delta, his3, trp1-90,1 ade2-101, ura3-52, leu2-3, -112, URA3::GAL-lacZ, LYS2::GAL(UAS)-HIS3, cyh.r. LacZ reporter assays were used for this strain. The collybistin library screen was carried out using this strain.

AH109 has the genotype MAT\textalpha, trp1-190, leu2-3, 112, ura3-52, his3-200, gal4\Delta, gal80\Delta, LYS2::GAL1_{UAS}\cdot GAL1_{TATA}\cdot HIS3, GAL2_{UAS}\cdot GAL2_{TATA}\cdot ADE2, URA3::MEL1_{UAS}\cdot MEL1_{TATA}\cdot lacZ. Nutritional growth reporter assays were used for this strain. Colonies were plated onto selective agar plates lacking adenine, histidine, leucine and tryptophan.

2.2.2 Yeast Expression Vectors

cDNAs encoding proteins used for yeast two-hybrid experiments were cloned into yeast expression vectors. The cloning of each of the constructs will be presented in the chapter in which they were used. Table 2.5 summarises the properties of the yeast expression vectors used throughout this project.

<table>
<thead>
<tr>
<th>Vector Name</th>
<th>Antibiotic Selectivity</th>
<th>Fusion Protein</th>
<th>Nutritional Selection Gene in Yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYTH9</td>
<td>Ampicillin</td>
<td>GAL4 BD amino acids 1-147</td>
<td>TRP1 Tryptophan</td>
</tr>
<tr>
<td>pYTH16</td>
<td>Ampicillin</td>
<td>GAL4 BD amino acids 1-147</td>
<td>TRP1 Tryptophan</td>
</tr>
<tr>
<td>pGBK7T</td>
<td>Kanamycin</td>
<td>GAL4 BD amino acids 1-147</td>
<td>TRP1 Tryptophan</td>
</tr>
<tr>
<td>pACT2</td>
<td>Ampicillin</td>
<td>GAL4 AD amino acids 768-881</td>
<td>LEU2 Leucine</td>
</tr>
</tbody>
</table>

**Table 2.5: Yeast expression vectors** – the antibiotic selectivity, fusion protein and nutritional selection of the GAL 4 yeast expression vectors.

2.2.3 Yeast Medium and Agar Plates

Yeast were grown and maintained in a commercially available YPD medium (YPD medium powder is a blend of yeast extract, peptone and dextrose, BD Biosciences). 25 g YPD medium was dissolved in 500 ml distilled water and autoclaved at 121°C for 15 min. Many yeast strains carry the ade2-101 mutation which means that they require adenine to grow. If there is insufficient adenine in the medium then the yeast colonies
appear red, which is thought to be due to an oxidised, polymerised derivative of 5-aminoimidazole ribotide which accumulates in vacuoles (Smirnov et al., 1967), therefore the medium was supplemented with 0.3 mM adenine as this appeared to help the culture grow faster and be transformed with greater efficiency. YPD agar contains the above in addition to agar, and YPD agar plates were used to maintain yeast strains at 4°C.

Minimal SD base is a blend of yeast nitrogen extract, ammonium sulphate and dextrose. Minimal SD agar contains the same as minimal SD base in addition to agar and was used to make selective agar dropout plates for yeast two-hybrid experiments. This was also supplemented with 0.3 mM adenine. Commercially available dropout supplements (BD Biosciences) were added to SD base to make the desired selective medium or agar plates. To make the agar plates, 32 g minimal SD agar was dissolved in 500 ml distilled water with 30 mg adenine and the appropriate amount of the required dropout supplement, autoclaved at 121°C for 15 min, cooled and poured into 90 mm diameter plastic Petri dishes.

2.2.4 Preparation of DNA for Yeast Transformations

If an integrated yeast strain was used, then only the prey cDNA had to be prepared (see 2.2.6). If the yeast strain was not integrated, then both prey and bait plasmids needed to be transformed into the yeast cells. The cDNA mixes were prepared 2-3 h before transformation and consisted of 1 µg of bait plasmid (if the yeast strain being used was not integrated), 1 µg of prey plasmid and 5 µl denatured single stranded carrier DNA (Herring Sperm DNA 11 mg/ml, Sigma), which were thoroughly mixed together. The carrier DNA was denatured by boiling it at 96°C for 10 min and rapidly cooling it on ice before use. The denatured DNA could be frozen and thawed 3-4 times without loss of activity. The cDNA mix was kept at room temperature until the competent cells were ready for transformation.

2.2.5 Transformation of yeast

100 ml of YPD medium was inoculated with a single yeast colony and grown overnight at 30°C with constant agitation (250 rpm). The following day, the density of the overnight culture was determined by diluting it 1:10 so that its OD_{600} reading was <1. At this range, 0.1 OD_{600} corresponds to ~3x10^6 cells/ml. Log phase growth is divided
into three phases based on the rate of cell division - early, mid and late, and cells are generally thought to be of a good density and viability during mid-log phase. A volume of the overnight culture was added to 200 ml YPD medium so that it measured 0.25 at OD$_{600}$. The day culture was grown until it reached a density of 0.65-0.70 at OD$_{600}$. This was determined to be mid-log phase for the yeast strains used in this study. The cell suspension was centrifuged at 3,000 rpm for 3 min at 20°C to harvest the yeast cells. The supernatant was decanted and the cells were resuspended in 25 ml distilled water and pooled together. The suspension was centrifuged as before and the resulting cell pellet was resuspended in 25 ml 1× lithium acetate/Tris-EDTA buffer (LiOAc 10× stock: 1 M, Sigma; TE buffer 100× stock: 1 M Tris-HCl, 0.1 M EDTA, pH 8.0, Sigma). The cell suspension was centrifuged as before, resuspended in 25 ml LiOAc/TE and centrifuged once more. The resulting pellet was resuspended in 1× LiOAc/TE (100 µl per transformation). 100 µl of the resulting suspension and 600 µl of 40% polyethylene glycol /LiOAc/TE (50% stock PEG 3350, Sigma; 1× LiOAc/TE) was added to each DNA mix. This was incubated at 30°C for 30 min and then the cells were heat shocked at 42°C for 20 min. The suspension was centrifuged at 13,000 rpm for 30 s. The resulting supernatant was decanted and the pellet was resuspended in 100 µl of pre-warmed distilled water. The yeast cell suspension was then evenly spread out onto the appropriate selective agar plate and incubated at 30°C until colonies emerged.

2.2.6 Integration of yeast strains

The use of a stably integrated yeast strain for a library screen and subsequent experiments simplifies the screening procedure. The library cDNA can be introduced into the yeast cell in a single transformation, and the recovery of the prey also becomes easier as it is the only episomal plasmid present (Fuller et al., 1998). The pYTH9 vector has a TRPI marker for integration at the trp1-901 locus. It also has a unique XbaI site for linearisation and targeted integration of the recombinant plasmid. Y190 and AH109 were both integrated with pYTH9-CB2SH3, pYTH9-CB2SH3ΔRhoGEF and pYTH9-CB2SH3ΔPH using the LiOAc/TE method described above. The integration DNA mix contained 10 µl linearised bait plasmid (0.5 µg/µl) and 5 µl of carrier DNA. The final cell suspension was then plated onto an agar plate lacking tryptophan.
2.2.7 Replica plating

Yeast colonies were lifted from agar plates onto filter paper (90 mm discs, Whatman #54) and either replicated onto another agar plate or the filters were used for LacZ assays, to see whether the proteins expressed by the yeast interacted with each other (see below).

Colonies were transferred from the plate onto a 90 mm diameter filter paper using a replica plating block. The paper was placed on the block and the plate gently inverted onto the paper, firmly tapped and the paper swiftly ripped away from the agar plate using forceps. The filter was inverted onto a selective plate and an impression of the colonies was made on the agar. These plates were then incubated at 30°C until the colonies grew back.

2.2.8 Freeze fracture LacZ assay

The LacZ reporter assay is designed to visualise the transcriptional activity of genes using a semi-quantitative colourimetric assay (Rupp, 2002). Induction of the LacZ gene produces β-galactosidase. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, BDH) is a chromogenic substrate for β-galactosidase. In the presence of β-galactosidase X-gal is cleaved and appears blue. Therefore an interaction between two proteins within the yeast colony will give a blue reaction, whilst colonies in which proteins do not interact will remain white.

Fig. 2.6: LacZ assay – X-gal is a colourless chromogenic substrate for β-galactosidase which is converted to a blue indolyl derivative through cleavage. The LacZ reporter gene product is β-galactosidase, therefore induction of the gene by two interacting proteins in the yeast two-hybrid system will result in blue colonies.
To freeze fracture the cells, the filter paper onto which the colonies had been lifted was immersed in liquid nitrogen for 5-10 s. The filter paper, colony side up, was thawed at room temperature for 1-2 min, and re-immersed in liquid nitrogen for a further 5-10 s. Once thawed the filter paper was placed, colony side up, in a Petri dish containing 2 ml LacZ assay reagent, ensuring there were no air bubbles and incubated at 37°C. The colonies were generally assayed for up to 3 h, and the colour change was monitored and graded after 30 min, 1 h and 2 h.

For the assay reagent (per 10 ml), 10 mg X-gal was completely dissolved in 100 μl N,N, dimethylformamide (DMF, Sigma) ensuring there were no aggregates left in the glass container. To this, 10 ml Z-buffer (Z-buffer: 60 mM Na₂HPO₄,2H₂O, 40 mM NaH₂PO₄, H₂O, 10 mM KCl, 1 mM MgSO₄,7H₂O) and 27 μl β-mercaptoethanol (Sigma) were added, the solution was thoroughly mixed and wrapped in foil to protect it from the light.

2.2.9 Isolation of plasmid DNA from yeast cells

In order to establish the identity of the library screen 'hit', the prey plasmid had to be isolated from the yeast colonies. Yeast colonies that gave a positive reaction in the LacZ assay were cultured and the prey was isolated so it could be analysed.

The positive colony was selected from the replica plate and inoculated in 2 ml YPD and grown overnight at 30°C with constant agitation (250 rpm). The next day, 1.5 ml of this culture was centrifuged at 13,000 rpm for 15 s to harvest the cells. The supernatant was decanted and the cells were resuspended in 250 μl of P1 resuspension buffer (containing RNase A). To isolate the plasmid DNA, 10 μl of lyticase (5 U/μl, Sigma) and 50-100 μl of glass beads (acid washed glass beads 425-600 microns, Sigma) were added to the cell suspension and incubated at 37°C for 30 min with vigorous shaking and periodic vortexing. The lyticase, glass beads and vortexing all contributed to breaking down the yeast cell wall, making access to the prey plasmid easier. The cell suspension was briefly centrifuged and decanted into a fresh microcentrifuge tube. 250 μl buffer P2 (lysis buffer) was added to the solution, gently mixed and incubated at room temperature for 5 min. Next, 350 μl buffer N3 (neutralisation buffer) was added, the solution was gently mixed and centrifuged for 10 min at 13,000 rpm. The supernatant was transferred to a QIAprep spin mini-prep column (Qiagen) and processed as
Chapter 2: Materials and Methods

described in method 2.1.11, however, the DNA was eluted in 20 μl EB buffer instead of 50 μl.

2.2.10 Nutritional growth assays

In addition to LacZ reporter gene assays, interactions between proteins were also tested using nutritional growth assays. The yeast strain AH109 was employed for these experiments as it is incapable of producing histidine and adenine, but carried two reporter genes HIS3 and ADE2 which, when induced were able to produce histidine and adenine respectively. Like the LacZ reporter gene, these genes were only transcribed if activated by the transcription factor which was reconstituted by two interacting proteins. Therefore, the transformed yeast were plated onto selective medium lacking histidine and adenine (as well as tryptophan and leucine to select for the bait and prey plasmids, respectively), which meant that the colonies only grew if there was an interaction between the two transformed proteins. Growth was monitored on a daily basis. This offered a more stringent selection mechanism for protein-protein interactions in yeast, as two reporter genes needed to be activated, instead of one as in Y190. However, nutritional assays are time consuming as it can take up 10 days for colonies to emerge.

2.3: Mammalian cell culture

2.3.1 Cell lines

A number of cell lines were used to investigate the properties and interactions of collybistin with other proteins.

<table>
<thead>
<tr>
<th>Cell Designation</th>
<th>Organism/Age/Organ</th>
<th>Morphology, Properties</th>
<th>ECACC #</th>
<th>ATCC #</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293</td>
<td>Homo sapiens, foetal, kidney</td>
<td>Adherent, epithelial, has transforming ability in nude mice,</td>
<td>85120602</td>
<td>CRL-1573</td>
</tr>
<tr>
<td>REF52</td>
<td>Rattus novegicus, foetal</td>
<td>Adherent, fibroblasts</td>
<td>Gift from P. Fort</td>
<td></td>
</tr>
<tr>
<td>NIH3T3</td>
<td>Mus musculus, embryo</td>
<td>Adherent, fibroblasts</td>
<td>93061524</td>
<td>CRL-1658</td>
</tr>
</tbody>
</table>

Table 2.6 : Mammalian cell lines - Properties of cell lines used throughout this project
2.3.2 Mammalian expression vectors

A number of mammalian expression vectors were used throughout this project and the cloning of individual constructs will be presented in the relevant results chapters. These expression vectors fuse a ‘tag’ onto the N- or C-terminus of the protein of interest, which allows detection when expressed in mammalian cells.

<table>
<thead>
<tr>
<th>Vector Name</th>
<th>Selection Antibiotic</th>
<th>Fusion protein for visualisation</th>
<th>Excitation/ Emission Spectra</th>
</tr>
</thead>
</table>
| pEGFP C1 (Clontech) | Kanamycin            | EGFP-enhanced green fluorescent protein is a red-shifted variant of wild-type GFP, which has been optimised for a brighter fluorescence. C1 and C2 fuse an EGFP tag to the N-terminus of the inserted protein, whilst N1 gives a C-terminal tag. | Excited at 488 nm  
Emits at 507 nm                                      |
| pEGFP C2 (Clontech) |                      |                                                                                                 |                                       |
| pEGFP N1 (Clontech) |                      |                                                                                                 |                                       |
| pDsRed N1 (Clontech) | Kanamycin            | A C-terminal DsRed tag. DsRed is a novel red fluorescent protein isolated from Discosoma sp., an indo-pacific sea anemone. | Excited at 558 nm  
Emits at 583 nm                                      |
| pRK5Myc         | Ampicillin           | A N-terminal Myc tag is fused to the inserted protein. The Myc tag has the sequence EQKLISEEDL and can be detected with the 9E10 antibody. | Excitation and emission depended on the secondary antibodies used, see Table 2.10 |
| pRK5Flag        | Ampicillin           | A N-terminal Flag tag is fused to the inserted protein. The Flag tag has the sequence DYKDDDDKGS and can be detected using an anti-Flag antibody. | Excitation and emission depended on the secondary antibodies used, see Table 2.10 |

Table 2.7: Mammalian expression vectors – the antibiotic selectivity and excitation and emission values of the fusion protein used for detection.
Chapter 2: Materials and Methods

2.3.3 Cell line growth and maintenance

The HEK293, NIH3T3 and REF52 cells were sustained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% (v/v) foetal calf serum (FCS, Invitrogen), 2 mM L-glutamine (Sigma), streptomycin (100 μg/ml) and penicillin (100 U/ml, Invitrogen). Cultures were grown in T70 tissue culture flasks (Greiner) pre-coated with poly-D-lysine (50 μg/ml, Sigma) and maintained at 37°C in a 5% CO₂/air atmosphere.

When cells reached 70% confluency, they were washed in Ca²⁺ and Mg²⁺ - free Hanks balanced salt solution (HBSS, Invitrogen), passaged in 2 ml trypsin/EDTA solution (0.5 g/l and 0.2 g/l respectively, Invitrogen) by mechanical agitation and harvested in DMEM containing 10% FCS. The cell suspension was centrifuged at 1000 g for 5 min to harvest the cells. The supernatant was discarded and the cells resuspended in medium (as above) and reseeded into flasks or onto glass coverslips precoated with poly-D-lysine (as above).

Excess cell pellets were frozen following the above protocol, but were resuspended in FCS supplemented with 10% DMSO. The cell suspension was placed in a 1 ml cryotube (Nalgene) and kept at -80°C for 24 h before being transferred to -150°C for storage.

2.3.4 Transfection methods

Electroporation

Electroporation is a method which uses high-voltage electric shocks to temporarily permeabilise the cell membrane, thus facilitating the entry of molecules such as DNA into cells. It can be used to transiently or stably transfect numerous cell lines. Cells were used for electroporation when the flasks were about 70% confluent, this provided enough cells for 3-4 transfections. The cells were washed briefly with 10 ml HBSS, and then passaged in 2 ml trypsin/EDTA solution (as before) by mechanical agitation and harvested in DMEM containing 10% FCS. The cell suspension was centrifuged at 1000 g for 2 min, the supernatant was removed and the cell pellet was resuspended in 25 ml of Optimem-1 with Glutamax-1 (Invitrogen), and centrifuged as before. Once again, the supernatant was removed, and this time the cell pellet was resuspended in Optimem-1 with Glutamax-1 (500 μl per transfection). 500 μl of cell suspension was added to an
Chapter 2: Materials and Methods

electroporation cuvette (BioRad 0.4 cm) together with the cDNA (total amount 2-4 μg), and gently mixed. The electroporator (BioRad) was set as shown in Table 2.8.

<table>
<thead>
<tr>
<th>Voltage</th>
<th>400 V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacitance (high)</td>
<td>125 μF</td>
</tr>
<tr>
<td>Resistance</td>
<td>Infinity</td>
</tr>
</tbody>
</table>

Table 2.8: Electroporator Settings

The cuvette was placed in the holder and the cells were pulsed twice, left in the cuvette for 10 min at room temperature and periodically shaken. The cells were then diluted in 5 ml of medium and 500 μl of this suspension was seeded onto poly-D-lysine-coated glass coverslips, 30 min later 2.5 ml of growth medium was added and the cells were incubated at 37°C in a 5% CO₂/air atmosphere.

Lipofection

Effectene (Qiagen) is a unique non-liposomal lipid formulation which is used to transfect DNA into cells with great efficiency. The DNA is condensed by interaction with the enhancer reagent in a defined buffer system. The effectene reagent is then added to produce a condensed DNA-effectene complex which can be directly added to the cells. Effectene was used to transfect REF52 and HEK293 cells that were seeded onto poly-D-lysine-coated glass coverslips and allowed to grow until 60% confluent. Each transfection complex was sufficient to transfect cells on two 22 mm² coverslips. The DNA (total amount 1 μg) was diluted in 40 μl of buffer and 8 μl of enhancer solution added. The mixture was briefly vortexed and incubated at room temperature for 5 min. 10 μl of effectene reagent was then added, vortexed for 10 s and following a 10 min incubation at room temperature, 1 ml of medium was added. The cells were washed in PBS (0.01 M phosphate buffer containing 2.7 mM KCl and 137 mM NaCl, pH 7.4) and incubated in fresh medium. 500 μl of the transfection complex was added drop wise to each coverslip. The cells were then incubated at 37°C in a 5% CO₂/air atmosphere overnight.
Chapter 2: Materials and Methods

Microinjection

Microinjection is a technique to physically introduce DNA, RNA or protein directly into a specific cell. The cell is manipulated by a blunt capillary so that gentle suction holds it in place. A micromanipulator – a very fine tipped pipette is then inserted into the cell and the DNA, RNA or protein can be injected directly into the nucleus or cytoplasm. The advantage of this technique is that a specific cell can be transfected and monitored. NIH3T3 cells were grown and maintained as previously described. Prior to microinjection, the cells were starved overnight by incubating them in DMEM containing 0.1% fatty-acid free BSA. The next day they were injected with plasmid constructs. Cells were fixed and probed with antibodies two hours after microinjection.

2.3.5 Immunolabelling for intracellular antigens

The cells were briefly washed with PBS at room temperature, fixed with 4% (w/v) paraformaldehyde/PBS (PFA was diluted in 1× PBS by heating to 65°C with continuous stirring, and adjusted to pH 7.4 using NaOH, Sigma) for 2-5 min at 4°C, quenched with 50 mM NH₄Cl (Sigma) for 10 min at room temperature and then permeabilised with 0.1% Triton-X-100 (Sigma) in FCS/BSA/PBS [10% (v/v) FCS/ 0.5% (w/v) BSA fraction V (Sigma) in PBS], for 10-15 min at room temperature. The cells were then washed in PBS followed by 2 washes in FCS/BSA/PBS. The cells were incubated in the primary antibody (diluted in FCS/BSA/PBS – see Table 2.9) for 1 h at room temperature. The cells were washed three times in FCS/BSA/PBS. This was followed by incubation in the secondary antibody (diluted in FCS/BSA/PBS – see Table 2.10) for 20-30 min at room temperature and the samples were covered in foil to protect them from the light. Following this, the cells were washed twice in FCS/BSA/PBS, then three times in PBS and mounted onto microscope slides with VECTASHIELD™ hard set mounting medium (Vector laboratories). This reagent prevents the rapid loss of fluorescence, retains anti-fading ability and minimises photo-bleaching, therefore, cells were able to withstand long exposures to the lasers of the confocal microscope.

2.3.6 Antibodies

A number of commercial primary and secondary antibodies were used throughout the project and are summarised in Tables 2.9 and 2.10.
Chapter 2: Materials and Methods

### Table 2.9: Primary antibodies – a list of the primary antibodies used throughout this project.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Type</th>
<th>Conc. used</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>9E10 Myc epitope</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>1:200</td>
<td>Sigma</td>
</tr>
<tr>
<td>FLAG epitope</td>
<td>Sheep</td>
<td>Polyclonal</td>
<td>1:200</td>
<td>Sigma</td>
</tr>
<tr>
<td>mAb7a</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>1:200</td>
<td>Gift from H. Betz</td>
</tr>
</tbody>
</table>

### Table 2.10: Secondary antibodies – a list of the secondary antibodies used throughout this project.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Type</th>
<th>Conc. used</th>
<th>Conjugate, Excitation and Emission Spectra</th>
<th>Manufacturer</th>
</tr>
</thead>
</table>
| Mouse IgG        | Goat     | Monoclonal | 1:200      | Alexa Fluor® 594  
Excited at 590 nm  
Emits at 617 nm   | Molecular Probes |
| Rabbit IgG       | Goat     | Monoclonal | 1:200      | Alexa Fluor® 488  
Excited at 495 nm  
Emits at 519 nm   | Molecular Probes |
| Sheep IgG        | Donkey   | Monoclonal | 1:200      | Cyanin 5 (Cy5)  
Excited at 650 nm  
Emits at 670 nm   | Jackson Immunoresearch |

2.3.7 Confocal microscopy

Cells were imaged using a Zeiss LSM510-META confocal microscope. Captured images were processed using LSM 5 image browser software and CorelDRAW graphics suite. The majority of the images presented in this thesis are of a single plane taken at the centre or surface of the cell. However some Z-stack images are also shown, and these were reconstituted using the LSM software.
Chapter 3: Collybistin and Gephyrin
Chapter 3: Collybistin and Gephyrin

3.1 Introduction

Gephyrin is the main constituent of the subsynaptic scaffold that provides support to glycine receptors at inhibitory synapses. The molecular mechanisms which underlie gephyrin activities are not fully understood, but a number of proteins appear to contribute to this subsynaptic support system. The membrane activation model was suggested by Kneussel and Betz (2000) to address the mechanisms involved in the clustering of glycine receptors at postsynaptic sites. One of the proteins they suggested was pivotal to this process was the gephyrin interactor collybistin.

3.1.1 Collybistin

Collybistin is a member of the diffuse B cell lymphoma-like (dbl-like) family of GDP-GTP exchange factors superfamily. These GEFs catalyse the GDP-GTP exchange reaction on small G proteins of the Rho-family (discussed in Chapter 4). The GTPases cycle between an inactive GDP-bound state and an active GTP-bound state and the GEFs act as molecular switches for the cycling. GEFs of this family are characterised by tandem DH-PH domains. The DH domain also referred to as the RhoGEF domain, is thought to catalyse the guanylate nucleotide exchange reaction, and the PH domain may be involved in anchoring the GEF to the membrane by binding to PI\(_{4,5}P_2\) or PI\(_{3,4,5}P_3\) (Kins et al., 2000).

3.1.1.1 Structure of collybistin

Collybistin was isolated as a gephyrin-interacting protein using the GAL4 yeast two-hybrid system. Initially two isoforms were identified - collybistin I contains an SH3 domain at the N-terminus, and a coiled coil C-terminus, whereas collybistin II lacks the SH3 domain and has an alternative, shorter C-terminus. Both splice variants contain the RhoGEF/PH moiety. The SH3 domain identified in collybistin I displays high homology to SH3 domains of cytoskeletal proteins (Kins et al., 2000). Co-expression of these collybistin variants with gephyrin in HEK293 cells gave two different phenotypes. Cells transfected with either collybistin I or collybistin II alone displayed cytoplasmic localisation. When collybistin I was co-transfected with gephyrin, it was redistributed to gephyrin-rich domains in the cytoplasm, which demonstrated the binding of the two proteins. In cells co-transfected with gephyrin and collybistin II, gephyrin was relocated to submembranous micro-clusters. HEK293 cells transfected with collybistin II,
Chapter 3: Collybistin and Gephyrin

gephyrin and glycine receptor α1 and β subunits displayed peripheral micro-aggregates of collybistin II and gephyrin, which also had immunoreactivity with antibodies specific for glycine receptors. This showed that collybistin was capable of forming gephyrin clusters at the cell surface which could recruit hetero-oligomeric glycine receptors (Kins et al., 2000). Subsequently, two additional isoforms of rat collybistin were identified, collybistin II containing a SH3 domain, and a rat isoform of hPEM-2, also containing a SH3 domain (Harvey et al., 2004b). These variants were created by the alternative splicing of the primary transcripts. The alternative C-termini were generated by the use of different cassette exons and were renamed CB1, CB2 or CB3. In addition, the variants are affixed with SH3+ or SH3- to denote the presence or absence of the SH3 domain, respectively (Harvey et al., 2004b). Fig. 3.1 shows schematic representations of these isoforms and Fig. 3.2 shows the amino acid alignment of the four rat collybistin variants and the human homologue hPEM-2.

Fig. 3.1: Collybistin variants - Schematic representations of the four rat collybistin variants and hPEM-2, the human homologue. The SH3 domain is represented in red, the RhoGEF in blue and the PH in green, in accordance with the amino acid alignments in Fig. 3.2. The different C termini are distinguished by colour, turquoise for CB1, purple for CB2 and pink for CB3 and hPEM-2.
Fig. 3.2: The amino acid alignment of four identified rat collybistin variants and the human homologue hPEM-2. The SH3 domain is red, the RhoGEF domain is blue and the PH domain is green. The different C-terminals are differentiated by colour, and any amino acid differences between the rat and human homologues are depicted in **bold**.
3.1.1.2 Expression and distribution of collybistin

Collybistin is predominantly expressed in the brain, although weak Northern hybridisation signals for CB1\textsubscript{SH3} and CB2\textsubscript{SH3} mRNA were detected in the heart and skeletal muscle (Kins \textit{et al.}, 2000). RT-PCR revealed that SH3 domain containing transcripts were found in greater abundance in rodent and human brains compared to transcripts lacking the SH3 domain coding exon. Both CB2 and CB3 C-termini were detected in rodents, whereas only CB3 was detected in humans (Harvey \textit{et al.}, 2004b). \textit{In situ} hybridisation studies revealed that collybistin was predominantly expressed during the second half of embryonic development. Moderate levels were detected at E7, and reached plateau by E15. Gephyrin expression was detected earlier, but this could be attributed to its role in metabolism. However, collybistin expression preceded that of the glycine receptor β subunit, which is in accordance with its role in the clustering of these receptors (Kneussel \textit{et al.}, 2001). Expression studies on adult tissue revealed that collybistin was expressed throughout the grey matter, but most prominently in the hippocampus and olfactory bulb. The distribution pattern of collybistin in the adult rat brain was similar to that of gephyrin (Kneussel \textit{et al.}, 2001).

3.1.1.3 GTPase Exchange Factors (GEFs)

The first mammalian GEF specific for the Rho family was identified from diffuse B cell lymphoma cells, and hence termed Dbl. Dbl shares a 240 amino acid sequence with the \textit{S. cerevisiae} protein Cdc24, which interacted with Cdc42 during budding and establishment of polarity in these cells. Dbl was found to be a GEF for human Cdc42, and so these two proteins became the founding members of the Dbl-like family of GEFs that catalyse Rho-family GTPases. Fig. 3.3 is a dendrogram representing the 69 members of the Dbl-like family identified in humans (reviewed in Rossman \textit{et al.}, 2005).
Fig. 3.3: A phylogenetic tree of the 69 human GEFs identified to date. The tree is based upon the alignment of the RhoGEF domains of each protein. hPEM-2, the human homologue of collybistin and neuronal GTPase exchange factor (NGEF), a collybistin interactor identified in the library screen presented in Chapter 5 are shown in red (adapted from Rossman et al., 2005).
Chapter 3: Collybistin and Gephyrin

RhoGEF domain

The RhoGEF domain, about 200 amino acids long, is the region required for GEF activity. The sequence homology between the RhoGEF domain exhibited by these GEFs is restricted to three highly conserved regions, and mutations within these regions compromise the GEF activity of the protein (Hoffman and Cerione, 2002). The RhoGEF domains display a varying amount of specificity for GTPases, some GEFs act on many GTPases, whilst others are specific for one only. The RhoGEF domain of collybistin shares significant homology with Vav1 and Tiam1 which are GEFs for Rac1 (Kins et al., 2000).

PH domain

The PH domain is located adjacent to the RhoGEF domain and is 100-120 amino acids long. Over 250 different proteins contain the PH domain, making it one of the most common domains found in human proteins (Lemmon et al., 2002). PH domains are generally associated with subcellular localisation of proteins through interactions with phosphoinositides. GEF PH domains may be involved in correct subcellular targeting, but have also been implicated in facilitating GEF activity and mediating interactions with other proteins (Rossmann et al., 2005).

SH3 domain

Kins et al. (2000) described two isoforms of collybistin - collybistin I contained a SH3 domain and a coiled coil region at the C-terminus, whereas collybistin II did not. This is the only structural difference noted between the two but it did lead to phenotypic differences when co-transfected into HEK293 cells with gephyrin (Kins et al., 2000). SH3 domains have been long identified as a ubiquitous protein-protein interaction domain. Studies indicate that the region bound by SH3 domains are proline rich and PXXP has been identified as the core binding domain (Mayer, 2001). In the case of collybistin, it was found that the isoform involved in submembranous gephyrin clustering in HEK293 cells did not contain the SH3 domain, therefore, the SH3 domain in collybistin appears to have a role in negative regulation (Mayer, 2001).

The importance of the SH3 domain in collybistin function was emphasised by the identification of a glycine to alanine mutation at position 55 (G55A) within the SH3 domain of hPEM-2 in a hyperekplexic patient (Harvey et al., 2004b).
Hyperekplexia or startle disease is a rare autosomal, dominant, motor disorder and its prevalence is not known with certainty. It is characterised by sudden muscle spasms in response to an unexpected tactile stimulus, which manifests as facial grimacing, clenched fists, hunched shoulders and exaggerated jerks of the limbs. Some patients also respond to unexpected noise or lights. They often become rigid, and as a result fall over. As their arms are rigidly held by their sides, they are unable to protect themselves and therefore the falls often result in major injuries. Umbilical and inguinal hernias are common in hyperekplexic patients. Infant and juvenile patients also exhibit sustained increased muscle tone (hypertonia) between attacks, but this appears to reduce with age. The symptoms of hyperekplexia resemble those observed with mild strychnine poison (Zhou et al., 2002). The majority of mutations isolated in hyperekplectic patients have been identified in the glycine receptor α1 subunit (Zhou et al., 2002). However they have not been restricted to glycine receptor subunits. In addition to the mutation identified in hPEM-2 (Harvey et al., 2004b), a mutation was also found in the gephyrin gene (Rees et al., 2003) which resulted in a N10Y amino acid substitution. In vitro studies showed that this mutant did not appear to disrupt glycine receptor-gephyrin interactions, or the collybistin-induced cell surface clustering of gephyrin. The functional effect of this gephyrin mutation remains elusive (Rees et al., 2003).

The G55A mutation identified in hPEM-2 may affect the correct folding of the protein, or interfere with its quaternary structure (Harvey et al., 2004b). Co-transfection of the collybistin mutant and gephyrin into HEK293 cells gave a similar phenotype as the transfection of collybistin without the SH3 domain and gephyrin. Introduction of this CB3_{SH3}G55A mutant into cultured cortical neurons resulted in the accumulation of endogenous gephyrin clusters in the proximal dendrites, and in a subset of cells CB3_{SH3}G55A and gephyrin formed large somatic and dendritic clusters that resulted in a complete loss of gephyrin clusters. This study highlighted the importance of the SH3 domain in collybistin function (Harvey et al., 2004b).

Collybistin is predicted to activate a GTPase, but which one is still unclear (Kins et al., 2000). The human homologue of collybistin, hPEM-2, is thought to activate the GTPase Cdc42 (Reid et al., 1999). Cdc42 activation is known to cause reorganisation of the actin cytoskeleton via Rac and other signalling kinases (see Chapter 4).
3.1.1.4 The membrane activation model

This model was proposed by Kneussel and Betz (2000) to address the molecular mechanisms underlying the clustering of glycine receptors at postsynaptic sites (see Fig. 1.3). Central to this model is gephyrin and its numerous interactors. They suggested that both Ca\(^{2+}\) influx and the local activation of PI3K, by an unknown presynaptic signal, could induce the accumulation and assembly of the gephyrin scaffold at postsynaptic sites. In this model, the accumulation of PI\(_{3,4,5}^3\), which results from the phosphorylation of PI\(_{4,5}^2\) by PI3K, recruits collybistin and profilin to the synaptic membrane, where they co-ordinate changes in the actin cytoskeleton. They proposed that collybistin activates Cdc42, which is known regulate the actin cytoskeleton (Hall, 1998).

3.1.2 Aims

The aims of the work presented in this chapter were to:

- Investigate how the SH3, RhoGEF and PH domains contribute to the overall function of collybistin.

- Establish an explanation for the functional differences seen between the two isoforms of collybistin as presented by Kins et al. (2000).
3.2 Methods

To study the interaction between gephyrin and collybistin, a number of GAL4 yeast two-hybrid and mammalian expression vector constructs were generated, as described below. These are summarised in Table 3.1 and 3.2, respectively. These constructs were also used in experiments that will be presented in subsequent chapters.

3.2.1 Yeast Expression Vectors

The yeast two-hybrid data presented in this chapter were generated as described in section 2.2.5. Briefly, constructs were transformed into Y190 cells using the LiOAc/TE method, plated onto agar plates lacking leucine and tryptophan and incubated at 30°C until colonies emerged. Colonies were tested for positive interactions using the freeze fracture LacZ assay (2.2.8). Experiments in AH109 cells were conducted as described in section 2.2.10, and colonies were plated onto selective dropout agar plates lacking leucine, tryptophan, histidine and adenine.

3.2.1.1 pYTH9-CB2SH3-

CB2SH3 was excised from pRK5Myc-CB2SH3 and cloned into the NcoI/EcoRI sites of pYTH9. The plasmid was sequenced using the forward GAL4BD and reverse pYTH9 primers. pYTH9-CB2SH3 was digested with XbaI and integrated into the trpI-901 locus of Y190. The integrated strain was grown and maintained on agar plates lacking tryptophan. The same procedure was employed to integrate pYTH9-CB2SH3 into AH109 yeast cells (see 2.2.6).

3.2.1.2 pYTH9-CB2SH3-ΔRhoGEF and pYTH9-CB2SH3-ΔPH

Two deletion mutant constructs of collybistin were generated using the Quikchange site-directed mutagenesis kit (Stratagene) in pYTH9-CB2SH3 (see 2.1.2). The first was a
deletion of the RhoGEF domain and was generated using the primer combination ΔRhoGEF1 and ΔRhoGEF2. The second was a PH domain deletion, generated using the primer combination ΔPH1 and ΔPH2. The plasmids were sequenced using the forward GAL4BD and reverse pYTH9 primers, and appropriate internal primers. The plasmids were subsequently digested with XbaI and integrated into the *trp1-190* locus of the yeast strain Y190 and AH109. The integrated strains were grown and maintained on agar plates lacking tryptophan.

3.2.1.3 pYTH16-RhoGEF and pYTH16-PH

The RhoGEF domain and the PH domain of collybistin were amplified from pRK5Myc-CB2SH3 using the primer combinations rRhoGEF1 and rRhoGEF2 and rPH1 and rPH2 respectively. Amplifications were performed for 21 cycles at 94°C for 1 min, 65°C for 1 min and 68°C for 2 min using *Pfu* Turbo proof-reading polymerase. The PCR products were cloned into the *SwaI* sites of pYTH16 and sequenced using the GAL4BD sequencing primer.

3.2.1.4 pGBKTV-hPEM-2

hPEM-2 was amplified from P0 human whole-brain cDNA using the primer combination hPEM-2-1 and hPEM-2-2. Amplifications were performed for 30 cycles at 94°C for 1 min, 65°C for 1 min and 68°C for 3 min using *Pfu* Turbo proof-reading DNA polymerase. The PCR product was cloned into the *SalI/EcoRI* sites of pGBKTV. The plasmid was sequenced using the forward GAL4BD and reverse pGBKTV primers.

<table>
<thead>
<tr>
<th>Bait</th>
<th>Prey</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYTH9-CB2SH3</td>
<td>pACT2-Gephyrin P1</td>
</tr>
<tr>
<td>pYTH9-CB2SH3-ΔRhoGEF domain</td>
<td>pACT2-CB2SH3+</td>
</tr>
<tr>
<td>pYTH9-CB2SH3-ΔPH domain</td>
<td></td>
</tr>
<tr>
<td>pYTH16-RhoGEF</td>
<td></td>
</tr>
<tr>
<td>pYTH16-PH</td>
<td></td>
</tr>
<tr>
<td>pGBKTV7-hPEM-2</td>
<td></td>
</tr>
</tbody>
</table>

*Table 3.1: A summary of the yeast two-hybrid vectors used in this chapter.*
3.2.2 Mammalian Expression Vectors

To study the functional consequences of interactions between collybistin and gephyrin, different mammalian expression constructs were made and transfected into HEK293 cells which were maintained as previously described. Various construct combinations were transfected into the cells using effectene (2.3.4). After 24 h cells were fixed, stained with the appropriate antibodies and imaged by confocal microscopy.

3.2.2.1 pRK5Myc-CB2sH3-

CB2sH3. cDNA was amplified from P0 rat brain first strand cDNA (Clontech) using the primer combination rCB1 and rCB4. Amplifications were performed for 30 cycles at 94°C for 1 min, 65°C for 1 min and 68°C for 3 min using Pfu Turbo proof-reading DNA polymerase. The PCR product was cloned into the BamHI/EcoRI sites of pRKSMyc so that the 9E10 epitope was fused to the N-terminus. The plasmid was sequenced using the pRK5 sequencing primer and appropriate internal primers.

Fig. 3.5: pRK5Myc-CB2sH3-. Schematic representation of the pRK5Myc-CB2sH3- construct which was used throughout this study. The Myc tag attached in frame is fused to the N-terminus of CB2sH3, which was cloned into the BamHI and EcoRI sites of the vector (indicated in red). All the pRK5Myc constructs were cloned in this way.

Constructs pRK5Myc-CB2sH3+, pRK5Myc-CB3sH3- and pRK5Myc-CB3sH3+ were generated using the same procedure as that described for pRK5Myc-CB2sH3-, however, plasmids were sequenced using a number of internal primers to ensure that the correct isoform was cloned into the vector.

3.2.2.2 pRK5Myc-CB2sH3-ΔRhoGEF and pRK5Myc-CB2sH3-ΔPH

Two deletion mutant constructs of collybistin were generated using the Quickchange site directed mutagenesis kit (Stratagene) in pRK5Myc-CB2sH3. (see 2.1.2). The first was a deletion of the RhoGEF domain and was generated using the primer combination ΔRhoGEF1 and ΔRhoGEF2. The second was a PH domain deletion, generated using
the primer combination ΔPH1 and ΔPH2. The plasmids were sequenced using the pRK5Myc sequencing primer and appropriate internal primers.

3.2.2.3 pEGFP-gephyrin

The entire coding region of rat gephyrin P1 isoform was amplified from P0 rat brain first-strand cDNA using the primer combination rGeph1 and rGeph2. Amplifications were performed for 30 cycles at 94°C for 1 min, 65°C for 1 min and 68°C for 3 min using *Pfu* Turbo proofreading DNA polymerase. The PCR product was digested with *BclII* and *XhoI* and cloned into the *BglII/SalI* sites of pEGFP C2, so that the EGFP tag was fused in-frame to the N-terminus. The plasmid was sequenced using the GFP seq1 sequencing primer and internal primers.

3.2.2.4 pDsRed-Glycine receptor β subunit

The large intracellular loop of the human glycine receptor β subunit was amplified from human whole-brain first-strand cDNA using the primer combination hβGlyR1 and hβGlyR2. Amplifications were performed for 30 cycles at 94°C for 1 min, 60°C for 1 min and 68°C for 3 min using *Pfu* Turbo proofreading DNA polymerase. The PCR product was cloned into the *EcoRI/BamHI* sites of pDsRed N1, so that the DsRed tag was fused in-frame to the C-terminus. The plasmid was sequenced using the DsRed sequencing primer.

<table>
<thead>
<tr>
<th>pRK5Myc-CB2SH3-</th>
<th>pRK5Myc-CB2SH3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRK5Myc-CB2SH3-ΔRhoGEF domain</td>
<td>pRK5Myc-CB2SH3-ΔPH domain</td>
</tr>
<tr>
<td>pEGFP-hPEM-2</td>
<td>pEGFP-Gephyrin</td>
</tr>
<tr>
<td>pDsRed-Glycine receptor β subunit intracellular loop</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2: A summary of the mammalian expression vectors used in this chapter.
3.2.3 The phosphoinositide specificity of the PH domain of collybistin

In collaboration with Dr. Venkateswarlu (University of Bristol), the phosphoinositide specificity of collybistin was investigated using a novel lipid-protein binding assay. PH domains are generally thought to be involved in the correct subcellular localisation of proteins via interactions with phosphoinositides. Dowler et al. (1999) developed an assay to assess the phospholipid binding properties of proteins. This was employed to establish the identity of the phosphoinositides which may interact with collybistin.

The phosphoinositides tested were PI3P, PI4P, PI5P, PI3,4P2, PI3,5P2, PI4,5P2 and PI3,4,5P3. These were dissolved in chloroform:methanol:water (1:2:0.8) at a range of concentrations between 1.8-100 pmol and 1 μl lipid solution was spotted onto Hybond-C extra membrane (Amersham Biosciences) and allowed to dry for 1 h at room temperature (Fig. 3.14A). The membrane was blocked in 3% (w/v) fatty acid-free BSA in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween-20) for 1 h and incubated overnight at 4°C in the same solution containing the GST-fusion proteins. The GST-fusion proteins used were centaurin-α1 (2 μg/ml), RhoGEF domain (2 μg/ml and 20 μg/ml) and RhoGEF-PH domain (2 μg/ml and 20 μg/ml). The membrane was washed six times over 30 min in TBST and incubated for 1 h in 1:1000 dilution of anti-GST monoclonal antibody (Sigma). The membrane was washed as before and incubated for 1 h with a 1:5000 dilution of HRP-conjugated secondary antibody (Pierce). The membrane was washed as before and the GST-fusion protein bound to the membrane by virtue of its interaction with the phospholipids was detected by enhanced chemiluminescence.
3.3 Results

3.3.1 Interactions between collybistin, hPEM-2 and gephyrin

3.3.1.1 Interactions in Yeast

The interactions between collybistin, gephyrin and hPEM-2 were initially investigated using the yeast two-hybrid system. Yeast expression constructs were generated and tested in both Y190 and AH109 yeast strains, using \textit{LacZ} gene activation and nutritional selection reporter assays. Fig. 3.6A-C and F-H show the negative controls conducted when investigating the interactions between pACT2-gephyrin and pYTH9-CB2\textsubscript{SH3}. or pGBK7-hPEM-2. In Y190 cells transformed with combinations of empty bait vector (pYTH16 and pGBK7) and pACT2-gephyrin (Fig. 3.6B&G), or empty prey vector (pACT2) and pYTH9-CB2\textsubscript{SH3}. or pGBK7-hPEM-2 (Fig. 3.6C&H) colonies remained white 3 h after the start of the \textit{LacZ} assay indicating that these constructs did not activate the \textit{LacZ} gene. Fig. 3.6D&E shows the \textit{LacZ} assays conducted on Y190 cells integrated with pYTH9-CB2\textsubscript{SH3}. and transformed with pACT2-gephyrin (D) or pACT2-CB2\textsubscript{SH3}+ (E). An intense blue colour was observed within 10 min of the start of the assay with gephyrin, and a blue colour was observed after 30 min with CB2\textsubscript{SH3}+. Fig. 3.6I shows the \textit{LacZ} assay observed upon co-transformation of pGBK7-hPEM-2 and pACT2-gephyrin into Y190 yeast cells. There were fewer colonies on the plate, although the majority showed an intense blue colour which was evident 15 min after the start of the assay, however some remained white. Fig. 3.6J shows the \textit{LacZ} assay observed upon co-transformation of pGBK7-hPEM-2 and pACT2-CB2\textsubscript{SH3}+ into Y190 yeast cells. An intense blue colour appeared 30 min after the start of the \textit{LacZ} assay. Gephyrin and collybistin interactions were monitored in AH109 cells in which positive interactions are detected by nutritional selection (section 2.2.10). Table 3.3, shows the rate of colony growth in AH109 cells transformed with gephyrin and various collybistin constructs.

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\textbf{Table 3.3: Nutritional growth assays in AH109}: Yeast cells were transformed as described and plated onto selective dropout medium lacking histidine, adenine, leucine and tryptophan. Growth was monitored on a daily basis.
Fig. 3.6: Filters of freeze fracture *LacZ* assays conducted on Y190 cells. A-C and F-H are negative controls for pYTH9-CB$_{2\_SH3}$ and pGBK7-hPEM-2, respectively. A and F are the empty bait and prey vectors, B and G are the empty bait vector against pACT2-gephyrin, C and H are of the bait vector against empty prey vector. These images show that these constructs did not autoactivate. CB2$_{2\_SH3}$ (D) and hPEM-2 (I) both show an intense colour when transformed with gephyrin, indicating that these proteins are interacting with each other. A positive interaction was also seen between CB2$_{2\_SH3}$ and CB2$_{2\_SH3}$ (E) and hPEM-2 and CB2$_{2\_SH3}$ (J) indicating that collybistin interacts with itself.
Chapter 3: Collybistin and Gephyrin

3.3.1.2 Interactions in HEK293 cells

HEK293 cells were co-transfected with pEGFP-gephyrin and either pRK5Myc-CB2SH3+ (Fig. 3.7A-C) or pRK5Myc-CB2SH3- (Fig. 3.7) as previously described. Cells successfully transfected with gephyrin fluoresced green and were therefore easily detectable by microscopy. Collybistin expression was visualised with the 9E10 Myc antibody, followed by an Alexa Fluor 594 conjugated secondary antibody. Cells were also transfected with pEGFP-hPEM-2 and a myc-tagged gephyrin which was detected using the 9E10 antibody as previously described (Fig. 3.7D-F).

Upon transfection into HEK293 cells, gephyrin forms very distinct cytoplasmic aggregates (see Fig. 1.2 on page 6 for example of gephyrin expression in a HEK293 cell). Recombinant collybistin has a diffuse distribution throughout the cytoplasm in HEK293 cells (see Fig. 3.9A for example).

However, when co-transformed with gephyrin, both the CB2 splice variants and hPEM-2 re-localised to the gephyrin aggregates, although there was still a slightly diffuse cytoplasmic distribution of collybistin. Fig. 3.7A shows the formation of intracellular gephyrin aggregates and Fig. 3.7B shows that CB2SH3+ had a slightly diffuse distribution throughout the cell, and also formed intracellular aggregates. Fig. 3.7C shows the co-localisation between the two proteins. A similar phenotype was observed in cells transfected with gephyrin (Fig. 3.7D) and hPEM-2 (Fig. 3.7E), in which hPEM-2 targeted to the intracellular aggregates formed by gephyrin (Fig. 3.7F).

HEK293 cells co-transfected with pEGFP-gephyrin and pRK5Myc-CB2SH3- displayed a different phenotype than those seen upon co-transfection with gephyrin and CB2SH3+ or hPEM-2 (Fig. 3.7). Fig. 3.8A-C, are confocal images of a single section taken through the centre of the cell. Gephyrin no longer formed distinct aggregates, instead a strong signal was observed at the cell periphery (Fig. 3.8A). CB2SH3- displayed a slightly diffuse distribution throughout the cell (Fig. 3.8B), however, there was some co-localisation observed between the two proteins (Fig. 3.8C, arrows). Fig. 3.8D-F are confocal images of a single section taken near the surface of the same cell. Here the submembranous microaggregates of gephyrin were clearly evident (Fig. 3.8D). CB2SH3- had a diffuse distribution here (Fig. 3.8E) but some co-localisation was evident (Fig. 3.8F, arrows).
Fig. 3.7: CB2_{SH3} - Single plane confocal images taken through the centre of HEK293 cells transfected with either pEGFP-gephyrin and pRK5Myc-CB2_{SH3} (A-C) or pCisMyc-gephyrin and pEGFP-hPEM-2 (D-F). The Myc tagged constructs were detected using monoclonal mouse 9E10 antibody (1:200) followed by Alexa Fluor 594 conjugated goat anti-mouse (1:200). A and D show that gephyrin forms characteristic cytoplasmic aggregates, to which CB2_{SH3} (B) and hPEM-2 (E) target. The merged images (C&F) show the co-localisation between the proteins (arrows). Scale bar: 10 μm
Chapter 3: Collybistin and Gephyrin

Fig. 3.8: CB2SH3 - Confocal images of a HEK293 cell transfected with pEGFP-gephyrin and pRK5Myc-CB2SH3, which was detected using mouse monoclonal 9E10 antibody (1:200) followed by Alexa Fluor 594 conjugated goat anti-mouse (1:200). A-C show a single plane imaged through the centre of the cell. Submembranous microaggregates of gephyrin can be seen at the periphery of the cell, some co-localised with collybistin (C, arrow). D-F show a single plane imaged near the surface of the same cell. There is a high concentration of gephyrin microaggregates throughout this region (F, arrow), some of which show co-localisation with collybistin. Scale bar: 10 μm
3.3.1.3 Interactions in cortical neurons

Fig. 3.9 B&C are of cortical neurons transfected with pRK5Myc-CB2SH3, and pRK5Myc-CB2SH3, respectively. These images show that the recombinant collybistin is diffusely distributed throughout the cell body and processes. These neurons were also probed for endogenous gephyrin using the mAb7A antibody, and puncta are observed along the periphery of the cell body and dendritic processes. There is also some co-localisation between the two proteins noted in the cell body. These images are courtesy of B. Lüscher, Penn State University.

**Fig. 3.9A:** A single slice confocal image of a HEK293 cell transfected with pRK5Myc-CB2SH3. Collybistin expression was detected with the 9E10 antibody (1:200) and Alexa Fluor 594 conjugated secondary antibody. When expressed alone, recombinant collybistin had a diffuse distribution throughout the cytoplasm. Scale bar: 10 µm

**Fig 3.9 B&C:** Confocal images of cortical neurons transfected with either pRK5Myc-CB2SH3 (B) or pRK5Myc-CB2SH3 (C). Collybistin expression was detected using an antimyc rabbit polyclonal (1:200) followed by an Alexa Fluor 488 conjugated secondary antibody (1:200). Endogenous gephyrin was detected using mAb7a (1:200) followed by Alexa Fluor 594 conjugated secondary antibody (1:200). Recombinant collybistin was diffusely distributed throughout the cell body and dendrites, whereas gephyrin puncta were observed at the periphery of the cell body and along the dendritic processes (arrows). Scale bar: 10 µm. Images B&C of cortical neurons courtesy of B. Lüscher, Penn State University.
3.3.2 Gephyrin and the RhoGEF domain

The interaction between gephyrin and the RhoGEF domain was initially investigated using the yeast two-hybrid system as previously described. Fig. 3.10B shows that the transformation of pACT2 into Y190 integrated with pYTH9-CB2SH3-ARhoGEF did not give a positive signal for LacZ induction, as the colonies remained white 3 h after the start of the LacZ assay. Transformation of pACT2-gephyrin also gave the same result—colonies remained white in the LacZ assay (Fig. 3.10C). Fig. 3.10D shows that the co-transformation of pYTH16-RhoGEF and pACT2 into Y190 cells gave a negative LacZ assay. However in Y190 cells co-transformed with pACT2-gephyrin and pYTH16-RhoGEF an intense blue colour was observed (Fig. 3.10E). The colour change was evident 30 min after the start of the assay. Nutritional selection studies gave similar results and are presented in Table 3.1 on page 57. The transformation of pACT2-gephyrin into pYTH9-CB2SH3-ARhoGEF does not facilitate the growth of colonies indicating that gephyrin interacts with the RhoGEF domain. Fig. 3.10F-H are single section confocal images taken through the centre of a HEK293 cell co-transfected with pEGFP-gephyrin and pRK5Myc-CB2SH3-ARhoGEF, protein expression was detected as previously described. Fig. 3.10F shows that gephyrin formed cytoplasmic aggregates—typical of gephyrin expression. Fig. 3.10G shows that CB2SH3-ARhoGEF was diffusely distributed throughout the cytoplasm of the cell. However, when the two images were merged, it was clearly evident that the two proteins did not co-localise (Fig. 3.10H).

3.3.3 Collybistin and gephyrin mutants A4 and A5

The collybistin binding site on gephyrin was mapped to a short peptide sequence of PFPLTSMDKA, which is found at the interface of the linker region and MoeA homology domain (Harvey et al., 2004b). Fig. 3.11 shows the transfection of two alanine block mutant constructs that span this region into HEK293 cells and their interaction with pRK5Myc-CB2SH3. Fig. 3.11A-C shows that the A4 mutant, which lacks PFPLT, retains an interaction with collybistin, but instead of forming submembranous microaggregates typically observed, it formed elongated cytoplasmic aggregates. Fig. 3.11D-F shows that the A5 mutant, which lacks the amino acids SMDKA, gave a slightly different phenotype to that of gephyrin A4. Gephyrin formed cytoplasmic aggregates but not submembranous microaggregates, and collybistin was diffusely distributed throughout the cytoplasm, only weakly targeting to the gephyrin aggregates.
Fig. 3.10: RhoGEF domain - Schematic representation of CB$_{2\text{SH3-ARhoGEF}}$ and pRK5Myc-CB$_{2\text{SH3-ARhoGEF}}$ vector (A). Negative LacZ assays of Y190 integrated with CB$_{2\text{SH3-ARhoGEF}}$ and transformed with either pACT2 (B) or pACT2-gephyrin (C) indicated that gephyrin interacts with the RhoGEF domain. This was confirmed by the robust LacZ assay observed in Y190 transformed with pYTH16-RhoGEF and pACT2-gephyrin (E). D shows that pACT2 and pYTH16-RhoGEF does not activate the LacZ gene. F-G are confocal images of a HEK293 cell transfected with pEGFP-gephyrin (F) and pRK5Myc-CB$_{2\text{SH3-ARhoGEF}}$ (G) detected with mouse anti-myc (1:200) followed by Alexa Fluor 594 conjugated secondary antibody (1:200). Gephyrin formed characteristic cytoplasmic aggregates (F, arrow), but the collybistin was diffusely distributed throughout the cytoplasm and did not target to the gephyrin (G). Deletion of the RhoGEF domain abolishes gephyrin-collybistin interactions and prevents the formation of submembranous microaggregates (H). Scale bar: 10 μm
Fig. 3.11: Gephyrin A4 and A5 mutants - Single plane confocal images of HEK293 cells transfected with pRK5Myc-CB2$_{SH3}$ and either pEGFP-gephyrin A4 (A-C) or pEGFP-gephyrin A5 (D-F). Collybistin expression was detected as described previously. The binding site of collybistin was mapped to the peptide sequence PFPLTSMDKA (Harvey et al., 2004), and mutant A4 is an alanine block mutation of the first five amino acids of this peptide and A5 is a mutation of the last five amino acids. Co-transfection of these mutants with CB2$_{SH3}$ blocked the formation of submembranous aggregates which are normally observed with gephyrin and CB2$_{SH3}$ expression. There is a difference in the distribution of collybistin within these cells. Gephyrin A4 (A) retains the ability to bind to collybistin (B) but formed elongated aggregates (C, arrows). Gephyrin A5 formed typical cytoplasmic gephyrin aggregates (D) but CB2$_{SH3}$ had a more diffuse distribution (E) and only appeared to weakly target to these gephyrin aggregates (F, arrow). Scale bar: 10 µm
3.3.4 Gephyrin and the PH domain

The interaction between gephyrin and the PH domain was initially investigated using the yeast two-hybrid system as previously described. Two different constructs of pYTH9-CB2_{SH3}-ΔPH were generated as described in 3.2.1.2. Fig. 3.12 shows these tested against pACT2 (D) and pACT2-gephyrin (E). However, they both gave robust LacZ assays against pACT2 with a blue colour appearing 10 min after the start of the assay. These constructs were also integrated into, and tested in AH109, which has a very tightly controlled ADE2 reporter gene, but colonies also grew on these plates, therefore, the pYTH9-CB2_{SH3}-ΔPH construct was not used for further investigations. Co-transformation of pYTH16-PH domain with either pACT2 (E) or pACT2-gephyrin (F) into Y190 cells gave negative LacZ assays. The colonies remained white 3 h after the start of the LacZ activation assay. There was no known interactor for the PH domain, therefore a positive control could not be conducted for this construct, however as it did not show signs of autoactivation it was used in further investigations.

Fig. 3.12F-H are confocal images of a HEK293 cell co-transfected with pEGFP-gephyrin and pRK5Myc-CB2_{SH3}-ΔPH, protein expression was detected as previously described. Fig. 3.12F shows that gephyrin formed cytoplasmic aggregates and smaller elongated microaggregates. Fig. 3.12G shows that CB2_{SH3}-ΔPH also formed cytoplasmic aggregates, and had a slightly diffuse distribution throughout the cytoplasm. Fig. 3.12H clearly shows co-localisation of the two proteins, but the submembranous microaggregates were not as prominent in comparison to those seen upon co-expression of gephyrin and CB2_{SH3}. Fig. 3.12I is a confocal image of a mouse cortical neuron transfected with pRK5Myc-CB2_{SH3}-ΔPH, protein expression was detected with 9E10 Myc antibody followed by Alexa Fluor 488 conjugated secondary antibody (Image courtesy of B. Lüscher). Distribution of endogenous gephyrin was visualised with mAb7a (a gift from H. Betz) followed by Cy3 conjugated secondary antibody. CB2_{SH3}-ΔPH expression was detected throughout the cell body and dendrites. Co-localisation can be seen with endogenous gephyrin within the cell body and proximal dendrites. The pattern of endogenous gephyrin staining differed to that observed in the untransfected cells within the image. The untransfected cells showed a punctate staining of gephyrin along the dendrites, whereas the transfected cells displayed heavy co-labelling in the cell body and proximal dendrites. Gephyrin immunoreaction was absent from the distal dendrites. 

67
Fig. 3.12: PH domain - Schematic representation of CB2\textsubscript{SH3}\textunderscore APH and pRK5Myc-CB2\textsubscript{SH3}\textunderscore APH vector (A). Y190 integrated with pYTH9-CB2\textsubscript{SH3}\textunderscore APH was transformed with either pACT2 (B) or pACT2-gephyrin (C). Both gave robust LacZ assays indicating that this construct autoactivates. Y190 cells were co-transformed with pYTH16-PH and either pACT2 (D) or pACT2-gephyrin (E). These gave negative LacZ assays indicating that this construct did not autoactivate and gephyrin does not bind to the PH domain. Confocal images of a HEK293 cell showing the interaction between pEGFP-gephyrin (F) and pRK5Myc-CB2\textsubscript{SH3}\textunderscore APH (G), which was detected using mouse anti-myc (1:200) followed by Alexa Fluor 594 conjugated secondary antibody (1:200). Collybistin and gephyrin exhibit intracellular co-localisation, and some cytoplasmic (arrows) but no submembranous microaggregates (H). A confocal image of a cultured cortical neuron transfected with pRK5Myc-CB2\textsubscript{SH3}\textunderscore APH, and probed with mAb7a to identify endogenous gephyrin (I). Gephyrin shows accumulation in cell body and proximal dendrites (arrow heads). Scale bar: 10 μm. Cortical neuron image courtesy of B. Lüscher, Penn State University.
Chapter 3: Collybistin and Gephyrin

Fig. 3.13 shows the results of a lipid based immunoassay used to identify the phosphoinositide specificity of the collybistin PH domain. Centaurin-α1, known to bind to PI3,4P2 and PI3,4,5P3 (Venkateswarlu et al., 1999) was used as a positive control to show concentration dependent binding to PI3,4P2 and PI3,4,5P3 (Fig. 3.13B). Fig. 3.13 C&D show that the RhoGEF domain of collybistin did not bind to any of the phosphoinositides tested. However, the RhoGEF-PH fusion protein gave a signal for PI3P. Fig. 3.13E shows that a signal was detected at higher phospholipid concentrations when 2 μg/ml protein used. Fig. 3.13F shows that a more robust signal was detected at most PI3P concentrations when 20 μg/ml RhoGEF-PH fusion protein was used.

3.3.5 Glycine receptor β subunit, gephyrin and CB2SH3.

Fig. 3.14 shows the interaction between the intracellular loop of the glycine receptor β subunit, gephyrin and collybistin. The glycine receptor β subunit is diffusely distributed when expressed alone in HEK293 cells (A), but upon co-expression with gephyrin it redistributes to the characteristic gephyrin cytoplasmic aggregates (B; Kirsch et al., 1995). Fig. 3.14C-F are Z-stack confocal images of a HEK293 cell transfected with DsRed glycine receptor β subunit (C), pEGFP-gephyrin (D) and pRK5Myc-CB2SH3- (E), which was visualised with 9E10 antibody followed by Cy5 conjugated secondary antibody. The glycine receptor β subunit and gephyrin were distributed at submembranous sites. Collybistin had a diffuse distribution throughout the cytoplasm. Areas in which the three proteins are co-localised appear white in the merged image (Fig. 3.14F).
### Chapter 3: Collybistin and Gephyrin

#### Fig. 3.13: Analysis of phosphoinositide binding of collybistin using *in vitro* lipid-protein binding assay.

A nitrocellulose sheet blotted with various amounts of phosphoinositides (A) was incubated with the purified GST-tagged proteins - centaurin-α 12 μg/ml (B), RhoGEF domain 2 μg/ml (C), RhoGEF domain 20 μg/ml (D), RhoGEF and PH domain 2 μg/ml (E), and RhoGEF and PH domain 20 μg/ml (F). After washing, the protein bound to lipids was identified using an anti-GST antibody. Images courtesy of Dr. Venkateswarlu, University of Bristol.

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A nitrocellulose sheet blotted with various amounts of phosphoinositides (A) was incubated with the purified GST-tagged proteins - centaurin-α 12 μg/ml (B), RhoGEF domain 2 μg/ml (C), RhoGEF domain 20 μg/ml (D), RhoGEF and PH domain 2 μg/ml (E), and RhoGEF and PH domain 20 μg/ml (F). After washing, the protein bound to lipids was identified using an anti-GST antibody. Images courtesy of Dr. Venkateswarlu, University of Bristol.
Fig. 3.14: Glycine receptor β subunit, gephyrin and CB2_{SH3}. The glycine receptor β subunit intracellular loop was diffusely distributed when transfected into a HEK293 cell (A). Upon co-transfection with gephyrin, it relocated to the intracellular aggregates typical of gephyrin expression (B). C-F are Z stacked confocal images of a HEK293 cell transfected with DsRed-glycine receptor β subunit intracellular loop (C), pEGFP-gephyrin (D) and pRK5Myc-CB2_{SH3} (E), which was visualised with 9E10 antibody, followed by Cy5 conjugated secondary antibody. The glycine receptor β subunit and gephyrin relocated to submembranous microaggregates whereas collybistin was diffusely distributed throughout the cytoplasm, however there are some areas where the three proteins co-localised, which appear white on the merged image (F). Scale bar: 10 μm
3.4 Discussion

From the studies presented in this chapter, it is evident that the interaction between gephyrin and collybistin is robust and vital for the trafficking and clustering of gephyrin at submembrane sites.

Kins et al. (2000) identified two collybistin variants. Collybistin I which contained an SH3 domain and C-terminus with a coiled coil domain, and collybistin II which had a shorter C-terminus and did not contain an SH3 domain. In addition to these, Harvey et al. (2004) presented two more isoforms of rat collybistin, a collybistin II variant containing an SH3 domain, and an isoform which contained an SH3 domain and a C-terminus with a strong similarity to that of the human homologue hPEM-2. They also showed that the most abundant form of collybistin in vivo was that harbouring the SH3 domain. The rat isoforms of collybistin were renamed CB1, CB2 or CB3 depending on the C-terminus and affixed with SH3+ or SH3- to show the presence or absence of the SH3 domain (see Fig. 3.1 on page 46).

3.4.1 Collybistin, hPEM-2 and gephyrin interactions

The interaction between collybistin and gephyrin was initially studied using the yeast two-hybrid system, and both LacZ induction and nutritional selection assays show that there is a robust interaction between the two proteins (Fig. 3.6 and Table 3.1). LacZ assays gave a very intense blue colour and under nutritional selection colonies began to emerge after three days. A similar result was observed between gephyrin and hPEM-2, which contains the SH3 domain and has a different C-terminus to collybistin. The LacZ assay gave an intense blue colour, which was indicative of a robust interaction between the two proteins. However, some of the colonies remained white, a phenomenon that has been seen previously upon expression of gephyrin and collybistin (R. Harvey, personal communication). This may be due to toxicity caused by either protein to the yeast cells resulting in the in vivo deletion of the GAL4 fusion protein but retention of the selectable nutritional marker.

Co-expression of CB2SH3- and CB2SH3+ in Y190, gave a positive LacZ assay reaction, indicating that collybistin interacts with itself (Fig. 3.6E). A similar interaction was seen between hPEM-2 and CB2SH3+ (Fig. 3.6J). The functional consequence of this interaction is not known, however some GEFs, for example Lsc/p115RhoGEF, have
been found to function as dimers, and this may be the case for collybistin (Eisenhaure et al., 2003).

It was evident that collybistin and gephyrin displayed a strong interaction in yeast therefore HEK293 cells were used to study the effects of collybistin-gephyrin interactions in mammalian cells. Transfection of gephyrin into these cells results in the formation of intracellular aggregates. In this study, gephyrin was co-transfected into HEK293 cells with either CB2\textsubscript{SH3\textsuperscript{+}} or CB2\textsubscript{SH3\textsuperscript{-}} and phenotypes similar to those presented by Kins et al. (2000) were observed. Gephyrin formed cytoplasmic aggregates to which both CB2\textsubscript{SH3\textsuperscript{+}} and hPEM-2 bound. Upon co-transfection of gephyrin and CB2\textsubscript{SH3\textsuperscript{-}}, gephyrin relocated to submembranous microaggregates. Fig. 3.14 shows that the glycine receptor β subunit intracellular loop (which contains the gephyrin binding site) has a diffuse distribution when transfected into HEK293 cells, however it targets to gephyrin aggregates when co-expressed with it. These aggregates are translocated to submembranous sites in the presence of CB2\textsubscript{SH3\textsuperscript{-}}, which shows that gephyrin alone does not have the ability to move glycine receptors, but relies upon its interaction with collybistin to achieve the trafficking of glycine receptors in this cell system. These results show that collybistin has the ability to translocate gephyrin from intracellular sites to submembranous positions, a process which appears to be negatively regulated by the SH3 domain.

3.4.2 The SH3 domain

A few GEFs have been shown to be negatively regulated by their own C- or N-termini. For example, Lsc/p115RhoGEF is a Rho-specific GEF essential for the normal function of T and B cell lymphocytes. This GEF homo-oligomerises through its C-terminus, and the deletion of this region results in a gain of function, indicating that the C-terminus may be involved in the negative regulation of this GEF, similar to that seen with collybistin and its SH3 domain (Eisenhaure et al., 2003), although the SH3 domain of collybistin is at the N-terminus. It has also been shown that the N-terminal truncation of some GEFs results in a constitutively active form, which is similar to the phenotype observed with collybistin. The removal of the N-terminal SH3 domain from collybistin results in a functionally active collybistin in HEK293, although the most abundantly found isoform of collybistin in both rodents and humans \textit{in vivo} is one which harbours the SH3 domain (Harvey et al., 2004b). The best studied GEF which is constitutively active when truncated at the N-terminus is Vav1. Removal of the first 66 amino acids
Chapter 3: Collybistin and Gephyrin

results in a constitutively active form (Katzav et al., 1991; Aghazadeh et al., 2000). It is thought that auto-inhibition is structural, and upon GEF activation, certain residues at the N-terminal are phosphorylated, thus destabilising the structure and removing inhibition (reviewed in Schmidt and Hall, 2002).

The importance of the collybistin SH3 domain was highlighted by the discovery of a mutation within this domain in a patient with hyperekplexia (Harvey et al., 2004b). The patient was diagnosed with hyperekplexia after birth, and suffered from long lasting seizures accompanied by a decline in psychomotor development. The seizures were epileptic and hyperekplectic in origin, and drug therapy (Phenobarbital and lamotrigine followed by clonazepam) did not alleviate the symptoms for very long. Eventually the patient developed a progressive epileptic encephalopathy and by the age of four years suffered from almost daily, prolonged seizures, which led to death at the age of four years and four months. The clinical symptoms presented in this patient appeared to be consistent with the mislocalisation of inhibitory amino acid neurotransmitter receptors. Transfection of the G55A mutant into cortical neurones resulted in the disruption and subsequent mislocalisation of GABA_A and glycine receptors (Harvey et al., 2004b).

The SH3 domain is important for correct collybistin function although the precise nature of its action remains unknown. SH3 domains have long been identified as a ubiquitous protein-protein interaction domain, found in hundreds of proteins from all species. These domains bind to proline rich ligands, PXXP has been identified as the core binding domain, with moderate specificity and affinity, and play a critical role in a wide range of biological processes (Mayer, 2001). These include the regulation of enzymes via intra-molecular interactions, increasing the local concentration or altering the subcellular localisation of components of signalling pathways and mediating the assembly of large multi-protein complexes.

SH3 domains have been implicated in the regulation of actin dynamics by members of the Wiskott-Aldrich syndrome protein (WASP) family proteins (see Chapter 4). WASP was originally identified as the gene mutated in Wiskott-Aldrich syndrome, and, along with its relatives, plays a pivotal role in cytoskeletal organisation. These proteins contain several modular domains, including a Cdc42 binding domain and a proline rich domain. This particular region can interact with other cytoskeletal proteins which contain SH3 domains to alter the actin cytoskeleton (Abdul-Manan et al., 1999). There
are also many other cytoskeletal proteins which have proline rich regions that may interact with SH3 domains, and Kins et al. (2000) stated that the SH3 domain shared more homology with SH3 domains from cytoskeletal proteins such as myosin and spectrin than those of GEFs. There may be other proteins that interact with collybistin that somehow induce a conformational change in the SH3 domain, thus allowing collybistin to function, or the SH3 domain may mediate interactions with other proteins that enhance collybistin function. It is evident that collybistin interacts with itself, and this may be an alternative explanation for how this protein functions, but the molecular mechanisms underlying the role of the SH3 domain remains unclear.

3.4.3 The RhoGEF domain

The negative LacZ assay reaction observed when gephyrin was expressed in Y190 integrated with pYTH9-CB2SH3ΔRhoGEF suggested that gephyrin binds to the RhoGEF domain. This was confirmed by the positive LacZ assay observed by the interaction between gephyrin and the isolated RhoGEF domain (Fig. 3.10C&E). A similar phenotype was observed under nutritional selection in AH109 cells. Co-transfection of pEGFP-gephyrin and pRK5Myc-CB2SH3ΔRhoGEF into HEK293 cells also supported the data generated using the yeast two-hybrid system. The interaction between CB2SH3- and gephyrin in HEK293 cells generally resulted in the formation of submembranous microaggregates, however this was abolished by the deletion of the RhoGEF domain. Gephyrin still formed characteristic cytoplasmic aggregates, but collybistin no longer targeted to them and had a diffuse distribution throughout the cytoplasm (Fig. 3.10H). Taken together, these data strongly suggest that the binding site for gephyrin on collybistin is found within the RhoGEF domain.

To identify the binding sites for collybistin on gephyrin, the gephyrin mutants A4 and A5 were used (Harvey et al., 2004b) These mutants, which are presented in Fig. 3.11, showed that disrupting the collybistin binding site resulted in a loss of submembranous microaggregates in HEK293 cells. The expression of these mutants in mouse cortical neurons had a detrimental effect on the distribution of endogenous gephyrin, and there was a significant loss of synaptic gephyrin clustering. This highlights the importance of collybistin in the trafficking of gephyrin to synaptic sites (Harvey et al., 2004b).
The finding that gephyrin binds to the RhoGEF domain is surprising because typically the RhoGEF domain of GEFs are involved in catalysing GTPase exchange activity. However, some GEFs also act as scaffolding proteins by bringing together different proteins to form multi-protein complexes. For example, Tiam1 participates in a complex which includes JIP2 and spinophilin to stimulate Rac1 activity in the MAPK signalling pathway, culminating in the activation of p38 MAPK and p70 S6 kinase (Buchsbaum et al., 2002, 2003). Likewise, collybistin could act as a scaffold protein, bringing gephyrin together with other proteins, in addition to having GEF activity. However, it is clear that the RhoGEF domain of collybistin serves at least two roles – mediating interactions with gephyrin and RhoGEF activity.

3.4.4 The PH domain

Possible interactions between the PH domain and gephyrin could not be investigated using the yeast two-hybrid system as deletion mutant CB2SH3-ΔPH activated reporter genes in the absence of gephyrin, and as there was no known positive control for the PH domain, it was not certain whether the pYTH16-PH domain construct was fully functional as this failed to interact with gephyrin. However, experiments in HEK293 cells yielded more interesting data. Co-transfection of CB2SH3-ΔPH and gephyrin resulted in the formation of large cytoplasmic aggregates, typical of gephyrin expression, to which CB2SH3-ΔPH targeted. However, the phenotype which is associated with CB2SH3-ΔPH and gephyrin is that of submembranous microaggregates. This implies that the PH domain is somehow involved in the trafficking of gephyrin from intracellular aggregates to the plasma membrane. Therefore the first conclusion is that CB2SH3-ΔPH retains the ability to interact with gephyrin (Fig. 3.12H). Transfection of either CB2SH3+ or CB2SH3- into cultured cortical neurones resulted in a diffuse distribution of collybistin throughout the neuron, and endogenous gephyrin puncta were detected at the periphery of the cell body and along the dendritic processes (Fig. 3.9B&C). However, in cultured cortical neurons transfected with CB2SH3-ΔPH, endogenous gephyrin no longer appeared as puncta along the dendritic processes but was bound to CB2SH3-ΔPH and appeared trapped within the cell soma and proximal dendrites (Fig. 3.12I). This trafficking mutant appeared to compete with endogenous collybistin and did not allow the translocation of gephyrin away from the cell body to distal dendrites (Harvey et al., 2004b). This implies that the PH domain may be involved in the targeting of gephyrin and collybistin to synaptic sites.
Chapter 3: Collybistin and Gephyrin

The phospholipid binding assay (Fig. 3.13) showed that the PH domain of collybistin binds to the phosphoinositide PI3P, and not to PI4,5P2 or PI3,4,5P3 as speculated in the membrane activation model (Kneussel and Betz, 2000). PI3P localisation is restricted to early endosomes and multivesicular bodies (MVB), and is involved in constitutive intracellular membrane trafficking (Gillooly et al., 2003). This is the mechanism by which membrane lipids and proteins are moved from the ER where they are synthesised, through the Golgi apparatus to their final site of function (reviewed in Roth, 2004). PI3P generally binds to FYVE or Phox homology (PX; Ellson et al., 2002) domains of proteins involved in Golgi-endosome transport, therefore its interaction with collybistin is quite novel, suggestive of a role for collybistin in gephyrin trafficking from the trans-Golgi network (Stenmark, 2000).

The precise functions of PH domains in GEFs are unknown. Historically, they are thought to mediate the correct subcellular localisation and membrane targeting of proteins through interactions with phosphoinositides, specifically PI4,5P2 and PI3,4,5P3 (Lemmon and Ferguson, 2000). Studies have shown that some PH domains associate with membranes through direct and specific interactions with phosphoinositides, however, this only applies to 10% of PH domains, the other 90% only bind to phosphoinositides weakly or with little specificity. PH domains with high affinity and specificity have been extensively studied and show that phosphoinositide binding by the PH domain is necessary to drive membrane targeting of that protein (Lemmon et al., 2002). However, there is very little evidence to suggest that this is the main role of the PH domains found within GEFs. Functions attributed to this domain include, mediating interactions with other proteins, facilitating GEF activity and membrane anchoring.

A large number of cytoskeletal proteins and various proteins associated with the control of the cytoskeleton contain PH domains. These domains can fall into any of the classes described above, and so the regulation of membrane association of each of these proteins is fundamentally different. Simple alterations in membrane lipid composition may affect membrane association of proteins with PH domains that have high specificity and affinity for phosphoinositides. Proteins that display low affinity and no specificity may require modulation of protein-protein interactions to affect membrane association. Proteins with PH domains that display some specificity but have low affinity may require changes in phosphoinositides and protein-protein interactions to affect membrane association (Lemmon et al., 2002). However, there is very little evidence to
suggest that a major role of the PH domain in GEFs is in membrane targeting via interactions with phosphoinositides. The PH domains of GEFs interact with phospholipids with very low affinity and low specificity. A number of GEFs do not appear to rely upon PH-phosphoinositide interactions for correct subcellular localization instead other domains and motifs control their cellular distribution. In the case of collybistin, the PH domain was found to bind to PI3P, thus suggesting this interaction mediates trafficking instead of membrane targeting (Lemmon et al., 2002).

Another possible role for the PH domains of GEFs is facilitating RhoGEF activity. Several studies have shown that the RhoGEF-PH fragments have greater exchange activity than the RhoGEF domain alone, suggesting that the two co-operate with each other to facilitate exchange activity (Liu et al., 1998; Rossman and Campbell, 2000). It is difficult to deduce how this occurs as the relative orientation between the RhoGEF and PH domains differs in each GEF. The PH domain of some GEFs participates in the GTPase-GEF interface, whereas others do not. The PH domain of the GEF Dbs interacts with bound Cdc42, and this is required for the efficient activation of Cdc42 and its associated cellular responses (Rossman et al., 2002, 2005).

The PH domain may also contribute to GEF activity by mediating interactions with other proteins and phospholipids. It is thought that ligand binding to the PH domain regulates the activity of the RhoGEF domain (Lemmon et al., 2002). It has been proposed that the PH domain of Vav1 binds to the PI3K product PI3,4,5P3, which enhances the ability of the RhoGEF domain to activate Rac/Rho GTPases. It was also noted that high concentrations of PI3,4,5P3 enhanced Vav1 activity in vitro, and that PI4,5P2 inhibits exchange activity. This suggests that the PH domain may exert a negative influence on Vav1 activity and that PI3,4,5P3 can relieve it (Han et al., 1998). Deletion of the PH domain from Vav1 resulted in a mutant with constitutive activity in vivo. The PH domain of Sos, another member of dbl-like family, was also reported to have an inhibitory effect upon its RhoGEF domain in vivo which was relieved upon deletion of the PH domain (Nimual et al., 1998). Based on this, it has been suggested that the PH domain blocks access for the Rho/Rac GTPase on the RhoGEF domain (Soisson et al., 1998; Worthylake et al., 2000).

The PH domain of Trio directly interacts with filamin and Tara, two proteins that are involved in cross linking filamentous actin (Bellanger et al., 2000; Seipel et al., 2001).
The PH domain of dbl interacts with ezrin, which is involved in joining the plasma membrane to the actin cytoskeleton (Vanni et al., 2004). It is becoming evident that, in addition to binding to phospholipids, PH domains also interact with other proteins, and may contribute to linking activated GTPases with various signalling cascades and specific cellular sites (Rossman et al., 2005). PH domains also show PH-PH interactions, and perhaps these could be responsible for the dimerisation of collybistin (Rossman et al., 2005).

Although, the PH domain is a pre-requisite for a protein in the dbl-like family of GEFs, its actual function remains elusive. It may be involved in facilitating GEF activity, regulating membrane targeting or mediating protein-protein interactions. In the case of collybistin, its absence results in a trafficking mutant which disrupts the translocation of gephyrin in both HEK293 cells and cultured cortical neurons.

3.4.5 Revision of the membrane activation model

Kneussel and Betz (2000) suggested that collybistin activates Cdc42 at the synapse causing local actin rearrangements which lead to the formation of the gephyrin scaffold, which in turn facilitated glycine receptor clustering. They identified two isoforms of collybistin, one with and one without the SH3 domain - a region which is imperative for the correct functioning of collybistin. In this chapter, it was shown that gephyrin binds to the RhoGEF domain of collybistin and that the disruption of collybistin-gephyrin interactions results in mislocalised gephyrin clusters in cultured neurons. Kins et al. (2000) suggested that the PH domain was required for the correct subcellular localisation of collybistin by binding to PI4,5P2 or PI3,4,5P3. Here, it was shown that the PH domain binds to PI3P and that its disruption results in a trafficking mutant that does not allow the correct translocation of gephyrin in HEK293 cells or cultured neurons.

The aim of the work presented in this chapter was to examine the interactions between collybistin and gephyrin and to relate these to the suggestions presented in the membrane activation model. The roles that the SH3, RhoGEF and PH domains contribute to the overall function of collybistin were investigated to establish the role played by it in the clustering of gephyrin. The schematic overleaf summarises the findings of this chapter.
3.5 Conclusions

- Most abundant isoform found in rats contain the SH3 domain
- Only isoform found in humans contain the SH3 domain
- Negatively regulates ability of collybistin to translocate gephyrin to submembranous sites
- Important for function in vivo
- May mediate protein-protein interactions

- Contains the gephyrin binding site
- Disruptions between collybistin and gephyrin association causes mislocalisation of gephyrin in HEK293 cells and cultured neurons
- May mediate GEF activity

- Deletion of PH domain results in a trafficking mutant
- Interacts with PI3P, which is involved in constitutive membrane trafficking
- May be involved in subcellular localisation
- May mediate protein-protein interactions
- May facilitate GEF activity
Chapter 4: Collybistin and Rho-family GTPases
4.1 Introduction

Small GTPases are guanosine nucleotide binding proteins which cycle between an inactive GDP-bound state and an active GTP-bound state. In the active state they are capable of recognising effector proteins, and by interacting with them, they coordinate a wide variety of cellular processes. The intrinsic or catalysed GTPase activity of these proteins ensures that they are only switched on for a fixed length of time before returning to the dormant, GDP-bound state.

The small GTPases are classified into six main families based upon structural and functional criteria. The founding group is the Ras-family, members of which generally regulate normal cell growth and differentiation, and are widely implicated in the development of cancer. The Rho-family act as molecular switches to control a wide range of essential biochemical pathways, especially those involving the cytoskeleton. The Rab-family are involved in the regulation of membrane traffic. The Arf/Sar-family are involved in the regulation of vesicle docking and fusion. The Ran-family are involved in most nuclear processes such as DNA replication, cell-cycle progression and RNA transcription (reviewed in Wennerberg and Der, 2004; Sorokina and Chernoff, 2005).

4.1.1 Rho-family GTPases

Collybistin has been proposed to be a GEF for the Rho-family of GTPases (Reid et al., 1999; Kins et al., 2000). These control a diverse range of cellular functions and are distinguished from other small GTPase families by the insertion of a well defined Rho-insert sequence within their GTPase domain (Valencia et al., 1991). To date, 23 members have been identified. They are implicated in a variety of functions, however, their one common role is in the establishment of polarity and polarised structures through dynamic regulation of the actin cytoskeleton. This trend is carried through all three eukaryote kingdoms from budding in *S. cerevisiae*, to pollen tube elongation in *Arabidopsis*, to the formation of complex structures like cochlear cilia in mammals (Wherlock and Mellor, 2002). In addition, Rho-family GTPases are also involved in the regulation of gene transcription, cell cycle progression, vesicular trafficking and receptor endocytosis and sorting (Bishop and Hall, 2000). The most extensively studied members of this family are RhoA, Rac1 and Cdc42.
Chapter 4: Collybistin and Rho-family GTPases

The GTPase activity of Rho-family G-proteins is carefully regulated by a number of distinct control mechanisms. They cycle between activated and inactivated forms (see Fig. 4.1). When inactive, they are GDP-bound and sequestered by guanine nucleotide dissociation inhibitors (GDIs) into pools of cytosolic proteins. The presence of a stimulus causes the GDI to dissociate from the Rho protein making way for the cognate GEF to catalyse the GDP to GTP exchange. To date, four GDIs have been identified, but the precise mechanism by which they function in vivo remains unclear. There is some in vitro data suggesting that they interact with moesin (an ezrin, radaxin, moesin (ERM) protein which binds to membrane spanning proteins). The interaction between RhoGDI and moesin may be involved in releasing the Rho protein from the GDI and stimulating GEF activity (Takahashi et al., 1997).

Once the GTPase is free of GDI, GDP-GTP exchange can be catalysed by GTPase exchange factors (GEFs – see Chapter 3). The activated GTPase subsequently binds to a range of effector proteins eliciting various biological responses. Active GTPases are switched off by GTPase activating proteins (GAPs), which enhance the intrinsic GTP hydrolysis activity of Rho proteins and convert them to the GDP-bound state for sequestration until they are required once more. Over 70 mammalian GAPs for Rho-family GTPases have been identified based upon a 150 amino acid conserved Rho-GAP domain. It has been shown that GAPs, which hydrolyse a range of GTPases in vitro, appear to be more selective in vivo, suggesting that the large number of GAPs may be required to provide specificity to Rho-family GTPase actions. For example, the protein p50RhoGAP can hydrolyse RhoA, Rac1 and Cdc42 in vitro, but only appears to hydrolyse RhoA in vivo (Ridley et al., 1993). Rho-GAPs have also been implicated in cellular processes associated with effector proteins and therefore may have a dual role in GTPase deactivation and mediating downstream functions. The Rho-GAP P190 has been implicated in regulating Rho function in cells which are undergoing cytoskeletal changes (Chang et al., 1995). The cycling of Rho-family GTPases is a carefully regulated process. This is reflected in the large number of regulatory proteins identified in comparison to the number of GTPases identified. It appears that the regulatory proteins may lend specificity to the selection of effector proteins by activated Rho proteins.
Chapter 4: Collybistin and Rho-family GTPases

Inactive Sequestered

Switched Off

Rho-GDP

Active

Switched On

GDI

GEF

Effectors

Kinases

Lipases

Scaffold proteins

Biological Responses

Rearrangement of actin cytoskeleton
G0 cell cycle progression
Cell-cell contacts
Secretion
Translation
Transcription

Fig. 4.1: GTPase activation cycle - A schematic model of the Rho-family GTPase activation cycle. GEFs catalyse the GDP to GTP reaction to give an active GTP-bound protein which can interact with a range of downstream effectors to cause a number of biological responses. GAPs enhance the intrinsic GTPase activity of the Rho protein to return it to the inactive GDP-bound state. GDIs sequester the Rho-GDP and regulate subcellular localisation.
Chapter 4: Collybistin and Rho-family GTPases

Historically, Rho-family GTPases were associated with cytoskeletal changes, whilst other members of the Ras-GTPase superfamily were attributed with other functions. However, Rho-family GTPases have now been implicated in a wide range of cellular functions. There is also growing evidence for their involvement in many diseases and they have become a potential target for new cancer therapies. The most widely studied GTPases are RhoA, Rac1 and Cdc42 and some of the biological functions they are involved in are summarised in Table 4.1.

<table>
<thead>
<tr>
<th>Function</th>
<th>RhoA</th>
<th>Rac1</th>
<th>Cdc42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulation of actin cytoskeleton</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>SRF transcription factor pathway</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>JNK/p38 kinase pathways</td>
<td>×</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>NF-κB transcription factor pathway</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Phagocytic NADPH oxidase complex</td>
<td>×</td>
<td>✔</td>
<td>×</td>
</tr>
<tr>
<td>G1 cell-cycle progression</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Cell-cell contacts</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Secretion in Mast cells</td>
<td>✔</td>
<td>✔</td>
<td>?</td>
</tr>
<tr>
<td>Cell polarity</td>
<td>×</td>
<td>×</td>
<td>✔</td>
</tr>
<tr>
<td>Transformation</td>
<td>✔</td>
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Table 4.1: A summary of the signalling pathways and functions that RhoA, Rac1 and Cdc42 are involved in (adapted from Bishop and Hall, 2000).

Rho-family GTPases exert their influence by interacting with a number of different types of effector proteins, to elicit reactions in a myriad of biological functions. These effectors range from kinases, which are components of larger signalling pathways, to scaffolding proteins. It appears that the majority of Rho proteins activate their effectors by disrupting intramolecular autoinhibitory interactions that expose functional domains within the effector protein (reviewed in Bishop and Hall, 2000).

4.1.2 RhoA, Rac1 and Cdc42 induced changes in actin structures

4.1.2.1 RhoA

The cell cytoskeleton is composed of actin filaments, intermediate filaments and microtubules. Actin exists in two forms, globular actin (g-actin) and filamentous actin (f-actin). G-actin polymerises to form f-actin, and this is a dynamic process involving
many different types of accessory proteins which facilitate polymerisation and depolymerisation (Diaz-Nido and Avila, 1997).

In eukaryotic cells, proteins of the Rho family are involved in the organisation of the actin cytoskeleton. Using cultured 3T3 fibroblast cells, the effect of each major Rho-family GTPase protein was investigated. Cells which are deprived of growth factors enter into G0 of the cell cycle and are said to be quiescent. Subsequent stimulation allows the cells to proliferate synchronously. In these studies, 3T3 cells were synchronised by serum starvation, which also created a low background in f-actin structures and experiments were carried out to elucidate the affect of different growth factors on these quiescent cells (Ridley and Hall, 1992; Ridley et al., 1992; Hall, 1998).

It was found that lysophosphatidic acid (LPA) induced the formation of stress fibres (axial bundles of f-actin underlying the cell body, see Fig. 4.2 for an example) through the activation of RhoA. RhoA is known to activate a number of effector proteins. It is proposed that the activation of two of these effectors, Rho of coiled-coil kinase (ROCK) and Diaphanous forming subfamily (Dia), are involved in the Rho-induced assembly of actin stress fibres and focal adhesions.

ROCKs are serine/threonine kinases (Matsui et al., 1996; Ishizaki et al., 2001) that have a number of target molecules, including myosin light chain (MLC, Amano et al., 1996) and myosin binding subunit (MBD, Kawano et al., 1999), which are likely to be integral components of actin-myosin filament assembly. Lin-11, Isl-3, Mec-3 domain containing kinase 1 (LIMK1) is a ROCK substrate which phosphorylates cofilin, an actin depolymerising factor which is then unable to bind to and depolymerise f-actin, resulting in the stabilisation of filamentous actin structures (Bamburg et al., 1999; Maekawa et al., 1999). Dia is a scaffold protein, which when activated induces the formation of stress fibres (Watanabe et al., 1997; Nakano et al., 1999; Watanabe et al., 1999). Dia interacts with profilin, which binds to g-actin, and somehow this relationship allows Dia to contribute to the polymerisation of actin and the subsequent organisation of f-actin into stress fibres (Wasserman, 1998). It is proposed that the combined activation of ROCK and Dia are required for Rho-induced stress fibres. Fig. 4.2 shows the signalling pathways thought to be involved in the Rho-mediated formation of actin stress fibres (Bishop and Hall, 2000).
Fig. 4.2: RhoA-induced stress fibres - A schematic representation of the signalling pathways involved in the formation of RhoA-induced stress fibres. Activated RhoA interacts with a number of effector proteins, of which ROCK and Dia are believed to be integral to the formation of stress fibres (arrow) and focal adhesions typical of RhoA (adapted from Bishop and Hall, 2000; image of a 3T3 cell showing RhoA-induced stress fibres taken from Hall, 1998)
4.1.2.2 Rac1 and Cdc42

The activation of Rac1 through growth factors such as platelet derived growth factor (PDGF), epidermal growth factor (EGF) and insulin causes *de novo* actin polymerisation and induces the formation of actin-rich lamellipodia (structures at the edge of the cell that are composed of a f-actin meshwork – see Fig. 4.3 for an example) and membrane ruffles associated with focal contacts. The formation of these structures can be prevented by the expression of dominant negative Rac1 (Nobes and Hall, 1995). Dominant negative Rac1 contains the mutation T17N (threonine to asparagine at position 17). The threonine at position 17 is thought to coordinate the binding of Mg$^{2+}$, which is essential for guanine nucleotide binding. Rac and Cdc42 containing this mutation have a lower affinity for GDP and GTP (Self and Hall, 1995). The dominant negative proteins are thought to be locked in an inactive state, thus inhibiting the activity of their respective endogenous protein by competing for exchange factor binding (Farnsworth and Feig, 1991).

Bradykinin causes *de novo* actin polymerisation and induces the formation of microspikes and filopodial extensions (long, thin protrusions at the periphery of cells, composed of f-actin bundles – see Fig. 4.3 for an example) by activating Cdc42 (Kozma *et al.*, 1995). Cross talk between these proteins has also been observed, and Cdc42 activates Rac1, so filopodia and lamellipodia can often be detected together in cells (Ridley *et al.*, 1992; Nobes and Hall, 1995; Kozma *et al.*, 1995; Machesky and Hall, 1997). The signalling pathways initiated by the activation of Rac1 and Cdc42 share many effector proteins and Fig. 4.3 is a summary of the proteins involved in Rac1 induced lamellipodia and membrane ruffles, and in the formation of microspikes and filopodia by Cdc42 activation.

A major Cdc42 effector protein involved in actin reorganisation is Wiskott-Aldrich Syndrome Protein (WASP - Derry *et al.*, 1994; Kirchhausen and Rosen, 1996). WASP, expressed in haematopoietic cells, is the product of the X-linked immunodeficiency gene found in Wiskott-Aldrich syndrome patients. The T-lymphocytes of these patients have severely disrupted microvilli, and the chemotactic properties of their macrophages and monocytes are abolished. WASP and its ubiquitously expressed isoform neuronally enriched WASP (N-WASP, Aspenstrom *et al.*, 1996; Kolluri *et al.*, 1996; Miki *et al.*, 1996), both have an effect on the regulation of the actin cytoskeleton.
Fig. 4.3: Reorganisation of the actin cytoskeleton by activated Rac1 and Cdc42 - A schematic representation of the signalling pathways involved in actin rearrangements induced by activated Rac1 and Cdc42. In fibroblasts, Rac1 induces the formation of lamellipodia (arrow) and membrane ruffles, whilst Cdc42 induces the formation of filopodia (arrow) and microspikes (adapted from Bishop and Hall, 2000; images of 3T3 cells showing Rac 1 and Cdc42 phenotypes, taken from Hall, 1998).
WASP and N-WASP interact with Cdc42, and when overexpressed with Cdc42, appear to give exaggerated microspikes and filopodia, suggesting that they somehow aid Cdc42 in the formation of these structures. WASP is known to bind to actin monomers, and WASP interacting protein (WIP) which binds to profilin, thereby contributing to actin polymerisation (Ramesh et al., 1997). Additionally, WASP binds to, and activates the Arp2/Arp3 complex (Rohatgi et al., 2001). This complex binds to actin monomers and serves as a nucleation site for the polymerisation of new actin filaments. *In vitro* studies have shown that GTP-Cdc42 induces actin polymerisation, and biochemical dissection of this process revealed that N-WASP and Arp2/Arp3 were involved (Zigmond et al., 1997; Ma et al., 1998). It appears that N-WASP and WASP were activated by Cdc42 and act as scaffold proteins by recruiting actin monomers and the Arp2/Arp3 complex to facilitate actin polymerisation (Fig. 4.3).

WASP like verpolin homologous protein (WAVE) is a protein that is found concentrated at membrane ruffles and has been precipitated with Rac1, although it is unclear whether the interaction is direct. Overexpression of WAVE has been shown to cause actin clusters, and a WAVE mutant has been shown to inhibit Rac1-induced ruffling, suggesting that there may be an *in vivo* link between the two proteins (Miki et al., 1998). WAVE has also been shown to interact with the Arp2/Arp3 complex, thus strengthening the case for its involvement in actin reorganisation (Machesky and Insall, 1998; Machesky et al., 1999).

Rac1 also interacts with phosphatidylinositol-4-phosphate 5-kinase (PI4P5K) in a GTP-independent manner. This interaction mediates the increase in PI4,5P2 levels required for the uncapping of actin filaments, a prerequisite for actin filament assembly (Hartwig et al., 1995; Tolias et al., 1998, 2000).

The p21 activated kinases (PAK) proteins are a highly conserved family of serine/threonine kinases activated by Rac1 and Cdc42. Mammals encode six proteins that are divided into two groups – A and B. All PAKs contain a N-terminal p21 GTPase binding domain (PBD) and a C-terminal kinase domain. In addition, the PAK-A proteins contain numerous SH3 domain recognising PXXP motifs. The PAK-A proteins were strongly activated upon the binding of Rac1 and Cdc42, whereas the PAK-B proteins showed greater specificity for Cdc42 than Rac1, but were not appreciably activated upon binding (reviewed in Hofmann et al., 2004). In fact, Cdc42
appears to be required for correct localisation than actual function. Microinjection of PAK into 3T3 cells induced the formation of lamellipodia, filopodia and microspikes, a phenotype observed when Cdc42 was injected into these cells (Sells et al., 1997; Daniels et al., 1998).

The molecular mechanisms by which PAKs exert their effects is not completely understood, although they do appear to phosphorylate a number of downstream substrates, including myosin light chain kinase (MLCK), LIMK1, and some GEFs (Edwards et al., 1999; Sanders et al., 1999). Some mammalian PAKs have functions independent of kinase activity. For example, PAK1 appears to act as a scaffold protein by uniting the GEF PAK interacting exchange factor α (αPIX) and Cdc42 in the polarisation of chemotaxing cells (Li et al., 2003). To date, very little is known about the downstream effects of PAK, but it appears to be an integral part of intracellular signalling, both dependent and independent of Rac1 and Cdc42 activation.

The Rho-family proteins play a regulatory role in cellular processes that involve actin, and to date RhoA, Rac1 and Cdc42 have been implicated in cell movement, axonal guidance, cytokinesis and processes requiring changes in cell shape and polarity.

4.1.3 Role of GTPase activity in the membrane activation model

Collybistin was initially identified as a dbl-like GTPase exchange factor as it contained the tandem DH-PH domains typical of these proteins (Kins et al., 2000). The dbl-like GEFs have been shown to catalyse the GDP-GTP exchange reaction on small GTPases of the Rho-family proteins which are well established as regulators of the actin cytoskeleton (reviewed in Hall, 1998). Kins et al. (2000) showed that the co-expression of collybistin I (CB1SH3+), and gephyrin in HEK293 cells resulted in the formation of intracellular aggregates, whereas the co-expression of collybistin II (CB2SH3-) resulted in the formation of submembranous microaggregates. In Chapter 3 of this study, it was shown that these phenotypes were a result of the presence or absence, respectively, of the SH3 domain and not any other part of the protein in which they differ. Kins et al. (2000) speculated that the molecular mechanisms underlying the collybistin II induced redistribution of gephyrin involved the activation of a Rho-family GTPase. In addition, the human isoform of collybistin – hPEM-2 - was identified as a GEF specific for Cdc42 (Reid et al., 1999). The authors used biochemical pull-down assays and cellular
phenotypic analysis to identify the GTPase activated by hPEM-2. Both assays indicated that Cdc42 was activated by hPEM-2. However, closer analysis of the data is not as convincing as it first appears. Fig. 4.4A&B are images published by Reid et al. (1999) which show that the cells are very small and appear to be of a low magnification, therefore the microspikes and filopodia cannot be easily visualised. However, the authors did show some biochemical evidence to suggest that Cdc42 rather than RhoA or Rac1 may be the target of hPEM-2 (shown in Fig. 4.4C-E). These Western blots are of GTPase activation pull-down assays in which cells were transfected with various GEFs and the activated GTPase was isolated in a pull-down assay using GST-bound downstream effectors (C21 for Rho and PAK for Rac and Cdc42). The GTPases were detected using specific antibodies. Although the authors stated that hPEM-2 activates Cdc42, there also appears to be a signal in the RhoA lane. Note, also that activated RhoA and Cdc42 were also detected in the negative control lanes in the absence of protein loading controls, which suggests that these results could be artifactual.

Fig 4.4: Images published by Reid et al. (1999) representing the activation of Cdc42 by hPEM-2. These purport to show the activation of Cdc42 by hPEM-2. NIH3T3 cells were transfected with Cdc42 G12V (A) or hPEM-2 (B) and the actin cytoskeleton was detected with phalloidin. They do show a similar phenotype. However, compared to the cells published by Hall (1998; see Fig. 4.3), these cells are very small and of a low magnification, therefore microspikes cannot be observed with confidence. C-E are images of the biochemical assays which indicate that Cdc42 is activated by hPEM-2 (red arrow), however a signal was also detected for RhoA (black arrow). Note that there is also unequal loading in the total Cdc42 lanes, therefore it is difficult to ascertain what the relative quantities of activated Cdc42 are.
The data presented by Reid et al. (1999), taken together with the sequence analysis of collybistin and the differences seen between gephyrin distribution in cells co-expressing gephyrin with the two different collybistin isoforms identified by Kins et al. (2000), led Kneussal and Betz (2000) to nominate Cdc42 as the substrate for collybistin in their membrane activation model. In this model, they suggested that collybistin-activated Cdc42 may constitute an important step in the chain of reactions that signal from the membrane to the subsynaptic cytoskeleton to direct gephyrin to appropriate postsynaptic sites.

4.1.4 Aims

In Chapter 3 of this study, it was shown that the RhoGEF domain of collybistin binds to gephyrin and the disruption of this interaction causes the mislocalisation of gephyrin in HEK293 cells and cultured neurons. This poses the questions of whether collybistin can activate a GTPase if the RhoGEF domain is involved in gephyrin interactions or whether collybistin has a dual role in trafficking gephyrin to the plasma membrane and activating a GTPase. Three different assays were employed in order to establish the identity of the GTPase activated by collybistin – a cell based phenotype assay, an exchange assay based on the yeast two-hybrid system, and a biochemical pull-down assay. To discover whether collybistin activated a GTPase, phenotypic analysis was conducted on cultured fibroblast cells. It is well established that the activation of a particular GTPase confers a specific phenotype upon the actin cytoskeleton of a fibroblast cell (for review see Hall, 1998). For example, RhoA induces the formation of stress fibres and focal adhesions, Rac1 activation results in lamellipodia and membrane ruffling and Cdc42 causes microspikes and filopodia (see section 4.1.2 for examples). In order to identify which GTPase was activated by collybistin, an exchange assay based upon the yeast two-hybrid system was conducted. This assay has been used previously to identify the GTPase for a novel GEF (De Toledo et al., 2000). Finally, a biochemical pull down assay was employed to verify whether Cdc42 was activated by collybistin. Due to the varied nature of the assays, each will be presented separately, with each section including the methods, results and discussion for that assay.

The aims of the work presented in this chapter were to:

- Establish whether collybistin activates a GTPase
- To identify whether the GTPase is Cdc42
4.2 Cellular Assays

The first method employed to identify the GTPase activated by collybistin was a cell phenotype assay. This assay is based on the changes observed in the structure of the actin cytoskeleton upon the activation of different GTPases, and two different rodent fibroblast cell lines (mouse NIH3T3 and rat REF52) were used. As collybistin is thought to be a GEF for the Rho-GTPases, it was predicted that the expression of collybistin in these cells would result in a morphological change that can be compared to the morphology of a cell activated by a known GTPase. The cell phenotypes that different GTPases induced were established using constitutively active GTPases or by drug incubations. For example, if collybistin activated Cdc42 then microspikes and filopodia should be evident.

4.2.1 Establishment of Cell Phenotypes

4.2.1.1 Drug incubations

NIH3T3 cells were maintained in DMEM supplemented with 5% FCS (see section 2.3.3). Cells were seeded onto poly-D-lysine-coated coverslips and grown until 40% confluent. To obtain quiescent cells, they were serum starved for 16 h. To observe changes in cell morphology caused by different drugs the cells were incubated with the following for between 10 min and 2 h: LPA (20 ng/ml), insulin (1 μg/ml) and bradykinin (100 ng/ml) which are known to activate Rho, Rac and Cdc42, respectively (Ridley, 1999). The actin structure was then observed using phalloidin staining.

4.2.1.2 Transfection of DNA constructs

To establish cell phenotypes in REF52 cells, the constitutively active forms of various GTPases were transfected by electroporation or effectene (see section 2.3.4) into the cells and allowed to grow overnight. The cells were fixed and stained with phalloidin to identify the actin structures, and to establish their morphology under the active form of the GTPase.

NIH3T3 and REF52 cells were transfected with either pRK5Myc-CB2SH3- or pRK5Myc-CB2SH3+. NIH3T3 cells were also microinjected with either pRK5Myc-CB2SH3- or pRK5Myc-CB2SH3+ (see section 2.3.4) Collybistin expression was detected using the 9E10 antibody against the Myc epitope as described in section 2.3.5.
4.2.1.3 Identification of actin structures using phalloidin

Phalloidin is a 789 Da cyclic peptide isolated from the death cap fungus \textit{(Amanita phalloides)}. It binds to and stabilises f-actin and by conjugating it to a detectable marker, it can be used to observe the actin cytoskeleton. For actin localisation, the cells were fixed, quenched and permeabilised as described in section 2.3.5. Cells were incubated in biotin conjugated phalloidin (1:100) for 1 h, followed by Alexa Fluor 488 conjugated biotin antibody (1:200; both from Molecular Probes) for 20 min at room temperature. The cells were subsequently washed as described in section 2.3.5, mounted onto microscope slides using glycerol jelly (Sigma) and viewed under a microscope (Nikon Eclipse E600).

4.2.2 Results

Initial experiments were carried out using NIH3T3 cells. Quiescent cells were incubated with drugs that are known to activate certain Rho-GTPases, thus conferring a particular morphology upon those cells. LPA was used to activate RhoA, which resulted in the formation of stress fibres. Insulin was used to activate Rac1, which induced the formation of lamellipodia and membrane ruffles, and finally Cdc42 was activated by incubating the cells in bradykinin to produce microspikes and filopodia (data not shown).

Fig. 4.5 shows confocal images taken 2 h after serum starved NIH3T3 cells were microinjected with pRK5Myc-CB2\textsubscript{SH3+} or pRK5Myc-CB2\textsubscript{SH3−} and were generated in collaboration with H. Mellor, (University of Bristol). These cells were probed with 9E10 antibody and phalloidin to identify collybistin expression and actin structure, respectively. The cells showed a positive staining for CB2\textsubscript{SH3+} (Fig. 4.5A) and CB2\textsubscript{SH3−} (Fig. 4.5D). The actin filaments were clearly stained with phalloidin (Fig. 4.5B&E). Both cells show spike-like outgrowths emanating from the cell surface. The actin also appeared to be concentrated around the periphery of the cell. Cells which did not express collybistin were also stained with phalloidin but had a different morphology. These cells had a much flatter appearance with the actin fibres distributed throughout the cell.
Fig. 4.5: NIH3T3 cells - Confocal images of serum starved NIH3T3 cells that were microinjected with either pRK5Myc-CB2_{SH3+} (upper panel) or pRK5Myc-CB2_{SH3-} (lower panel). Cells were fixed and stained 2 h after microinjection. Collybistin was detected with 9E10 antibody followed by Alexa Fluor 594 conjugated secondary antibody (A and D). Actin structures were identified using Alexa Fluor 488 phalloidin (B and E). Expression of both collybistin constructs resulted in the formation of spike-like protrusions on the surface of the cell (arrows). The actin appears to be concentrated around the periphery of the cell when compared to an untransfected cell, seen in panels B and E. The merged images show that although there is some co-localisation of collybistin with the actin structures, it is mainly localised within the cell, and not in the protrusions which appear to be rich in actin. Both collybistin constructs display similar phenotypes. The identity of the GTPase activated by collybistin cannot be determined from these images, but collybistin does appear to be inducing actin cytoskeletal rearrangements in these cells. Scale bar: 10 μm. Image courtesy of H. Mellor.
Subsequent experiments were carried out on REF52 cells as they did not need to be serum starved to detect changes in cell morphology. Cells were transfected with constitutively active RhoA, Rac1 and Cdc42, and showed the expected morphologies – stress fibres, lamellipodia and filopodia, respectively (data not shown).

The cells were then transfected with pRK5Myc-CB2SH3- or pRK5Myc-CB2SH3+, and the morphology of the transfected cells was compared to that of untransfected cells. Collybistin expression was detected with 9E10 antibody (1:200) followed by TRITC-conjugated secondary antibody (1:200). The untransfected cell had a flat appearance, densely packed with actin filaments which ran parallel to each other throughout the cytoplasm. There was no evidence of filopodia or other fine actin structures at the cell periphery (Fig. 4.6A).

The cell transfected with pRK5Myc-CB2SH3- had a different morphology. This cell showed positive staining for collybistin expression and the actin cytoskeleton was clearly stained with phalloidin (Fig. 4.6B). In this cell, the actin fibres did not appear to be densely packed throughout the cell as in the untransfected cell, instead the periphery of the cell had adopted a ruffled morphology, and a number of spike-like protrusions were seen at the lower edge of the cell.

The cell transfected with pRK5Myc-CB2SH3+ was also different to the untransfected cell (Fig. 4.6C). This cell did not have any obvious filopodia, but showed signs of curtain-like membrane ruffling. The centre of the cell was not as densely packed with actin filaments, however, not all the actin had relocated as it had in the cell expressing CB2SH3-. 
Chapter 4: Collybistin and Rho-family GTPases

An untransfected REF52 cell. All cells were stained with biotin conjugated phalloidin and Alexa Fluor 488 conjugated biotin antibody to identify the actin cytoskeleton. This cell has a fairly dense cytoskeletal structure, with many f-actin strands aligned through the cell (arrow). However, the cell does not appear to have any filopodia extending out at its surface, or show extensive membrane ruffling. The cell is quite flat in appearance. Scale bar: 10μm

A REF52 cell transfected with pRK5Myc-CB2SH3. The cell was probed with a 9E10 antibody followed by TRITC-conjugated secondary antibody to identify expressed collybistin (red fluorescence). The actin cytoskeleton was stained using phalloidin (green fluorescence). The cell displays a different morphology to the untransfected cell in A. Membrane ruffles and filopodia have formed (arrows), and the interior of the cell does not appear to be as densely packed with actin filaments. Scale bar: 10μm

A REF52 cell transfected with pRK5Myc-CB2SH3. The cell was probed with a 9E10 antibody followed by TRITC-conjugated secondary antibody to identify expressed collybistin (red fluorescence). The actin cytoskeleton was stained using phalloidin (green fluorescence). The cell displays a different morphology to the untransfected cell. Membrane ruffles can be seen (arrows), although filopodia are not obvious in this cell. Scale bar: 10μm

Fig. 4.6: REF52 cells - Confocal images of REF52 cells transfected with pRK5Myc-CB2SH3, or pRK5Myc-CB2SH3+.
4.2.3 Discussion

Two cell types were used in this assay, and both displayed mixed phenotypes when expressing collybistin. NIH3T3 cells did not express collybistin when transfected by electroporation or using effectene, however, microinjection of the collybistin plasmids did result in its expression. These cells adopted a very different morphology to that of untransfected cells. They had many spike-like protrusions emanating from the surface, indicative of Cdc42 activation. However, the edges of the cells took on a slightly ruffled appearance, suggesting that Rac1 was also activated, either directly, or downstream of Cdc42. Therefore, these studies cannot show for certain whether collybistin activates Rac1, Cdc42, both or neither.

The REF52 cells also displayed a rearrangement of their actin cytoskeleton upon collybistin expression. The cytoplasm of the untransfected cell was packed full of actin fibres running in parallel to each other. In the cells expressing collybistin, the majority of these actin structures had dissolved and relocated to the cell periphery which had adopted a curtain-like ruffle, indicative of Rac1 activation (Ridley et al., 1992). These cells also had filopodial like extensions, which were very clear in the cell transfected with CB2sh3 and are indicative of Cdc42 activation (Kozma et al., 1995; Hall, 1998). Therefore, it can not be ascertained which GTPase is activated by collybistin, as the cells display mixed phenotypes.

It is known that Cdc42 and Rac1 share a number of effector proteins that are involved in cytoskeletal changes (see Fig. 4.3; Bishop and Hall, 2000). These proteins are involved in a complicated pathway, and there may be cross-talk between the two GTPases (Burridge and Wennerberg, 2004). Many of the effector proteins shared by Rac1 and Cdc42 are involved in the rearrangement of the cytoskeleton. In addition, Cdc42 has been shown to activate Rac1, therefore the phenotypes observed in this study could be due to the activation of both GTPases, or the cross-talk between effector proteins. Using this system, the identity of the GTPase activated by collybistin could not be determined. Rac1 and Cdc42 cannot be ruled out, as phenotypes typical for both were observed, nor can any other Rho-GTPase which may give these phenotypes in fibroblasts. There is also the possibility that not all Rho-GTPases have been identified and that collybistin may activate one of these.
4.3 Yeast Exchange Assay

The yeast exchange assay (YEA) is based upon the yeast two-hybrid interaction system and is a rapid qualitative test to identify the GTPase for a particular GEF (De Toledo et al., 2000). It works on the premise that GTPases do not bind to their effectors until they are activated by an exchange factor. Accordingly, the GTPase is cloned into a bait plasmid and its known effector protein into a prey plasmid. Only if the two proteins interact will the reporter gene be induced. When expressed in yeast, the wild-type mammalian GTPase is mainly bound to GDP, or weakly bound to its cognate effector. The presence of an exchange factor, which is cloned into a third vector whose expression can be controlled using nutritional parameters, will enable the GTPase to become GTP-bound and thus allow interaction with the effector protein and the subsequent induction of the reporter gene, which in this assay is the \textit{LacZ} gene. The \textit{LacZ} activation assay (section 2.2.8) can be used to see whether the GEF activates the GTPase (see Fig. 4.7).

4.3.1 Yeast strains

Two yeast strains were used in this assay, TAT7 and 12.1 (De Toledo et al., 2000). TAT7 has the genotype Mata, trp1, his3, leu2, ura3, ade2, LYS::(LexAop)4-HIS3, URA3::(LexAop)8-\textit{LacZ}). It was used to carry out all the activation tests and was grown at 30°C. The yeast strain 12.1 was obtained by mating TAT7 with YMP483 which bears the \textit{cdc24-5} thermo-sensitive mutation (Chenevert et al., 1994). This strain was used to carry out experiments which involved Cdc42. In TAT7, Cdc42 is catalysed by the yeast Rho-GEF Cdc24 into the GTP-bound state, which results in the activation of the \textit{LacZ} gene, thus giving false positive interactions. In the mutated strain the GEF ability of Cdc24 is compromised at 30°C but cells can grow at 25°C, allowing the assay for GDP-GTP exchange on Cdc42 to occur (Chenevert et al., 1994; De Toledo et al, 2000). This strain was grown at 25°C.

4.3.2 Yeast Constructs

The open reading frames of the GTPases were cloned into the bait vector pLexA and the downstream effector proteins were cloned into the prey vector pGAD, and are shown in Table 4.2. ROCK, kinectin and PAK are all established downstream effectors for their respective GTPases. ROCK and PAK have been discussed in 4.1.
When expressed in yeast, wild-type GTPases are mainly bound to GDP. They do not interact with their downstream target proteins (or very poorly if they do), therefore displaying negative LacZ reporter gene activity.

When the expression of an exogenous GEF is induced, the GTPase is converted from the GDP-bound state to the GTP-bound state.

The GTP-bound GTPase can then bind to the target protein, thus allowing the Lex A and GAL4 AD to initiate LacZ activity.

**Fig. 4.7: Schematic Representation of the YEA.** An assay based upon the yeast two-hybrid system to identify the GTPase activated by a particular GEF. The GEF is cloned into a vector, and is only expressed in the absence of methionine in the medium. The GTPase and effector protein are cloned into bait and prey vectors, respectively. Only if the GEF activates the GTPase, will it bind to the effector protein and induce the reporter gene.
Kinectin is an integral membrane protein which binds to kinesin and is the membrane anchor for kinesin driven vesicle movement (Hotta *et al.*, 1996). Cdc42GV12 is the constitutively-active form of Cdc42. The glycine at position 12 has been mutated to valine and this keeps Cdc42 in the GTP-bound state by either decreased GTPase activity or insensitivity to GAPs, and was used as a positive control (Nobes and Hall, 1995).

<table>
<thead>
<tr>
<th>GTPase in pLex (Bait)</th>
<th>Downstream effector in pGAD (Prey)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RhoA</td>
<td>ROCK</td>
</tr>
<tr>
<td>Rac1</td>
<td>Kinectin</td>
</tr>
<tr>
<td>RhoG</td>
<td>Kinectin</td>
</tr>
<tr>
<td>Cdc42Hs</td>
<td>PAK</td>
</tr>
<tr>
<td>TC10</td>
<td>PAK</td>
</tr>
<tr>
<td>TCL</td>
<td>PAK</td>
</tr>
<tr>
<td>Cdc42GV12</td>
<td>PAK</td>
</tr>
</tbody>
</table>

**Table 4.2: YEA constructs** - A list of the bait and prey vectors available for testing in the YEA to identify the GTPase activated by collybistin.

The catalytic region of the Cdc42 exchange factor αPIX was cloned into the EcoRI/SalI sites of the vector pRSMT1. GEF expression is under the control of the Met25 promoter, and will only be transcribed if methionine is absent from the medium on which the yeast are grown. Full-length and truncated forms (RhoGEF domain only, and RhoGEF-PH tandem domain) of CB2<sub>SH3+</sub> and CB2<sub>SH3</sub> were cloned into the EcoRI and SalI sites of pRSMT1.

4.3.3 Transformations and assay

Plasmids were transformed into the yeast strains using the LiOAc/TE method described in section 2.2.5. Co-transformed yeast were plated onto selective dropout medium agar plates lacking leucine and tryptophan. For the three-hybrid experiments, co-transformed yeast were plated onto selective dropout medium plates lacking leucine, tryptophan, uracil and either with or without 2 mM methionine. The tryptophan selected for the GTPase, the leucine selected for the downstream effector and uracil selected for the GEF expressing vector. The presence of methionine in the medium suppressed the expression of the GEF, and these transformations were used as negative controls for the activation of the GTPase. The yeast which were plated onto medium lacking
methionine expressed the GEF since this was under the control of the Met promoter. If the GEF catalysed the GTPase, then the activated GTPase would bind to its effector and induce expression of the reporter gene. However, if the GEF did not catalyse the GTPase then it would remain in the GDP-bound state and the reporter gene would not be expressed. Transformed TAT7 yeast were incubated at 30°C and transformed 12.1 yeast were incubated at 25°C until colonies emerged. Yeast colonies were then lifted onto filter paper and screened for protein-protein interactions using the freeze fracture assay for \textit{LacZ} activation described in section 2.2.8.

4.3.4 Results

A number of positive and negative controls were required to eliminate any false positive interactions. These are included in the tables for each of the GTPases. Initially the GTPase (bait) and downstream effector (prey 1) constructs were co-transformed into the relevant yeast strains and assayed for an interaction. In these experiments, a negative result was expected as the GTPase should be bound to GDP and without an active GEF, should not be able to bind to the effector. Initially TC10 and TCL were transformed into TAT7 with their downstream effector PAK, but both gave positive reactions for the \textit{LacZ} activation assay. This suggested that the GTPases were active, either by being constitutively bound to GTP or were activated by a GEF that was endogenously expressed by the yeast (data not shown). An alternative explanation for this observation is that both TC10 and TCL have been implicated in participating in Cdc42 activation pathways, and therefore may have been activated by endogenous Cdc42. Consequently, both GTPases were tested in yeast strain 12.1. Tables 4.3 and 4.4 show the expected and actual results of the YEA for TC10 and TCL in yeast strain 12.1, Tables 4.5-4.7 are the YEA results for RhoA, Rac1 and RhoG in TAT7 and Table 4.8 shows the YEA results for Cdc42 in yeast strain 12.1.

TC10 and PAK were transformed into 12.1, and plated onto selective agar lacking leucine and tryptophan. Colonies grew as expected, however they gave a blue colour in the \textit{LacZ} assay. The yeast were also co-transformed with TC10, PAK and \textit{CB2\textsubscript{SH3}} and plated onto selective agar with and without methionine. In the presence of methionine colonies grew and became blue in the \textit{LacZ} assay. In the absence of methionine colonies failed to grow (Table 4.3).
Chapter 4: Collybistin and Rho-family GTPases

<table>
<thead>
<tr>
<th>Bait</th>
<th>Prey 1</th>
<th>Prey 2</th>
<th>Plated onto</th>
<th>Expected result</th>
<th>Actual result</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCL</td>
<td>PAK</td>
<td>—</td>
<td>-Leu/-Trp</td>
<td>Colonies should grow, but give a negative LacZ assay as TCL should be GDP-bound and therefore unable to interact with PAK</td>
<td>Colonies did grow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Positive LacZ assay +++</td>
</tr>
<tr>
<td>TCL</td>
<td>PAK</td>
<td>CB2SH3-</td>
<td>-Leu/-Trp/-Ura + 2mM Met</td>
<td>Colonies should grow but there should not be a positive interaction as GEF expression should be suppressed by the presence of methionine</td>
<td>Colonies did grow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Positive LacZ assay +++</td>
</tr>
<tr>
<td>TCL</td>
<td>PAK</td>
<td>CB2SH3-</td>
<td>-Leu/-Trp/-Ura/-Met</td>
<td>Colonies should grow, and should only give a positive reaction if collybistin activates TCL</td>
<td>Colonies did not grow</td>
</tr>
</tbody>
</table>

Table 4.3 YEA conducted in 12.1 to establish whether collybistin activated TCL.

TCL and PAK were co-transformed into 12.1, and plated onto selective agar lacking leucine and tryptophan. Colonies grew as expected, however they gave a blue colour in the LacZ assay. The yeast were also co-transformed with TCL, PAK and CB2SH3-, and plated onto selective agar with and without methionine. In the presence of methionine colonies grew and became blue in the LacZ assay. In the absence of methionine colonies failed to grow (Table 4.4).

<table>
<thead>
<tr>
<th>Bait</th>
<th>Prey 1</th>
<th>Prey 2</th>
<th>Plated onto</th>
<th>Expected result</th>
<th>Actual result</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCL</td>
<td>PAK</td>
<td>—</td>
<td>-Leu/-Trp</td>
<td>Colonies should grow, but the LacZ gene should not be activated as TCL should be GDP-bound, thus unable to interact with PAK</td>
<td>Colonies did grow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Positive LacZ assay +++</td>
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<td>CB2SH3-</td>
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<td>Colonies should grow, and only give a positive reaction if collybistin activates TCL</td>
<td>Colonies did not grow</td>
</tr>
</tbody>
</table>

Table 4.4 YEA conducted in 12.1 to establish whether collybistin activated TCL.
Yeast strain TAT7 was co-transformed with RhoA and ROCK and plated onto selective agar lacking leucine and tryptophan. Colonies grew as expected, however they gave a blue colour in the \textit{LacZ} assay. The yeast were also co-transformed with RhoA, ROCK and CB2\textsubscript{SH3}, and plated onto selective agar with and without methionine. In the presence of methionine, colonies grew and became blue in the \textit{LacZ} assay. In the absence of methionine, the colonies also grew and gave a blue colour in the \textit{LacZ} assay (Table 4.5).

<table>
<thead>
<tr>
<th>Bait</th>
<th>Prey 1</th>
<th>Prey 2</th>
<th>Plated onto</th>
<th>Expected result</th>
<th>Actual result</th>
</tr>
</thead>
<tbody>
<tr>
<td>RhoA</td>
<td>ROCK</td>
<td>—</td>
<td>-Leu/-Trp</td>
<td>Colonies should grow, but the \textit{LacZ} gene should not be activated as the RhoA should be GDP-bound, thus unable to interact with ROCK</td>
<td>Colonies did grow Positive \textit{LacZ} assay +++</td>
</tr>
<tr>
<td>RhoA</td>
<td>ROCK</td>
<td>CB2\textsubscript{SH3}</td>
<td>-Leu/-Trp/-Ura + 2mM Met</td>
<td>Colonies should grow but there should not be a positive interaction as GEF expression should be suppressed by the presence of methionine</td>
<td>Colonies did grow Positive \textit{LacZ} assay +++</td>
</tr>
<tr>
<td>RhoA</td>
<td>ROCK</td>
<td>CB2\textsubscript{SH3}</td>
<td>-Leu/-Trp/-Ura/-Met</td>
<td>Colonies should grow, and only give a positive reaction if collybistin activates RhoA</td>
<td>Colonies did grow Positive \textit{LacZ} assay +++</td>
</tr>
</tbody>
</table>

\textbf{Table 4.5} YEA conducted in TAT7, to establish whether collybistin activated RhoA.

Yeast strain TAT7 was co-transformed with Rac1 and kinectin and plated onto selective agar lacking leucine and tryptophan. Colonies grew as expected, and remained white in the \textit{LacZ} assay. The yeast were also co-transformed with Rac1, kinectin and CB2\textsubscript{SH3}, and plated onto selective agar with and without methionine. A very small number of colonies emerged in the presence of methionine and these remained white in the \textit{LacZ} assay. However, colonies failed to grow in the absence of methionine (Table 4.6).
Bait | Prey 1 | Prey 2 | Plated onto | Expected result | Actual result
--- | --- | --- | --- | --- | ---
Racl | Kinectin | - | -Leu/-Trp | Colonies should grow, but the \( \text{LacZ} \) gene should not be activated as Racl should be GDP-bound, thus unable to interact with kinectin. | Colonies did grow |
Racl | Kinectin | CB2\(_{SH3}\) | -Leu/-Trp/-Ura + 2mM Met | Colonies should grow but there should not be a positive interaction as GEF expression should be suppressed by the presence of methionine | Colonies did grow (very few) |
Racl | Kinectin | CB2\(_{SH3}\) | -Leu/-Trp/-Ura/-Met | Colonies should grow, and only give a positive reaction if collybistin activates Racl | Colonies did not grow |

Table 4.6 YEA conducted in TAT7, to establish whether collybistin activated Rac1.

Yeast strain TAT7 was co-transformed with RhoG and kinectin, and plated onto selective agar lacking leucine and tryptophan. Colonies grew as expected and remained white in the \( \text{LacZ} \) assay. The yeast were also co-transformed with RhoG, kinectin and CB2\(_{SH3}\), and plated onto selective agar with and without methionine. In the presence of methionine, colonies grew, but became blue in the \( \text{LacZ} \) assay. In the absence of methionine, the colonies also grew and gave a blue colour in the \( \text{LacZ} \) assay (Table 4.7).

| Bait | Prey 1 | Prey 2 | Plated onto | Expected result | Actual result
--- | --- | --- | --- | --- | ---
RhoG | Kinectin | - | -Leu/-Trp | Colonies should grow, but the \( \text{LacZ} \) gene should not be activated as RhoG should be GDP-bound, thus unable to interact with kinectin. | Colonies did grow |
RhoG | Kinectin | CB2\(_{SH3}\) | -Leu/-Trp/-Ura + 2mM Met | Colonies should grow but there should not be a positive interaction as GEF expression should be suppressed by the presence of methionine | Colonies did grow |
RhoG | Kinectin | CB2\(_{SH3}\) | -Leu/-Trp/-Ura/-Met | Colonies should grow, and only give a positive reaction if collybistin activates RhoG | Colonies did grow |

Table 4.7 YEA conducted in TAT7, to establish whether collybistin activated RhoG.
Table 4.8 describes the results of the YEA for Cdc42 conducted in yeast strain 12.1, and Fig. 4.8 shows the freeze fracture \textit{LacZ} assays for these experiments. Yeast strain 12.1 was co-transformed with Cdc42 and PAK and plated onto selective agar lacking leucine and tryptophan. Colonies grew and remained white in the \textit{LacZ} assay (Fig. 4.8A). Yeast were also co-transformed with Cdc42GV12, which is the constitutively active mutant of Cdc42 and PAK, and colonies grown on selective agar lacking leucine and tryptophan gave a blue colour in the \textit{LacZ} assay (Fig. 4.8B). The interaction observed here was robust and the change in colour was evident within 15 min of the start of the assay. A known GEF (\textit{αPIX}) was available for Cdc42, therefore yeast were transformed with Cdc42, PAK and \textit{αPIX} and plated onto selective agar lacking leucine, tryptophan and uracil and either with or without methionine. In the presence of methionine, colonies grew and remained white in the \textit{LacZ} assay as \textit{αPIX} expression was suppressed (Fig. 4.8C). However, in the absence of methionine, colonies became blue in the \textit{LacZ} assay (Fig. 4.8D). The yeast were then co-transformed with Cdc42, PAK and CB2\textsubscript{SH3}+ or CB2\textsubscript{SH3}−. In the presence of methionine the colonies grew and remained white in the \textit{LacZ} assay for both collybistin isoforms (Fig. 4.8E&G). However, in the absence of methionine, colonies failed to grow for both collybistin isoforms (Fig. 4.8F&H; Table 4.8). These experiments were also conducted using truncated forms of collybistin, and although colonies grew in the absence of methionine they failed to give a positive \textit{LacZ} reaction indicating that collybistin does not activate Cdc42.
<table>
<thead>
<tr>
<th>Bait</th>
<th>Prey 1</th>
<th>Prey 2</th>
<th>Plated onto</th>
<th>Expected result</th>
<th>Actual result</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdc42</td>
<td>PAK</td>
<td>—</td>
<td>-Leu/-Trp</td>
<td>Colonies should grow, but give negative <em>LacZ</em> as Cdc42 should be GDP-bound and, therefore, be unable to interact with PAK</td>
<td>Colonies did grow</td>
<td>Fig. 4.8A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Negative <em>LacZ</em> assay</td>
<td></td>
</tr>
<tr>
<td>Cdc42</td>
<td>GV12</td>
<td>PAK</td>
<td>-Leu/-Trp</td>
<td>Colonies should grow and give a positive <em>LacZ</em> reaction as V12 is the constitutively active mutant of Cdc42 therefore should bind to PAK</td>
<td>Colonies did grow</td>
<td>Fig. 4.8B</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Positive <em>LacZ</em> assay ++</td>
<td></td>
</tr>
<tr>
<td>Cdc42</td>
<td>PAK</td>
<td>αPIX</td>
<td>-Leu/-Trp/ -Ura + 2mM Met</td>
<td>Colonies should grow but there should not be a positive interaction as GEF expression should be suppressed by the presence of methionine in the medium</td>
<td>Colonies did grow</td>
<td>Fig. 4.8C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Negative <em>LacZ</em> assay</td>
<td></td>
</tr>
<tr>
<td>Cdc42</td>
<td>PAK</td>
<td>αPIX</td>
<td>-Leu/-Trp/ -Ura/-Met</td>
<td>Colonies should grow, and there should be a positive reaction as αPIX is known to activate Cdc42</td>
<td>Colonies did grow</td>
<td>Fig. 4.8D</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Positive <em>LacZ</em> assay ++</td>
<td></td>
</tr>
<tr>
<td>Cdc42</td>
<td>PAK</td>
<td>CB2SH3+</td>
<td>-Leu/-Trp/ -Ura + 2mM Met</td>
<td>Colonies should grow but there should not be a positive interaction as GEF expression should be suppressed by the presence of methionine</td>
<td>Colonies did grow</td>
<td>Fig. 4.8E</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Negative <em>LacZ</em> assay</td>
<td></td>
</tr>
<tr>
<td>Cdc42</td>
<td>PAK</td>
<td>CB2SH3+</td>
<td>-Leu/-Trp/ -Ura/-Met</td>
<td>Colonies should grow, and there should be a positive reaction if collybistin activates Cdc42</td>
<td>Colonies did not grow</td>
<td>Fig. 4.8F</td>
</tr>
<tr>
<td>Cdc42</td>
<td>PAK</td>
<td>CB2SH3-</td>
<td>-Leu/-Trp/ -Ura + 2mM Met</td>
<td>Colonies should grow but there should not be a positive interaction as GEF expression should be suppressed by the presence of methionine</td>
<td>Colonies did not grow</td>
<td>Fig. 4.8G</td>
</tr>
<tr>
<td>Cdc42</td>
<td>PAK</td>
<td>CB2SH3-</td>
<td>-Leu/-Trp/ -Ura/-Met</td>
<td>Colonies should grow, and there should be a positive reaction if collybistin activates Cdc42</td>
<td>Colonies did not grow</td>
<td>Fig. 4.8H</td>
</tr>
</tbody>
</table>

Table 4.8 YEA conducted in 12.1 to establish whether collybistin activated Cdc42. Cdc42 did not appear to autoactivate, and gave expected results against its known GEF αPIX. Note that full length collybistin did not grow, but the truncated versions of collybistin grew and gave negative *LacZ* assays.
The colonies grew, but did not give a blue colour as Cdc42 was in the GDP-bound state and therefore could not interact with PAK.

The colonies grew and gave a blue colour as Cdc42 G12V is the constitutively active form of Cdc42, thus would have been GTP-bound and able to interact with PAK.

The colonies grew but did not give a blue colouring as αPIX expression was suppressed in the presence of methionine, thus Cdc42 remained bound to GDP.

The colonies grew and gave a blue colouring as αPIX was expressed, and catalysed Cdc42 to the GTP-bound state enabling it to interact with PAK.

Colonies grew but remained white

Colonies did not grow

Colonies grew but remained white

Colonies did not grow

**Fig. 4.8: Freeze fracture LacZ assays of the YEA for Cdc42** - These filters show the results obtained when yeast strain 12.1 was used for the YEA for Cdc42. The plasmids transformed are indicated on the individual panels, and yeast were either plated onto selective agar lacking leucine and tryptophan (-L/-T), or lacking leucine, tryptophan, and uracil, and supplemented with 2 mM methionine (-L/-T/-U) or lacking methionine (-L/-T/-U/-M). These yeast were grown at 25°C. The blue colouring indicates an interaction between the Cdc42 and its downstream effector PAK.
4.3.5 Discussion

The GTPase constructs available to test for collybistin GEF activity were RhoA, RhoG, Rac1, TCL, TC10 and Cdc42 (summarised in Table 4.9). By plating yeast co-transformed with just the GTPase and its downstream effector onto medium lacking leucine and tryptophan, yeast colonies should grow, but not induce the expression of the LacZ gene as the two proteins should be unable to bind to each other as the GTPase should be GDP-bound. However, for RhoA, TC10 and TCL blue colonies were observed, suggesting that the GTPase was in its GTP-bound state and therefore, able to bind to its downstream effector, thus inducing the expression of the LacZ reporter gene. Possible explanations for this observation are that the GTPases were either activated by a GEF endogenously expressed by the yeast, or, in the case of TC10 and TCL, were activated by endogenous Cdc42. TC10 and TCL were subsequently tested in 12.1, which should have eliminated the effect of Cdc42, however here co-transformation of the GTPase and downstream effector also gave a positive LacZ reaction, suggesting that the GTPases were switched on by an endogenous GEF, or that they may be constitutively active; consequently these constructs could not be used to verify the identity of the GTPase activated by collybistin.

However, Rac1, RhoG and Cdc42 gave the predicted response in the corresponding experiments and were therefore tested against collybistin. Unfortunately, a GEF for Rac1 and RhoG was unavailable, therefore the two-hybrid system could not be verified for these GTPases in this study, but they have been described elsewhere (De Toledo et al., 2000). Nevertheless, experiments in which collybistin was co-transformed with the GTPase and its effector were performed. These triple transformations were plated onto selective dropout medium lacking leucine, tryptophan, uracil and either with or without 2mM methionine. The presence of methionine should have suppressed the expression of collybistin and the absence of methionine should have induced the expression of collybistin. If collybistin had the ability to catalyse GDP-GTP exchange on those GTPases, then blue colonies would have been observed. In the Rac1 YEA very few colonies grew on the selective agar containing methionine and these remained white in the LacZ assay. Colonies did not emerge on the selective agar lacking methionine. In the RhoG YEA, colonies grew on both the selective agar plates, however, both sets of colonies also gave positive LacZ assays indicating that RhoG was bound to kinectin, in a manner that did not appear to be dependent on the expression of collybistin.
Chapter 4: Collybistin and Rho-family GTPases

Therefore the YEA could not be used to verify whether collybistin activated one of these two GTPases. The final GTPase tested was Cdc42, the GTPase proposed to be activated by collybistin and hPEM-2 (Reid et al., 1999, Kneussel and Betz, 2000). A known GEF for Cdc42 (αPIX) was available for testing in the three-hybrid system and these experiments worked as expected. In the presence of methionine, αPIX was not expressed, therefore a negative LacZ reaction was seen (Fig. 4.4C). In the absence of methionine, αPIX was expressed and activated Cdc42, therefore a positive LacZ reaction was seen (Fig. 4.4D). Given that the three-hybrid system was successful for αPIX, CB2SH3- and CB2SH3+ were co-transformed with Cdc42 and PAK instead of αPIX. For both collybistin isoforms yeast colonies grew on selective agar containing methionine and gave negative LacZ reactions (Fig. 4.4E&G). On selective agar lacking methionine, the expression of collybistin would have been induced by the Met promoter, and if collybistin activated Cdc42 a positive LacZ reaction should have been observed. Unfortunately, colonies did not grow in these experiments, however, when truncated collybistin was transformed with Cdc42 and PAK, colonies did grow on agar lacking methionine, but these remained white in the LacZ assay indicating that collybistin does not activate Cdc42. Collybistin appears to affect the growth of the yeast in the YEA, as colonies failed to grow for four of the six GTPases which were tested. The two GTPases which did grow, RhoA and RhoG, gave positive LacZ assays even when collybistin expression was prevented by the presence of methionine in the medium, indicating that these GTPases may be activated by GEFs endogenous to the yeast. Yeast express six Rho-family GTPases, Rho1p-5p and Cdc42, and the GEFs for one of these may also be able to activate RhoA and RhoG (Wherlock and Mellor, 2002).

<table>
<thead>
<tr>
<th></th>
<th>Transformed with effector Plated on -L/-T</th>
<th>Transformed with effector and collybistin Plated on -L/-T/-U</th>
<th>Transformed with effector and collybistin Plated on -L/-T/-U/-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>RhoA</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>RhoG</td>
<td>—</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Rac1</td>
<td>—</td>
<td>—</td>
<td>DNG</td>
</tr>
<tr>
<td>TC10</td>
<td>+++</td>
<td>+++</td>
<td>DNG</td>
</tr>
<tr>
<td>TCL</td>
<td>+++</td>
<td>+++</td>
<td>DNG</td>
</tr>
<tr>
<td>Cdc42</td>
<td>—</td>
<td>—</td>
<td>DNG</td>
</tr>
</tbody>
</table>

Table 4.9: Summary of the YEA for the GTPases tested against collybistin;—: negative LacZ assay, +++: grading of positive LacZ assay, DNG: colonies did not grow

Although this yeast based assay has been successful in the identification of GTPases for some GEFs (De Toledo et al., 2000), it was not the most ideal system for collybistin.
4.4 Cdc42 Activation Assay

In order to establish whether collybistin activated Cdc42 as suggested in the membrane activation model, a commercially available assay was used (Upstate, USA). Like the yeast exchange assay, this assay also exploits the tight interaction formed between an activated GTPase and its downstream effector protein. However, this assay is carried out in cultured mammalian cells and relies on affinity precipitation and detection of proteins. Activated GTPases were affinity purified by the specific binding of the activated GTPase to its effector protein which was immobilised on an agarose resin. This was subsequently processed and the activated GTPase detected using a specific antibody.

4.4.1 Sample preparation

HEK293 cells were grown, maintained and transfected with effectene as previously described (section 2.3). Cells were transfected with pRK5Myc-CB2SH3+, pRK5Myc-CB2SH3-, pEGFP-hPEM-2 or constitutively active Cdc42 (Cdc42G12V). All reagents were stored on ice and all steps were carried out at 4°C. Cell lysates were prepared by detaching and lysing the cells using Mg^{2+} lysis buffer (25 mM HEPES, 50 mM NaCl, 1% Igepal CA-630, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol, 10 μg/ml aprotinin and 10 μg/ml leupeptin; pH 7.5, Upstate). The lysates were centrifuged at 14,000 g for 5 min at 4°C to remove all the cell debris. The supernatant was extracted to a pre-chilled micro-centrifuge tube and stored on ice or snap frozen in liquid nitrogen and stored at -80°C until required.

4.4.2 Activation assay

4.4.2.1 Positive and Negative Controls

Positive and negative controls for the activation assay were prepared by loading 0.5 ml of untransfected cell lysate (containing 10 mM EDTA) with either 100 μM GTPγS (positive control) or 10 mM GDP (negative control). The samples were incubated at 30°C for 30 min with constant agitation. The samples were then quenched with 60 mM MgCl₂.
4.4.2.2 Cdc42 Pull Down Assay

10 µl of PAK-1 PBD agarose (downstream effector of activated Cdc42) was added to 0.5 ml of each cell extract and incubated at 4°C for 45 min with gentle agitation. The beads were then briefly centrifuged, and the pellet washed three times with lysis buffer. The pellet was then resuspended in 40 µl of 2× Laemmli reducing sample buffer, supplemented with 2 µl of 1 M dithiothreitol and boiled for 5 min. The samples were then briefly centrifuged, the beads and supernatant were thoroughly mixed and 20 µl of each sample loaded onto a polyacrylamide gel for electrophoresis (5% stacking gel and 12% resolving gel; Sambrook et al., 1989).

4.4.3 Gel Electrophoresis, Western Blot and Detection.

Each gel had 10 wells, one of which was loaded with biotinylated protein molecular weight marker (New England Biolabs). Two gels were run concurrently and the samples were distributed accordingly. The gel electrophoresis was carried out at 80 volts, 300 mA for 2 h (Tris-glycine electrophoresis buffer: 25 mM Tris-base, 190 mM glycine, 0.1% SDS, pH 8.3). The proteins were then transferred to nitrocellulose membrane at 40 volts, 300 mA for 1 h (Tris-glycine transfer buffer: 25 mM Tris-base, 190 mM glycine, 0.1% SDS, 20% methanol, pH 6.8). Once transfer was complete, the membrane was washed in distilled water twice and then incubated in freshly prepared PBS containing 3% nonfat dry milk (PBS-MLK) for 30 min at room temperature with constant agitation. The membrane was then incubated in 1 µg/ml Cdc42 antibody diluted in PBS-MLK (supplied as part of the kit) at 4°C overnight with gentle agitation. The following day, the membrane was washed twice in PBS-0.05% Tween20 and then incubated in the secondary antibody for 1 h at room temperature with constant agitation. The secondary antibody used was goat anti-mouse horse radish peroxidase (HRP)-conjugated antibody, diluted 1:2000 in PBS-MLK. An anti-biotin HRP-conjugated antibody was also added to this buffer to detect the protein markers. The membrane was then washed three times in PBS-0.05% Tween20. The secondary antibody was detected using a luminol-based chemi-luminescent HRP detection system. 4 µl of 30% (w/v) hydrogen peroxide (H₂O₂, Sigma) was added to 10 ml ECL buffer solution (100 mM Tris-HCl, pH 8.5, 4°C; 90 mM p-coumaric acid/DMSO; 250 mM luminol/DMSO). The HRP linked to the secondary antibodies hydrolysed H₂O₂ to release H₂O and O₂. The O₂ oxidised luminol to emit a luminescence which was captured by ECL.
hyperfilms (Amersham). Samples were exposed to the film for time ranges of 1 min-1 h, and subsequently developed using standard protocols.

4.4.4 Results and Discussion

The Cdc42 activation assay kit contained enough reagents for three experiments only. Each experiment was carried out using lysates from cells transfected with pRK5Myc-CB2sH3, pRK5Myc-CB2sH3, and pEGFP-hPEM-2, untransfected cells and the positive and negative controls. A number of problems were encountered with this assay. The main one being the inability of the Cdc42 antibody provided in the kit to detect Cdc42 on the nitrocellulose membrane. It did not detect the positive control, which should have elicited a strong Cdc42 activation. The membranes were counterstained with Ponceau stain (0.1% Ponceau-S, 1% acetic acid in distilled water) which showed that the transfer of proteins had occurred. This assay was not followed up due to time constraints, and the need to pursue other avenues of investigation.
4.5 Discussion and Conclusions

The aims of the work conducted in this chapter were to establish whether collybistin activated a GTPase, and if so, the identity of that GTPase. In the membrane activation model, Kneussel and Betz (2000) had suggested that collybistin activated Cdc42, which caused rearrangement of the actin cytoskeleton at the synapse, and aided the clustering of gephyrin at postsynaptic sites. They based this on the structural homology between collybistin and other dbl-like GEFs, the redistribution of gephyrin seen when co-expressed with collybistin II in HEK293 cells, and the reports by Reid et al. (1999) which stated that hPEM-2, the human homologue of collybistin, activated Cdc42.

Three different assays were employed in an attempt to identify the GTPase activated by collybistin, each with their own set of methodological problems.

The first assay was a cell based phenotype assay which relied upon the cytoskeletal changes elicited in fibroblast cells by the activation of GTPases. Two different fibroblast cell lines were used – NIH3T3 and REF52. The NIH3T3 cells were unable to express proteins that had been transfected by electroporation or effectene, but were successfully microinjected with the plasmid. Phenotypic analysis of both cell lines showed that there was a definite rearrangement of the actin cytoskeleton upon expression of CB2_{SH3} and CB2_{SH3+}. Phenotypes consistent with particular GTPases were observed in both cell types. Microspikes and membrane ruffling were observed, indicative of Cdc42 and Rac1 activation, respectively. These data suggest that collybistin does activate a GTPase, although this is not influenced by the SH3 domain. In HEK293 cells, gephyrin distribution differed in the presence and absence of the SH3 domain (see Chapter 3, Kins et al., 2000). These data also suggest that collybistin may activate more than one GTPase. Some GEFs are specific towards one GTPase, for example p115RhoGEF only appears to activate RhoA (Eisenhaure et al., 2003), whereas other GEFs are able to activate a number of GTPases, for example Vav has been shown to activate Cdc42, Rac1 and RhoA (Abe et al., 2000). Using the cell based system, it was evident that collybistin expression affected the actin cytoskeleton of fibroblasts, which is indicative of GTPase activation (Hall, 1998), however, the identity of the GTPase remains elusive.

The second assay was conducted to try and elucidate the identity of the GTPase activated by collybistin. The YEA is based on the yeast two-hybrid system and
exploited the properties of GTPase activation. A number of problems were encountered with this assay. Many of the GTPase constructs provided gave false positive interactions, therefore could not be used with confidence. Another drawback was the number of GTPases that were available for testing, only six of the 23 identified GTPases could be tested, as downstream effectors are not known for some, or the GTPase was unsuitable for testing in this assay. Those which were available did not give any positive interactions for collybistin, and it may be that collybistin activates one of the other GTPases. Although the GTPase activated by collybistin could not be identified using this assay, a number could be eliminated, including Cdc42. This is contrary to the membrane activation model, which suggests that Cdc42 is activated by collybistin at the synapse.

The final method used was a biochemical affinity purification assay, in which activated Cdc42 should bind to PAK and be detected by an antibody specific to Cdc42. However the Cdc42 specific antibody did not detect the positive controls, although proteins had transferred to the membrane.

There is the possibility that collybistin activates one or more of the 23 Rho-family GTPases identified to date. These have been classified into six subfamilies based upon their amino acid structure and biological function. RhoA has 2 highly homologous proteins RhoB and RhoC. The Rac1-subfamily includes Rac1, Rac2, Rac3 and RhoG. The Cdc42-subfamily contains the most members including Cdc42, G25K TC10, TCL, Wrch1 and Chp/Wrch2. The Rnd-subfamily contains Rnd1, Rnd2 and RhoE/Rnd3. The RhoBTB-subfamily has 3 members – RhoBTB1, RhoBTB2 and RhoBTB3. The most recently identified subfamily is the Miro-family containing Miro1 and Miro2. RhoD, Rif and Rho/TTF are also members of the Rho-GTPases family but do not belong to any of the recognised subfamilies. Fig. 4.9 shows a phylogenetic tree of the Rho-family GTPases (adapted from Wennerberg and Der, 2004).

Phenotypic profiles for other members of the Rho-family have been reported, and some of these proteins do induce changes in the actin cytoskeleton. The Rho-like proteins form stress fibres and focal adhesions similar to those induced by RhoA (Chardin, 1988; van Golen et al., 2000). Rac2 and Rac3 form lamellipodia and membrane ruffles akin to those observed with Rac1 (Didsbury et al., 1989; Haataja et al., 1997).
Interestingly, RhoG induces the formation of lamellipodia, membrane ruffles and filopodia, which is similar to the phenotype induced by collybistin (Gauthiere-Rouviere et al., 1998). Not all members of the Cdc42-like family have phenotypes like Cdc42. Chp forms lamellipodia (Aronheim et al., 1998), TCL forms membrane ruffles and Wrch induces long thin filopodial extensions that are distinct to those of Cdc42 (Tao et al., 2001). TC10 induces the formation of lamellipodia and membrane ruffles, which can be inhibited by dominant negative Rac1 and Cdc42, which suggests that TC10 may act downstream of these GTPases, or that they share effector proteins (Vignal et al., 2000). Rif forms long, highly dynamic, actin rich filopodial extensions (Ellis and Mellor, 2000). The RhoBTB and Miro proteins do not appear to have any effect on the actin cytoskeleton (Ramos et al., 2002; Fransson et al., 2003).

Fig. 4.9: Phylogenetic tree of the Rho-family GTPases. The Rho GTPases are divided into six main branches: Rho-like, Rac-like, Cdc42-like, Rnd, RhoBTB and Miro. In addition, there are also RhoD, Rif and RhoH/TTF which are in distinct subfamilies. The other members of the Ras superfamily - Arf1, Ran1, Rab1A, H-Ras and RasA are in grey (adapted from Wennerberg and Der, 2004).
The findings of this chapter were:

- Collybistin does appear to activate a GTPase as evidenced by the actin rearrangements observed upon the expression of recombinant collybistin in NIH3T3 and REF52 fibroblast cells

- The identity of the GTPase is unknown, but it does not appear to be Cdc42.

In Chapter 3, it was shown that the RhoGEF domain of collybistin binds to gephyrin. Gephyrin-collybistin interactions are imperative for the correct localisation of gephyrin clusters in cultured neuronal cells. It was also shown that removal of the PH domain from collybistin results in a trafficking mutant, whereby gephyrin accumulates in the cell body and proximal dendrites in cultured cortical cells (Chapter 3 of this study; Harvey et al., 2004b). These findings, taken with the elusive nature of the GTPase identity, suggest that collybistin may have a secondary role in activities at the synapse, and maybe far more involved in trafficking gephyrin to the synapse. There is no solid evidence to suggest that collybistin is required at synaptic sites for GTPase related control of the actin cytoskeleton. There is also very little evidence to suggest that actin rearrangements are required at postsynaptic sites, as suggested by the membrane activation model. In view of this, a collybistin library screen was conducted to identify any proteins that may interact with collybistin and aid it in the trafficking and clustering of gephyrin. This proved to be more fruitful, and therefore the decision was made to follow up this line of enquiry. The results of these studies are presented in the next chapter.
Chapter 5: Collybistin Interacting Proteins
5.1: Introduction

The previous two chapters have questioned the validity of the membrane activation model (Kneussel and Betz, 2000) which suggests that synaptically located collybistin activates Cdc42, which, in turn, regulates the actin cytoskeleton, thus facilitating the accumulation and clustering of gephyrin and glycine receptors at inhibitory synapses.

Chapter 3 of this study showed that gephyrin binds to the RhoGEF domain of collybistin, the region which is normally associated with the GDP-GTP exchange activity on small G proteins. It was shown that a RhoGEF domain deletion mutant ceased to interact with collybistin in HEK293 cells. Harvey et al. (2004b) demonstrated that the disruption of collybistin-gephyrin interactions resulted in the accumulation of gephyrin within the cell body and proximal dendrites of cultured cortical neurons. It is evident, therefore, that the RhoGEF domain of collybistin may have more than one function – it binds to gephyrin, and also has GDP-GTP exchange activity. The identity of the GTPase catalysed by collybistin was not established in Chapter 4, however, it is clear that collybistin does cause actin rearrangements in fibroblast cells, indicative of GTPase activation.

Chapter 3 also showed that the deletion of the PH domain resulted in a trafficking mutant of collybistin. This mutant retained its ability to bind to gephyrin, but instead of forming submembranous microaggregates in HEK293 cells, it appeared to trap gephyrin within the cytoplasm. This mutant also impeded the trafficking of endogenous gephyrin when transfected into cultured cortical neurons, suggesting that collybistin may play a role in trafficking gephyrin to postsynaptic sites (Harvey et al., 2004b). PH domains are generally thought to regulate the subcellular localisation of proteins through interactions with phosphoinositides. However, the PH domain of collybistin binds to PI3P, which is associated with constitutive membrane traffic. The PH domains of some GEFs have been implicated in facilitating GEF activity and mediating protein-protein interactions (reviewed in Rossman et al., 2005). The collybistin PH domain deletion mutant impeded the trafficking of gephyrin, and as it is not involved in gephyrin binding, it may mediate interactions with other proteins that facilitate the transport of gephyrin from the cell soma to distal dendrites.
SH3 domains are ubiquitous protein-protein interaction domains which bind to proteins with the core motif PXXP (Mayer, 2001). The SH3 domain of collybistin negatively regulates the ability of collybistin to translocate gephyrin to submembranous sites when transfected into HEK293 cells. The majority of collybistin isoforms identified in rat harbour the SH3 domain, as does the human homologue of collybistin – hPEM-2, which implies that this region is of some functional importance. This was emphasised by the discovery of a lethal mutation within the SH3 domain in a hyperekplexic patient (Harvey et al., 2004b).

Proteins do not function as separate entities, rather, they are constituents of large multi-protein complexes. There is a wealth of evidence to show that a number of different classes of proteins are involved in the trafficking and postsynaptic clustering of excitatory amino acid receptors (reviewed in Sheng and Hyoung Lee, 2003; Esteban, 2003). Many proteins are not constrained to one particular function, but are involved in many processes. This may be the case with collybistin. It could have a role in the synaptic clustering of gephyrin as proposed by Kneussel and Betz (2000), and may also be involved in the trafficking of gephyrin to the synapse as indicated by the data presented in Chapter 3 and by Harvey et al. (2004b).

The available literature provides evidence that gephyrin binds to collybistin (Kins et al., 2000), and at best provides a tenuous link to the activation of Cdc42 by collybistin. Therefore, it was a progressive step to investigate whether collybistin interacted with other proteins. To achieve this a yeast two-hybrid cDNA library screen was conducted using CB2$_{SH3}$ as bait.

5.1.1 Aims

The aim of this chapter was:

- To identify proteins that interact with collybistin, and have the potential to aid it in the trafficking and/or clustering of gephyrin.
5.2: Methods

Fig. 5.1 is a schematic representation of the steps involved in conducting the yeast two-hybrid library screen, and the methods required for each step are presented in chapter 2. Procedures which were specific to the library screen are presented in this section.

Fig. 5.1: A flowchart outlining the yeast two-hybrid library screen process.
Chapter 5: Collybistin Interacting Proteins

5.2.1 Library transformation

A single colony of Y190 integrated with pYTH9-CB2\textsubscript{SH3} was inoculated in 100 ml YPD medium and cultured at 30°C overnight. The advantage of using this integrated strain for the library screen was that the library cDNA was introduced into the yeast cell in a single transformation, and recovery of the prey plasmid was simplified as it was the only episomal plasmid present (Fuller et al., 1998). The starter culture was diluted in 400 ml YPD to an OD\textsubscript{600} reading of 0.25 and grown until it reached 0.7. The cells were harvested by centrifugation at 3,000 rpm for 3 min at 20°C. The subsequent pellet was resuspended in distilled water and centrifuged as before. The pellet was washed twice in LiOAc/TE solution (see 2.2.6) to make the yeast cells competent to receive DNA. The washed pellet was resuspended in 1 ml LiOAc/TE. The DNA mix (see 5.2.2) and 9 ml 40% PEG solution was added to the cell suspension, and gently mixed. The cells were incubated at 30°C for 30 min followed by heat shock at 42°C for 20 min. The cell suspension was centrifuged as before, and the pellet was resuspended in 6 ml distilled water and plated onto the selective medium plates (see 5.2.3) and incubated at 30°C until colonies emerged.

5.2.2 Library screen DNA mix

For the library screen DNA mix 100 µl library cDNA (1µg/µl; BD Matchmaker cDNA library) was added to 400 µl freshly denatured carrier DNA, 56 µl LiOAc (10×) and 5.6 µl TE buffer. The library screen cDNA selected for the screen was whole human brain tissue isolated from a 60 y old Caucasian male and cloned into pACT2.

5.2.3 Library screen agar plates

150 mm diameter plates were used for the library screen. The transformed yeast were plated onto agar lacking histidine, leucine and tryptophan (see 2.2.3). The agar was supplemented with 10 mM 3- amino-1,2,4,-triazole (3-AT) dissolved in sterile water and filtered into the cooled agar (<50°C) to counteract the effect of the ‘leaky’ H\textit{I}S\textit{I}3 gene. The native H\textit{I}S\textit{I}3 promoter contains an upstream activating sequence (UAS) recognised by the transcription factor GCN4 and two TATA boxes – one of which is regulated by GCN4 (TR) and one which is constitutively active (TC; Iyer and Struhl, 1995). TC is not regulated by native GCN4-binding UAS, GAL1-UAS or artificial UAS\textsubscript{G} constructs (Mahadevan and Struhl, 1990). However, in Y190 the H\textit{I}S\textit{I}3 gene is under the control of
the GAL1-UAS and a minimal promoter which contains both HIS3 TATA boxes (Flick and Johnston, 1990). Consequently, Y190 exhibits a significant level of constitutive ‘leaky’ expression of the HIS3 gene (due to the TC). 3-AT is a histidine analogue and competitive inhibitor of the HIS3 gene product (IGP-dehydratase), and at the correct concentration it will inhibit growth (Kishore and Shah, 1988; Alexandre et al., 1993). Therefore it was added to the selective dropout agar plates to increase the stringency of the screen. A solution of X-α-gal (80 mg/l) was spread onto the agar plates to aid in the identification of any potential interactors.

5.2.4 Colony selection

Colony growth was monitored daily. Any colonies that appeared blue or were comparatively large were re-streaked onto agar plates lacking leucine and tryptophan and were grown for 3-4 days. Colonies were then replica plated onto agar plates lacking leucine and tryptophan (see 2.2.7). LacZ assays were carried out on colonies lifted onto filter paper (see 2.2.8). The assays were graded at 30 min intervals on the intensity of the colour change. Colonies that became blue were then selected from the corresponding replica plates and the prey plasmid was rescued from the yeast (see 2.2.10). The bait plasmid did not pose any problems when recovering the prey plasmid as it was integrated into the yeast genome. The prey plasmid was retransformed into E.coli and mini-prepped. To establish the size and identity of the cDNA insert, the plasmid DNA was digested with EcoRI and XhoI, which flank the insert and sequenced using the forward GAL4AD and reverse pACT2 M2 primers. The identity of the hit was revealed by carrying out a BLAST search of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). The plasmid was also back checked against pYTH16 and pYTH9-CB2SH3. to verify the interaction.

5.2.5 Analysis of chosen ‘hits’

Once the initial identification of the interactors had been conducted, some were chosen for further analysis. The selected hits were tested against pYTH9-CB2SH3ΔRhoGEF, pYTH16-RhoGEF, pYTH16-PH and pGBK7-hPEM-2 (see section 3.2) to identify potential binding sites for these proteins on collybistin. These studies were carried out in Y190 and AH109 yeast cells as previously described.
Chapter 5: Collybistin Interacting Proteins

The amino acid sequences of these proteins were also analysed for any known protein domains using the protein family (Pfam) website (http://pfam.wustl.edu/). Pfam is a collection of multiple sequence alignments and hidden Markov models (HMM) covering many protein families. HMM are statistical models developed to determine hidden patterns using visible patterns - the multiple sequence alignments were used to create HMM profiles, which were then used to identify protein domains in uncharacterised sequences (Ramanathan and Davison, 2002). The analysis presented here was carried out using Pfam version 17.0 (March 2005), which contains the alignments of 7868 protein families based on SwissProt 46.0 and Sp-TrEMBL protein sequence database. Pfam allocated each identified protein domain within a given amino acid sequence an E-value. This number represents the statistical analysis and probability of that particular domain. The smaller the value, the higher the likelihood of the amino acid sequence containing that domain. The E-value of the protein domains of each of the hits will be presented in the following results section.

5.3 Results

A CB2SH3 library screen was conducted to identify any potential interactors that may contribute to the role collybistin plays in the trafficking and clustering of gephyrin. Colonies that appeared to be blue or were considerably large were selected. All the selected plasmids were rescued from the yeast cells, digested with EcoRI/XhoI to elucidate the size of the cDNA insert, DNA sequenced and identified by searching the NCBI database. A total of 47 clones were isolated from the library screen including kinases, hypothetical proteins, chaperone proteins, adhesion proteins, intracellular receptors and gephyrin, which is a known interactor of collybistin. However, a GTPase was not isolated in the library screen, but this could be due to the transient nature of the interaction between a GEF and its GTPase. A complete list of the hits isolated in the library screen can be found in appendix 1. Table 5.1 summarises the hits that were selected for further analysis. In addition to the CB2SH3 screen presented in this chapter, a CB2SH3+ screen was also conducted, which yielded a large number of clones, one of which, KIF3A, will be presented in this chapter.
<table>
<thead>
<tr>
<th>Sequence ID</th>
<th>Chromosome location</th>
<th>Synonyms</th>
<th>GenBank accession numbers</th>
<th>X-gal assay</th>
<th>Insert size kb</th>
<th>In frame</th>
<th>Backcheck against pYTH16</th>
<th>Backcheck against pYTH9-CB2SH3-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuronal Guanosine nucleotide Exchange Factor (NGEF) – Rho-family GEF</td>
<td>2q37</td>
<td>Ephexin</td>
<td>NM_019850 NP_062824.1</td>
<td>++</td>
<td>1.7</td>
<td>Yes</td>
<td>Negative</td>
<td>++</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor - associated protein - like 2 (GABARAPL2) – involved in intra-Golgi transport</td>
<td>16q22.2-q24.1</td>
<td>Golgi associated ATPase Enhancer of 16 kDa GATE-16</td>
<td>NM_007285 NP_009216.1</td>
<td>+</td>
<td>1.0</td>
<td>Yes</td>
<td>Negative</td>
<td>+</td>
</tr>
<tr>
<td>Homo sapiens kinesin family member 5C (KIF5C) – motor protein</td>
<td>2q23.3</td>
<td>KINN, NKHC, NKHC2, NKHC-2</td>
<td>NM_004522 NP_004513.1</td>
<td>+++</td>
<td>4.0</td>
<td>Yes</td>
<td>Negative</td>
<td>+++</td>
</tr>
</tbody>
</table>
| Gephyrin (GPHN);* | 14q23.3 | - | NM_020806 NP_065857.1 | ++++ | 3.1 | No* | Negative | +++

Table 5.1: A summary of the selected proteins isolated from the mammalian brain cDNA library screen performed using pYTH9-CB2SH3- as bait. Plasmid cDNAs for potential interactors were rescued from the yeast, digested with EcoRI/XhoI to determine its insert size, and DNA sequenced for identification and to ensure it was in the correct reading frame. The preys were backchecked against pYTH16 and pYTH9-CB2SH3-, in order to eliminate any false positives and ensure the veracity of the interaction. In total, 47 clones were isolated from the library screen, however only those which were selected for further analysis are presented above. Note that gephyrin, which is a known interactor of collybistin, was also isolated from the library screen. LacZ assays were graded upon the intensity of the colour change: - no colour after 3h; ++++ colour change seen within 30 min of assay and a very intense colour after 3 h. *common for collybistin YTH screens.
5.3.1 NGEF

5.3.1.1 LacZ assays in Y190

Neuronal specific GTPase Exchange Factor (NGEF) was chosen for further analysis, and Fig. 5.2A shows the amino acid sequence of this protein, with the portion recovered in the library screen underlined. Initial indications were that this protein did not interact with empty pYTH16, thus excluding the possibility of it being a false positive, and gave a ++ strength interaction with pYTH9-CB2SH3. (see Table 5.1).

Subsequent experiments in Y190 showed that pACT2-NGEF expressed with either pYTH9-CB2SH3 or pGBK7-hPEM-2 colonies began to change from white to blue 1 h after the start of the assay, and when the assays were terminated after 3 h an intense blue colour was observed (Fig. 5.2C&F). Fig. 5.2D&E show that the colonies remained white when pACT2-NGEF was co-transformed with either pYTH16 or pGBK7, respectively. NGEF also gave a fairly strong blue colour when expressed with pYTH9-CB2SH3-ARhoGEF, which is the RhoGEF domain deletion mutant of collybistin (Fig. 5.2G). The Y190 colonies co-transformed with pYTH16-RhoGEF and pACT2-NGEF remained white 3 h after the start of the LacZ assay (Fig. 5.2H). The colonies also remained white when co-transformed with pYTH16-PH and pACT2-NGEF (Fig. 5.2I). All the assays presented in Fig. 5.2 were stopped after 3 h.

5.3.1.2 Growth assays in AH109

Table 5.2 shows the rate of colony growth for pACT2-NGEF when transformed into AH109 integrated with either pYTH9-CB2SH3 or pYTH9-CB2SH3-ARhoGEF. AH109 cells were also co-transformed with pACT2-NGEF and either pYTH16, pYTH16-RhoGEF or pYTH16-PH. Cells were plated onto selective dropout medium lacking histidine, adenine, leucine and tryptophan. Plates were monitored daily for emerging colonies and yeast were lifted after eight days.

Colonies of cells expressing pYTH9-CB2SH3 and pACT2-NGEF began to emerge on day five. These were very small in size, but by day eight were large enough to be lifted. Colonies of cells expressing pYTH9-CB2SH3-ARhoGEF and pACT2-NGEF also appeared five days after transformation and were lifted on day eight.
Chapter 5: Collybistin Interacting Proteins

Fig. 5.2: NGEF - Amino acid sequence of NGEF with the portion recovered in the library screen underlined (A). Model of NGEF Pfam protein domains and their E-values - RhoGEF, PH and SH3 (B). Interactions of pACT2-NGEF in yeast (Y190) with pYTH9-CB2SH3 (C), pYTH16 (D), pGBKKT7 (E), pGBKKT7-hPEM-2 (F), pYTH9-CB2SH3-ΔRhoGEF (G), pYTH16-RhoGEF domain (H) and pYTH16-PH domain (I). Positive interactions are indicated by the blue colouring.
The AH109 cells co-transformed with pACT2-NGEF and either pYTH16, pYTH16-RhoGEF or pYTH16-PH were incubated for 14 days, however, colonies did not emerge on any of these plates.

| Table 5.2: Growth assays for NGEF in AH109: Yeast were transformed as described and plated onto selective dropout medium lacking histidine, adenine, leucine and tryptophan. |
|-------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| BAIT                    | Prey: pACT2-NGEF   | Days post transformation |
|                         | pYTH16             | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   |
|                         | pYTH9-CB2SH3       | -   | -   | -   | -   | -   | -   | -   | -   |
|                         | pYTH9-CB2SH3ΔRhoGEF| -   | -   | -   | (-+) | +   | +   | ++  |
|                         | pYTH16-RhoGEF      | -   | -   | -   | -   | -   | -   | -   | -   |
|                         | pYTH16-PH          | -   | -   | -   | -   | -   | -   | -   | -   |

5.3.2 GATE-16

5.3.2.1 LacZ assays in Y190

Golgi associated ATPase Enhancer of 16 kDa (GATE-16) was chosen for further analysis, and Fig. 5.3A shows the amino acid sequence of this protein, with the portion recovered from the library screen underlined. Initial indications were that this protein did not interact with empty pYTH16, thus excluding the possibility of it being a false positive, and gave a + strength interaction with pYTH9-CB2SH3 (see Table 5.1), which is relatively weak compared to other interactors.

Subsequent experiments in Y190 showed that pACT2-GATE-16 expressed with either pYTH9-CB2SH3 or pGBK7-hPEM-2 colonies began to change from white to blue 1.5 h after the start of the assay, and when the assay was terminated after 3 h, a blue colour was observed (Fig. 5.3C&F). Fig. 5.3D&E show that colonies remained white when pACT2-GATE-16 was co-transformed with either pYTH16 or pGBK7, respectively. GATE-16 also showed a relatively weak blue colour when expressed with pYTH9-CB2SH3ΔRhoGEF, which is the RhoGEF domain deletion mutant of collybistin (Fig. 5.4G). The Y190 colonies co-transformed with pYTH16-RhoGEF and pACT2-GATE-16 remained white 3 h after the start of the LacZ assay (Fig. 5.3H). The colonies also remained white when co-transformed with pYTH16-PH and pACT2-GATE-16 (Fig. 5.3I). All the assays presented in Fig. 5.3 were terminated at 3 h.
**Chapter 5: Collybistin Interacting Proteins**

### A

MKWMFKEDHSLEHRCVESAKIRAKYPDRVPVIVEKVSGQIVDIDKRKYLVPSDITVAQPMWIIRKRIQLPSEKAIFLFVDKTVPQSSLTMGQLYEKEKDGEDFLYVAYSGENTFGF

### B

<table>
<thead>
<tr>
<th>Domain; amino acids</th>
<th>Pfam Accession Number</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP1 LC3: 13-116</td>
<td>PF02991</td>
<td>1.2E-79</td>
</tr>
</tbody>
</table>

### C

![Image](image_url)

**Fig. 5.3: GATE-16** - Amino acid sequence of GATE-16 with the portion recovered in the library screen underlined (A). Model of GATE-16 Pfam protein domain - MAP1 LC3 (B). Interactions of pACT2-GATE-16 in yeast (Y190) with pYTH9-CB2SH3 (C), pYTH16 (D), pGBK7 (E), pGBK7-hPEM-2 (F), pYTH9-CB2SH3ΔRhoGEF (G), pYTH16-RhoGEF domain (H) and pYTH16-PH domain (I). Positive interactions are indicated by the blue colouring.
Chapter 5: Collybistin Interacting Proteins

5.3.2.2 Growth assays in AH109

Table 5.3 shows the rate of colony growth for pACT2-GATE-16 when transformed into AH109 integrated with either pYTH9-CB2SH3 or pYTH9-CB2SH3ΔRhoGEF. AH109 cells were also co-transformed with pACT2-GATE-16 and either pYTH16, pYTH16-RhoGEF or pYTH16-PH. Cells were plated onto selective dropout medium lacking histidine, adenine, leucine and tryptophan. Plates were monitored daily for emerging colonies and were lifted after 10 days.

Colonies of cells expressing pYTH9-CB2SH3 and pACT2-GATE-16 began to emerge on day seven. These were small in size, but by day 10 were large enough to be lifted. Colonies of cells expressing pYTH9-CB2SH3ΔRhoGEF and pACT2-GATE-16 appeared eight days after transformation and were lifted on day 10. The AH109 cells co-transformed with pACT2-GATE-16 and either pYTH16, pYTH16-RhoGEF or pYTH16-PH were incubated for 14 days, however, colonies did not emerge on any of these plates.

<table>
<thead>
<tr>
<th>Prey: pACT2-GATE-16</th>
<th>Days post transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>pYTH16</td>
<td>-</td>
</tr>
<tr>
<td>pYTH9-CB2SH3</td>
<td>-</td>
</tr>
<tr>
<td>pYTH9-CB2SH3ΔRhoGEF</td>
<td>-</td>
</tr>
<tr>
<td>pYTH16-RhoGEF</td>
<td>-</td>
</tr>
<tr>
<td>pYTH16-PH</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.3: Growth assays for GATE-16 in AH109: Yeast were transformed as described and plated onto selective dropout medium lacking histidine, adenine, leucine and tryptophan.

5.3.3 KIF5C

5.3.3.1 LacZ assays in Y190

KIF5C, a neuronal specific motor protein of the kinesin superfamily, was chosen for further analysis, and Fig. 5.4A shows the amino acid sequence of this protein, with the portion recovered from the library screen underlined. Initial investigations revealed that pACT2-KIF5C did not interact with pYTH16, thus excluding the possibility that it was a false positive interaction, and gave a +++ strength interaction with pYTH9-CB2SH3. (see Table 5.1).
Chapter 5: Collybistin Interacting Proteins

Subsequent experiments in Y190 showed that pACT2-KIF5C expressed with either pYTH9-CB2SH3 or pGBK77-hPEM-2 colonies began to change from white to blue 1 h after the start of the assay, and when the assay was terminated after 3 h, an intense blue colour was observed (Fig. 5.4C&F). Fig. 5.4D&E show that the colonies remained white when pACT2-KIF5C was co-transformed with either pYTH16 or pGBK77, respectively. KIF5C also showed a fairly strong blue colour when expressed with pYTH9-CB2SH3ΔRhoGEF, which is the RhoGEF domain deletion mutant of collybistin (Fig. 5.4G). The Y190 colonies co-transformed with pYTH16-RhoGEF and pACT2-KIF5C remained white 3 h after the start of the \textit{LacZ} assay (Fig. 5.4H). The colonies also remained white when co-transformed with pYTH16-PH and pACT2-KIF5C (Fig. 5.4I). All the assays presented in Fig. 5.4 were terminated at 3 h.

5.3.3.2 Growth assays in AH109

Table 5.4 shows the rate of colony growth for pACT2-KIF5C when transformed into AH109 integrated with either pYTH9-CB2SH3 or pYTH9-CB2SH3ΔRhoGEF. AH109 cells were also co-transformed with pACT2-KIF5C and either pYTH16, pYTH16-RhoGEF or pYTH16-PH. Cells were plated onto selective dropout medium lacking histidine, adenine, leucine and tryptophan. Plates were monitored daily for emerging colonies and were lifted after eight days.

Colonies of cells expressing pYTH9-CB2SH3 and pACT2-KIF5C began to emerge on day five. These were small in size, but by day eight were large enough to be lifted. Colonies of cells expressing pYTH9-CB2SH3ΔRhoGEF appeared six days after transformation and were lifted on day eight. The AH109 cells co-transformed with pACT-KIF5C and either pYTH16, pYTH16-RhoGEF or pYTH16-PH were incubated for 14 days, however, colonies did not emerge on any of these plates.

<table>
<thead>
<tr>
<th>Prey: pACT2-KIF5C</th>
<th>Days post transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>BAIT</td>
<td></td>
</tr>
<tr>
<td>pYTH16</td>
<td>-</td>
</tr>
<tr>
<td>pYTH9-CB2SH3</td>
<td>-</td>
</tr>
<tr>
<td>pYTH9-CB2SH3ΔRhoGEF</td>
<td>-</td>
</tr>
<tr>
<td>pYTH16-RhoGEF</td>
<td>-</td>
</tr>
<tr>
<td>pYTH16-PH</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.4: Growth assays for KIF5C in AH109: Yeast were transformed as described and plated onto selective dropout medium lacking histidine, adenine, leucine and tryptophan.
Chapter 5: Collbystin Interacting Proteins

### Table 5.4: KIF5C - Amino acid sequence of KIF5C with the portion of the cargo binding domain recovered in the library screen underlined (A). Model of KIF5C Pfam protein domain and E-values - motor domain, and also the neck and cargo binding domains (B). Interactions of pACT2-KIF5C in yeast (Y190) with pYTH9-CB2_Shi3. (C), pYTH9-CB2_Shi3, (D), pYBH7 (E), pYBH7-hPEM-2 (F), pYTH9-CB2_Shi3,ΔRhoGEF (G), pYTH16-RhoGEF domain (H) and pYTH16-PH domain (I). Positive interactions are indicated by the blue colouring.

#### A
MADPAECSIKVMCRFRPLNEAEILRGDKFIPKFKGDETWIGQGKPYVFDRVLPPNTTQEVYNAIIQIKDVLVEQYNTIFAYGQTSGKTHMCKLHDQQLMGRIHAIHDFHISMDENLEFHIKVSTFYIELL

#### B
<table>
<thead>
<tr>
<th>Domain; amino acids</th>
<th>Pfam Accession Number</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinesin Motor Domain; 14-328</td>
<td>PF00225</td>
<td>1.8e-170</td>
</tr>
</tbody>
</table>

#### C
![Fig. 5.4: KIF5C](image)

```
Fig. 5.4: KIF5C - Amino acid sequence of KIF5C with the portion of the cargo binding domain recovered in the library screen underlined (A). Model of KIF5C Pfam protein domain and E-values - motor domain, and also the neck and cargo binding domains (B). Interactions of pACT2-KIF5C in yeast (Y190) with pYTH9-CB2_Shi3, (C), pYTH9-CB2_Shi3, (D), pYBH7 (E), pYBH7-hPEM-2 (F), pYTH9-CB2_Shi3,ΔRhoGEF (G), pYTH16-RhoGEF domain (H) and pYTH16-PH domain (I). Positive interactions are indicated by the blue colouring.
```
5.3.4 KIF3A

KIF3A was one of the proteins isolated in the CB2\textsubscript{SH3} library screen. It is a motor protein of the kinesin superfamily and was chosen for further analysis as it is related to KIF5C (identified in the CB2\textsubscript{SH3} screen), and may have a plausible functional role with collybistin. Fig. 5.5A shows the amino acid sequence of this protein, with the portion recovered in the library screen underlined. In Y190 cells expressing pACT2-KIF3A with either with pYTH9-CB2\textsubscript{SH3} or pGBKT7-hPEM-2 colonies began to change from white to blue 30 min after the start of the assay, and when the assay was terminated after 3 h, an intense blue colour was observed (Fig. 5.5C&F). Fig. 5.5D&E show that the colonies remained white when pACT2-KIF3A was co-transformed with either pYTH16 or pGBKT7, respectively.

\[
\begin{align*}
\text{A} & \quad \text{MPINKSEKPESDWVYVVCBPI} \text{N} \text{ERKSMCYKQAVSVDEMRGTTITVHKTDSSNEP} \text{PKTFTFD} \text{TVFGPE} \text{SKQLD} \text{VNYLTAIPFIISSVLEGYN} \text{TIFAYGQCTGT} \text{CTFTMEGVRAIPE} \text{L} \text{I} \text{P} \text{MSFAHIFGHI} \text{AK} \text{AE} \text{GD} \\
& \quad \text{TRFLVRVS} \text{LYMEEVRLDL} \text{GKDOTQ} \text{RELP} \text{VD} \text{G} \text{YIKD} \text{SAYV} \text{VNAD} \text{M} \text{DTI} \text{ML} \text{GHKNR} \text{SVGA} \\
& \quad \text{TNN} \text{HES} \text{S} \text{R} \text{SH} \text{AIT} \text{IT} \text{IC} \text{ESE} \text{C} \text{G} \text{I} \text{D} \text{G} \text{M} \text{H} \text{R} \text{M} \text{G} \text{K} \text{L} \text{H} \text{L} \text{V} \text{D} \text{L} \text{A} \text{S} \text{E} \text{R} \text{Q} \text{A} \text{T} \text{G} \text{T} \text{G} \text{Q} \text{R} \text{L} \text{E} \text{AT} \text{K} \text{I} \text{N} \text{L} \text{S} \text{L} \text{S} \text{L} \\
& \quad \text{G} \text{N} \text{V} \text{I} \text{A} \text{S} \text{L} \text{D} \text{G} \text{K} \text{S} \text{TH} \text{Y} \text{P} \text{R} \text{N} \text{S} \text{K} \text{R} \text{L} \text{D} \text{Q} \text{S} \text{L} \text{G} \text{N} \text{S} \text{K} \text{T} \text{M} \text{C} \text{A} \text{N} \text{I} \text{G} \text{P} \text{A} \text{D} \text{Y} \text{N} \text{D} \text{E} \text{T} \text{I} \text{S} \text{T} \text{L} \text{R} \text{Y} \text{A} \text{N} \text{R} \text{A} \text{K} \text{N} \text{I} \text{K} \text{N} \text{K} \text{A} \\
& \quad \text{I} \text{N} \text{E} \text{D} \text{P} \text{K} \text{D} \text{A} \text{L} \text{L} \text{R} \text{Q} \text{F} \text{K} \text{E} \text{RI} \text{E} \text{L} \text{K} \text{K} \text{L} \text{E} \text{E} \text{G} \text{I} \text{S} \text{G} \text{S} \text{I} \text{D} \text{G} \text{E} \text{E} \text{E} \text{D} \text{D} \text{D} \text{E} \text{E} \text{G} \text{E} \text{V} \text{G} \text{D} \text{E} \text{G} \text{E} \text{K} \text{R} \text{K} \text{R} \text{G} \text{K} \text{K} \text{V} \text{S} \text{P} \text{D} \text{K} \text{M} \\
& \quad \text{E} \text{M} \text{Q} \text{A} \text{K} \text{I} \text{D} \text{E} \text{E} \text{R} \text{K} \text{A} \text{E} \text{T} \text{K} \text{L} \text{D} \text{M} \text{E} \text{E} \text{E} \text{E} \text{R} \text{N} \text{K} \text{A} \text{E} \text{L} \text{K} \text{R} \text{E} \text{K} \text{D} \text{L} \text{L} \text{K} \text{A} \text{Q} \text{Q} \text{E} \text{H} \text{Q} \text{S} \text{L} \text{E} \text{L} \text{K} \text{L} \text{S} \text{A} \text{L} \text{E} \text{K} \text{V} \text{I} \text{G} \text{G} \text{V} \text{D} \text{L} \text{L} \text{A} \text{K} \text{A} \\
& \quad \text{E} \text{E} \text{E} \text{Q} \text{K} \text{L} \text{E} \text{E} \text{S} \text{N} \text{M} \text{E} \text{E} \text{E} \text{R} \text{R} \text{K} \text{A} \text{E} \text{Q} \text{L} \text{R} \text{L} \text{E} \text{E} \text{E} \text{K} \text{Q} \text{R} \text{L} \text{D} \text{I} \text{E} \text{E} \text{K} \text{T} \text{Y} \text{T} \text{S} \text{L} \text{Q} \text{E} \text{A} \text{Q} \text{G} \text{K} \text{T} \text{K} \text{L} \text{K} \text{K} \text{V} \text{W} \text{T} \text{M} \text{L} \text{M} \text{A} \text{A} \text{S} \text{E} \\
& \quad \text{A} \text{L} \text{D} \text{Q} \text{Q} \text{E} \text{H} \text{Q} \text{R} \text{E} \text{I} \text{E} \text{G} \text{L} \text{E} \text{R} \text{N} \text{Q} \text{L} \text{S} \text{R} \text{E} \text{L} \text{R} \text{L} \text{I} \text{D} \text{N} \text{F} \text{I} \text{P} \text{R} \text{D} \text{Y} \text{Q} \text{E} \text{N} \text{Y} \text{H} \text{W} \text{N} \text{D} \text{I} \text{G} \text{E} \text{W} \text{L} \text{K} \text{C} \text{A} \text{V} \text{T} \text{G} \text{N} \text{M} \text{R} \\
& \quad \text{K} \text{T} \text{P} \text{P} \text{D} \text{K} \text{E} \text{K} \text{D} \text{P} \text{F} \text{E} \text{V} \text{D} \text{L} \text{S} \text{H} \text{V} \text{L} \text{A} \text{Y} \text{E} \text{E} \text{S} \text{L} \text{R} \text{Q} \text{S} \text{L} \text{K} \text{L} \text{E} \text{R} \text{P} \text{T} \text{S} \text{G} \text{K} \text{A} \text{R} \text{P} \text{T} \text{K} \text{G} \text{R} \text{K} \text{R} \text{S} \text{A} \text{K} \text{P} \text{E} \text{T} \text{V} \text{I} \text{D} \text{L} \text{L} \\
\end{align*}
\]

Fig. 5.5: KIF3A - Amino acid sequence of KIF3A with the portion recovered in the library screen underlined (A). Model of KIF3A Pfam protein domain and E-value - motor domain, and also the neck and cargo binding domains (B). Interactions of pACT2-KIF3A in yeast (Y190) with pYTH9-CB2\textsubscript{SH3}. (C), pYTH16 (D), pGBKT7 (E) and pGBKT7-hPEM-2 (F). Positive interactions are indicated by the blue colouring.
Chapter 5: Collybistin Interacting Proteins

5.4 Discussion

A total of 47 clones were isolated from the CB2SH3 library screen, one of which was gephyrin. Three proteins were selected for further analysis as they gave robust interactions with CB2SH3 or had a plausible biological interaction with collybistin. A GAL4 yeast two-hybrid library screen was also carried out using CB2SH3 as bait, however this yielded many artifactual sequences harbouring SH3 domain binding PXXP motifs. Therefore only the CB2SH3 screen was followed up. However, one protein isolated in the CB2SH3 screen was included in this thesis as it is related to one of the CB2SH3 hits. The proteins which were selected for further analysis were NGEF, GATE-16, KIF5C, and KIF3A which was isolated from the CB2SH3 screen.

5.4.1 NGEF and collybistin

NGEF, like collybistin, is a member of the Dbl-like family of GEFs (Rodrigues et al., 2000). It is 710 amino acids long (Fig. 5.2A), and like other members of this family, contains the characteristic tandem RhoGEF and PH domains. NGEF also has a C-terminal SH3 domain (Fig. 5.2B). NGEF showed a strong blue colour with both CB2SH3 and hPEM-2 in the LacZ assay, which is indicative of an interaction. A positive interaction was also seen between the RhoGEF domain deletion mutant and NGEF, suggesting that NGEF does not bind to the RhoGEF domain of collybistin, this was confirmed by the white colonies observed when NGEF was co-expressed with just the RhoGEF domain. A negative LacZ assay was also observed for NGEF and the PH domain. Nutritional section studies concurred with the results from the LacZ assays. From these data it is evident that collybistin interacts with NGEF, however, the functional consequence of this interaction is not understood.

There is very little data available about human NGEF, however there are some for the murine and rat homologues. Throughout this section the mouse homologue will be referred to as Ngef (Rodrigues et al., 2000), and the rat homologue as ephexin (Shamah et al., 2001), as cited in the literature.

The expression pattern of Ngef and ephexin revealed that this protein is brain specific. Within the murine brain, Ngef is predominantly expressed in the caudate nucleus, and to a lesser extent in the hippocampus and amygdala. Expression patterns suggest a role in
early development as the highest level of expression was detected at day E7 followed by weaker signals at days 11, 15 and 17 (Rodrigues et al., 2000).

Ephexin was initially identified in a yeast two-hybrid screen as an interactor of the EphA4 receptor (Shamah et al., 2001). Ephexin differs from Ngef at the N-terminus, which is highly hydrophobic, suggesting that it may be involved in targeting or anchoring the protein to the inner surface of the plasma membrane. The classic RhoGEF/PH domain, SH3 domain and C-terminus were identical to those of Ngef. The greatest levels of ephexin expression were detected in the brain and spinal cord with lower levels in the testis, kidney and liver. These transcripts were found to be slightly larger than those found in the CNS, suggesting alternatively spliced variants. Ephexin was not detected in the heart, lung, skeletal muscle or spleen. As with Ngef, expression levels of ephexin also varied through development. It was first detected at E15, increasing rapidly to reach a peak at P10, and then gradually declining. The expression profile of ephexin mimics that of EphA receptors thus implying ephexin is important for Eph receptor function (Shamah et al., 2001).

Ephrin is the ligand which activates Eph receptors and the Eph receptor/ephrin system regulates many cellular functions that depend upon cytoskeletal remodelling such as axon guidance and synaptic plasticity. There are eight distinct ligands identified in mammals, and these are divided into two sub-groups. Ephrin-A (ephrin A1-A5) is anchored to the membrane by glycosylphosphatidylinositol (GPI), and ephrin-B (ephrin B1-B3) contains a transmembrane domain. The Eph receptors are one of the largest receptor tyrosine kinase families discovered. To date, there are 14 known receptors, which are divided into two sub-groups. EphA receptors (EphA1-8) are generally activated by ephrin-A ligands, and EphB receptors (EphB1-4 and EphB6) are generally activated by ephrin-B ligands. The influence of Eph/ephrin activation differs depending on cell type, but they are generally associated with mediating repulsion cues between neighbouring cells, or with the regulation of the neuronal growth cone. However, in some cases Eph/ephrin activation can lead to increased adhesion or attraction between cells (reviewed in Huot, 2004).

Ephexin interacts with the EphA4 receptor and is implicated in the function of these proteins in axon guidance. In the absence of ephrinA, ephexin catalyses GDP-GTP exchange on RhoA, Cdc42 and to a lesser extent Rac 1. Upon activation of EphA
receptors, the effect of ephexin on Cdc42 and Rac1 decreases and subsequently PAK1 is
down-regulated, whereas its activity upon RhoA increases. To see the effects of
ephexin on growth cone motility, a mutant which no longer possesses GEF activity, or
has the ability to bind potentially important SH3 co-regulators but still binds to EphA
receptors (thus uncoupling endogenous ephexin from EphA receptors) was used in
retinal ganglion cells (RGC). The authors (Shamah et al., 2001) showed that the mutant
suppressed EphA-mediated growth cone collapse and proposed that the function of
ephexin was to mediate EphA regulated growth cone motility through the action of
Rho-family GTPase signalling pathways. Correct growth cone motility is imperative
for neuronal development as it is a specialised structure found at the tip of migrating
neurites which reacts to external guidance cues in order to lay down the map of the
nervous system (Jockusch et al., 1997; Gordon-Weeks, 2004).

The interaction between NGEF and collybistin is of particular interest as it raises the
possibility that a dimeric complex may be required for collybistin to act as a GEF. In
Chapter 3 it was shown that collybistin has the ability to bind to itself although the
functional significance of this remains unknown. It was also shown that the RhoGEF
domain was the binding site for gephyrin, thus suggesting at least two roles for this
region. It could be possible that a collybistin homodimer or heterodimer (formed with
NGEF) may be required for GEF activity, whilst a single collybistin molecule suffices
for gephyrin interactions. The GEF Lsc/p115RhoGEF homo-oligomerises through its
C-terminal coiled-coil domain, and removal of this domain results in a gain of function
(Eisenhaure et al., 2003). It may also be possible that NGEF somehow disables the
negative regulation conferred upon collybistin by its SH3 domain. Although a GEF has
been identified as a potential interactor of collybistin, it is not evident what its role
could be.

5.4.2 GATE-16 and collybistin

GATE-16 was selected because it shares 57% amino acid identity with GABA<sub>A</sub>
receptor associated protein (GABARAP), a protein that is thought to link GABA<sub>A</sub>
receptors to microtubules via the γ2 subunit, although it is scarcely detected at synaptic sites but is
heavily implicated in the trafficking of these receptors (reviewed in Lüscher and Keller,
2004). GATE-16 is 117 amino acids long (Fig. 5.3A) and has a basic N-terminus, and a
C-terminus light chain 3 domain (LC3 – which is a subunit of the neuronal microtubule
Chapter 5: Collybistin Interacting Proteins

associated proteins MAP1A and MAP1B – Fig. 5.3B; Mann and Hammarback, 1994). It is involved in intra-Golgi transport (Legesse-Miller et al., 1998), and may aid collybistin in transporting gephyrin from the Golgi apparatus to synaptic sites.

GATE-16 showed a fairly weak blue colour change with both CB2SH3 and hPEM-2 in the LacZ assay, however, this is still indicative of an interaction, albeit a transient or weak one. A positive interaction was also seen between the RhoGEF domain deletion mutant and GATE-16, suggesting that GATE-16 does not bind to the RhoGEF domain of collybistin. This was confirmed by the white colonies observed when GATE-16 was co-expressed with just the RhoGEF domain. A negative LacZ assay was also observed for GATE-16 and the PH domain. Nutritional selection studies concurred with the results of the LacZ assays. From these data it is evident that collybistin interacts with GATE-16, however, it appears to be a rather weak interaction. The functional consequence of this interaction is not clear, but may be involved in the trafficking of gephyrin from the Golgi to synapses.

Northern blot analysis revealed that GATE-16 is constitutively expressed in all human and murine tissues tested. High levels of expression were detected in brain, heart, testis, prostate, ovary, spleen and skeletal muscle and lower levels in lung, thymus and small intestine tissue (Xin et al., 2001). GATE-16 was localised to the Golgi apparatus (Sagiv et al., 2000) and was initially isolated using a functional assay to identify cytosolic factors involved in intra-Golgi transport. It was found to be involved in the docking and fusion of transport vesicles to their target membrane (Legesse-Miller et al., 1998). Subsequently, it was shown to enhance the ATPase activity of N-ethyl-maleimide-sensitive factor (NSF). NSF and α-soluble NSF attachment protein (α-SNAP) catalyse the disassembly of SNAP receptor (SNARE) complexes after a round of fusion and also recruit GATE-16 to Golgi specific v-SNARE (GOS-28) which protects GOS-28 from proteolysis (Sagiv et al., 2000; Muller et al., 2002). Therefore, GATE-16 seems to provide protection to Golgi-SNARE complexes in membrane fusion events.

GABARAP and LC3 have been implicated in a ubiquitin-like conjugation system involved in autophagocytosis. Autophagocytosis is a non-selective mechanism by which cytoplasmic proteins and organelles are sequestered and degraded by lysosomes. There are a number of systems and pathways involved in the degradation of proteins. The yeast systems have been extensively studied and parallels can be drawn between
Chapter 5: Collybistin Interacting Proteins

these and mammalian systems. Two of these (Atg12 and Atg7/LC3) are essential in
yeast and are conserved in man (reviewed in Elazar et al., 2003; Mizushima et al., 2003;
Tanida et al., 2004). In vitro studies have shown that GATE-16, GABARAP and LC3
act as modifiers in these systems, thus having a role in autophagocytosis (Tanida et al.,

In 2000, Okazaki et al. showed that GATE-16 interacts with the Unc-51-like kinase
ULK1. This kinase was initially discovered as the human homologue of the C.elegans
kinase Unc-51 (Kuroyanagi et al., 1998). ULK1 is a 112.6 kDa protein and shares 41%
amino acid similarity with Unc-51 and 21% with Apg1, the yeast homologue which is
involved in autophagocytosis. ULK1 is a serine/threonine kinase and is ubiquitously
expressed in human tissue including skeletal muscle, brain, heart, lung, pancreas,
placenta, liver and kidney. Human ULK1 function is unclear, however, it has been
shown that expression of Unc-51 mutants result in defects in axonal elongation, with
most neurons exhibiting enlarged axon diameters, premature axon termination, or
abnormal vesicles and cisternae-like structures within the axon (Hedgecock et al., 1985;
McIntire et al., 1992). The mouse homologue of Unc-51 was shown to be involved in
axonal elongation in cerebellar cells (Tomoda et al., 1999). The interaction of ULK1
with GATE-16 and GABARAP indicates that it may play a role in vesicular transport.
To support this theory, the interaction between GABARAP and ULK1 was studied
(Okazaki et al., 2000). Both proteins display strong co-localisation in the rat
hippocampus, olfactory bulb and cerebellum, areas rich in GABA_A receptor γ2 subunits,
which are essential for the clustering of functional GABA_A receptors at postsynaptic
sites (Essrich et al., 1998). Starvation induces autophagocytosis in cells as the proteins
that are degraded can be used to provide amino acids for metabolic processes including
gluconeogenesis (Blommaart et al., 1997). Insulin inhibits autophagocytosis (Pfeifer,
1978), and is required for the clustering of functional GABA_A receptors (Wan et al.,
1997). Therefore, it appears that both autophagocytosis and GABA_A receptor
trafficking are regulated by vesicle trafficking involving GABARAP or a related protein
which is controlled by an insulin signal through an as yet unknown mechanism. By
considering these facts, it can be speculated that GABARAP and ULK1 are involved in
the trafficking of GABA_A receptors or other vesicle transport that is crucial for axonal
elongation.

137
5.4.3 KIF5C and collybistin

The last protein selected from the CB2SH3- library screen was kinesin family 5C (KIF5C) as it had a strong interaction with collybistin and also has a biologically plausible function. KIF5C is a neuronal-specific heavy chain of the motor protein kinesin-1 and is involved in the intracellular trafficking of organelles and membrane proteins along microtubule tracks within the axon and dendrites of neurons. It is 957 amino acids long with a predicted molecular weight of 107 kDa (Fig. 5.4A). KIF5C is composed of three distinct domains, a N-terminal motor domain, a central neck region and a C-terminal cargo binding domain (Fig. 5.4B). Collybistin binds to the C-terminal cargo binding domain. KIF5C showed a very strong blue colour with both CB2SH3- and hPEM-2 in the LacZ assay which is indicative of a robust interaction. A positive interaction was also seen between the RhoGEF domain deletion mutant and KIF5C, suggesting that KIF5C does not bind to the RhoGEF domain of collybistin. This was confirmed by the white colonies observed when KIF5C was co-expressed with just the RhoGEF domain. A negative LacZ assay was also observed for KIF5C and the PH domain. Nutritional selection studies confirmed the results of the LacZ assays. From these data it is evident that collybistin interacts with KIF5C, and may form part of the mechanism by which collybistin traffics gephyrin to the synapse.

Kinesin-1 is a holoenzyme composed of two motor-containing heavy chains and two light chains which mediate interactions with cargo proteins (reviewed in Hirokawa and Takemura, 2005). KIF5C can form homo- and heterodimers with KIF5A (which is also neuron specific) and KIF5B which is ubiquitously expressed. Expression profiles of KIF5C revealed that there is a high level of expression in the cortex, hippocampus, cerebellum and spinal cord. It is highly expressed in motor neurons, in both cranial nerves and the spinal cord. Subcellular studies show that the protein is found in the cell body, axon and dendrites (Kanai et al., 2000). The expression of KIF5C in motor neurons increases developmentally and prominent expression was observed two weeks post-natally, although all KIF isoforms were expressed from E13 onwards (Kanai et al., 2000).

Generally, kinesin-1 transports cargo towards the plus end of microtubules. In neurons this is normally away from the cell body, through axons and distal dendrites. Proximal dendrites, however, have mixed microtubule polarity, therefore transport is not
Chapter 5: Collybistin Interacting Proteins

necessarily away from the cell body (reviewed in Bush et al., 1996; Hirokawa and Takemura, 2005). Kinesins are known to transport various different types of cargoes and this superfamily of motor proteins and their functions will be discussed further in Chapter 6. However, one cargo which is worth mentioning here is glutamate receptor interacting protein-1 (GRIP1), which binds directly to KIF5 (Setou et al., 2002). GRIP1 is a scaffolding protein which contains seven PSD-95/Discs large/ZO1 (PDZ) domains and can mediate interactions with a number of proteins including the GluR2 subunit of the AMPA receptor and is thought to be involved in the clustering of AMPA receptors. All three KIF5 isoforms contain the GRIP binding region, therefore this interaction does not appear to be specific for a particular isoform. However, the interaction between kinesin and GRIP1 is specific for KIF5, as other major neuronal KIFs such as KIF17, KIF1A and KIF1Bβ do not interact with it (Setou et al., 2002).

5.4.4 KIF3A and collybistin

KIF3A was isolated in the CB2SH3+ library screen and was selected for further analysis as it gave a strong interaction with collybistin, is related to KIF5C and had a plausible biological function. KIF3A is 699 amino acids long (Fig. 5.5A) and has a predicted molecular weight of ~80-85 kDa. It contains a N-terminal motor domain, a central coiled-coil neck region and a C-terminal globular domain which mediates binding to cargo and accessory subunits (Fig. 5.5B). Collybistin binds to the C-terminal cargo binding domain. KIF3A showed a very intense blue colour with both CB2SH3- and hPEM-2 in the LacZ assay, which is indicative of a robust interaction. From these data it is evident that collybistin interacts with KIF3A, and may be involved in the trafficking of gephyrin by collybistin (Kondo et al. 1994; Hirokawa, 2000).

The kinesin-2 family contains many members including mammalian KIF3, sea urchin KRP85/95 (Cole et al., 1993), C.elegans Osm3 (Signor et al., 1999) and KIF17 (Setou et al., 2000). Three isoforms of KIF3 have been identified in vertebrates – KIF3A, KIF3B and KIF3C (Kondo et al., 1994; Yamazaki et al., 1995; Yang and Goldstein; 1998). All have a homologous N-terminal motor domain, a long coiled-coil neck region and a small globular tail region. KIF3B is ~95 kDa and has a distribution pattern similar to KIF3A. KIF3C is ~90 kDa and is mainly detected in the brain, spinal cord and retina, although traces were detected in other tissues investigated including lung, pancreas and kidney (Muresan et al., 1998).
KIF3A and KIF3B form a heterodimer which exhibits plus end directed microtubule sliding (reviewed in Hirokawa, 2000). This motor complex associates with the accessory protein kinesin associated protein (KAP3), and the heterotrimeric complex exists as a functional unit (Fig. 5.6). There are two known isoforms of KAP3 – KAP3A and KAP3B. Both have an α helical structure and bind to the tail region of the KIF3A/KIF3B heterodimer. KAP3 is ~100 kDa and therefore larger than the two motor subunits it associates with. KAP3 does not interfere with the ATPase activity of KIF3 and is thought to have a role in cargo binding (Yamazaki et al., 1996). The three proteins associate together in a ratio of 1:1:1. KIF3A also dimerises with KIF3C, although KIF3C does not form dimers with either itself or KIF3B. KIF3B and KIF3C are closely related, sharing 66% amino acid identity (Yang and Goldstein, 1998).

The kinesin-2 holoenzyme is involved in the intracellular transport cargo of vesicles and the intra-flagellar transport to form cilia in various structures. Studies on KIF3 and its many homologues in different species have revealed a number of processes in which this kinesin plays a vital role (reviewed in Hirokawa, 2000). Its conservation throughout different species also highlights the importance of this kinesin family. It has been implicated in processes such as axonal and dendritic transport, left/right asymmetry in development and formation of cilia and flagella.

One process of a kinesin-2 family member which is of particular interest is that of KIF17 and its involvement in the dendritic transport of vesicular NMDA glutamate receptors. KIF17 is a kinesin-2 motor containing chain and binds to the first PDZ domain of mLIN 10, a constituent of a multi-protein complex that includes the NMDA receptor NR2B subunit (Setou et al., 2000). KIF17 functions as a homodimer and
mediates fast plus-ended transport along microtubules. It appears to be specific for
dendritic transport, and has not been detected in axons. The protein complex also
includes the proteins mLIN 2 (CASK) and mLIN 7 (MALS/Velis) as well as the NR2B
subunit. This complex is involved in the polarised protein localisation pathways in
neurons and epithelia (Rongo et al., 1998). KIF17 has been shown to transport NR2B
containing vesicles down dendrites and to synaptic sites. The expression of these two
proteins appears to be tightly regulated to efficiently transport and target the receptor
subunit to its final synaptic position (Guillaud et al., 2003). The interaction between
neuronal receptors and kinesin appears to be important for the formation of synapses.

5.5 Conclusions

The membrane activation model states that collybistin activates Cdc42 at synapses,
resulting in the local rearrangement of the actin cytoskeleton which facilitates the
accumulation of gephyrin and the subsequent clustering of glycine receptors (Kneussel
and Betz, 2000). In Chapter 3 of this thesis, it was shown that the RhoGEF domain of
collybistin was the binding site for gephyrin. The membrane activation model also
suggests that the PH domain of collybistin is involved in membrane targeting by
binding to PI4,5P2 and PI3,4,5P3. However, in Chapter 3 it was shown that the PH
domain binds to PI3P and its deletion resulted in a trafficking mutant. This led to the
hypothesis that collybistin may have a role in the trafficking of gephyrin. Therefore, the
aim of this chapter was to identify other collybistin-interacting proteins and to achieve
this, a brain cDNA library screen was conducted using CB2_{SH3} as bait. Three
interesting proteins were identified in this screen and one in the screen conducted using
CB2_{SH3} as bait:

- NGEF; A dbl-like GEF, whose rat homologue has been implicated in controlling
growth cone dynamics during development.

- GATE-16; Involved in intra-Golgi trafficking and its close relative, GABARAP,
is implicated in GABA\textsubscript{A} receptor trafficking. It is possible that collybistin and
GATE-16 co-operate to transport gephyrin and glycine receptors from the Golgi
apparatus into the cytoplasm where they can then be transported into the axon or
to dendrites.
Chapter 5: Collybistin Interacting Proteins

- KIF5C (CB2_{SH3} screen) and KIF3A in the CB2_{SH3+} screen. Collybistin binds to the cargo binding domain of both subunits, suggesting that collybistin is one of the many proteins that these motor proteins transport along microtubules.

Kinesins are involved in the transport of AMPA and NMDA receptors along microtubules, and it is possible that collybistin may mediate an interaction between these motor proteins and gephyrin, to transport glycine receptors to the synapse. Therefore the decision was made to investigate the interaction between kinesins and collybistin further. This work is presented in Chapter 6.
Chapter 6: Collybistin and Kinesins
Chapter 6: Collybistin and Kinesins

6.1 Introduction

6.1.1 Microtubules and molecular motors

Neurons have highly polarised structures, comprising a cell body, a long thin axon and a number of short dendrites. The axons of some neurons can be up to a metre long and, as the majority of protein synthesis occurs in the cell body, the neuron has developed a sophisticated method by which proteins can be carried along the microtubule tracks from the site at which they are transcribed and synthesised to their functional position. Microtubules are hollow tubes, 24 nm in diameter that are formed of 13 protofilaments, each composed of aligned globular heterodimers consisting of α and β tubulin subunits (Diaz-Nido and Avila, 2004). To date, six α tubulin and six β tubulin genes have been identified in mammals (Dutcher, 2001). Microtubules are polarised structures as the dimers all have the same orientation with β tubulin exposed at the 'plus' end and α tubulin exposed at the 'minus' end. Microtubule assembly is a dynamic process which occurs by the association and dissociation of tubulin monomers at each end. This occurs at a faster rate at the plus-end compared to the minus-end (Howard and Hyman, 2003).

Tubulin is a GTP-binding protein, and GTP-tubulin has the ability to polymerise into microtubules. Once incorporated, GTP-tubulin is hydrolysed to GDP-tubulin. When the concentration of soluble GTP-tubulin drops below a threshold level, microtubules rapidly depolymerise, and there is the possibility that the microtubule will incorporate GTP-tubulin again, and stop depolymerising. Therefore, the microtubules are in a cycle of shrinking and growing, and this fluctuation in microtubule length is known as dynamic instability (Kirschner and Mitchison, 1986). A third tubulin subunit, γ tubulin, is not incorporated into the microtubule filaments, but is found in the microtubule organising centre (MTOC) or centrosome, where it is presumed to act as a seed for the initiation of microtubule polymerisation (Oakley, 1992).

Microtubules have an intrinsic polarity and generally within the axon and distal dendrites of a neuron, the fast growing plus-ends are orientated away from the cell body, whilst the slow growing minus-ends are anchored in the MTOC within the cell body (Baas et al., 1988; Craig and Banker, 1994). In proximal dendrites, which are about 75 µm from the cell body, microtubules have a mixed orientation (Baas et al.,
1989). Microtubules provide a track upon which cargo can be transported, although they do not reach the interface between the plasma membrane and cytoplasm. This region is rich in actin filaments which are thought to be the tracks for the final delivery of surface molecules (reviewed in Bush et al., 1996).

Molecular motors are proteins that utilize energy from ATP hydrolysis to generate force for directed movement along the cytoskeleton. There are three main types of molecular motors, myosin, dynein and kinesin. Myosin is responsible for the movement of organelles along actin filaments, and in axons, myosin V is associated with the transport of synaptic vesicles containing synaptophysin (Prekeris and Terrian, 1997). Dynein and kinesin interact with microtubules and are involved in the trafficking of a wide range of organelles and macromolecular complexes to and from the cell body. Dynein is mainly associated with minus-end transport along microtubules (King, 2000) whilst kinesin is chiefly associated with plus-ended transport.

6.1.2 The kinesin superfamily

Over 300 proteins belonging to the kinesin superfamily have been identified in all species investigated to date (http://www.nroweb.ors/kinesin/MotorSeqTable.html). Recently, the kinesins have been reclassified in order to simplify the subgroup names (see Table 6.1; Lawrence et al., 2004). There are now 14 recognised families of kinesins based upon the phylogenetic analyses of each motor domain sequence (Dagenbach and Endow, 2004). Individual isoforms still retain their original names and these will be referred to in this chapter. Kinesin holoenzymes will be referred to by their new names, for example, conventional kinesin will be referred to as kinesin-1, heterotrimeric kinesin as kinesin-2, and C-type kinesin as kinesin-13.

6.1.3 The general structure of kinesins

All kinesins share a modular structure which consists of a conserved motor domain, attached through an α-helical coiled-coil region to a variety of divergent tail domains (Fig.6.1; reviewed in Hirokawa and Takemura, 2005). In most families, this motor domain is located at the N-terminus, although kinesin-13 members have a centrally located motor domain and kinesin-14 members have a C-terminal located motor domain. The motor domain contains the ATP and microtubule binding sites which are essential for the translocation of kinesin along microtubules. The coiled-coil region acts
as a linker between the motor and tail and is often involved in kinesin dimerisation. While the motor domain is the most conserved region between kinesins, the tail region is the most diverse in both structure and function. Generally it is thought to be involved in binding accessory proteins that comprise a kinesin holoenzyme or associating with cargo molecules directly.

Fig. 6.1: Domain alignments - A schematic representation of the domain structures of the major kinesin heavy chains. The motor domain is represented in light blue, with the ATP binding consensus sequence as the thin red line, and the microtubule binding consensus sequence as the thick yellow line. The dimerisation domains, which are generally located in the neck region are in green, the forkhead-associated domains in purple and the pleckstrin homology domains in orange. The cargo binding domain is generally at the opposite terminus to the motor domain (adapted from Hirokawa and Takemura, 2005).
<table>
<thead>
<tr>
<th>Standardised Family Name</th>
<th>Previous Names</th>
<th>Example Sequences - Mammalian</th>
<th>Example Sequences - Non Mammalian</th>
<th>Properties and Functions</th>
</tr>
</thead>
</table>
| Kinesin-1                | KHC, N-I, Kinesin I, conventional kinesin | KIF5A KIF5B KIF5C | KHC (Dm) UNC-116 (Ce) | N-terminal motor domain, consists of four subunits, two heavy chains and two light chains; involved in the intracellular transport of cargo including GluR2 containing AMPA receptors. 
*see subsequent text*
| Kinesin-2                | KRP85/95, N-IV, Kinesin-II, heterotrimeric | KIF3A KIF3B KIF17 | KRP85/95 (Sp) KRP-85/95 (Ce) OSM-3 (Ce) | Plus-ended transport, N-terminal motor domains, involved in anterograde flagellar transport and axonal vesicle transport, including transport of NR2B containing NMDA receptors |
| Kinesin-3                | Unc-104/Kif1, N-III, Unc104, Monomeric | KIF1A KIF1B KIF13 | KIN Unc104 (Ce) | Involved in the transport of various membranous organelles.
Monomeric |
<p>| Kinesin-4                | Chromokinesin/Kif4, N-V, Chromokinesin | KIF4 KIF21 | Chromokinesin (Gg) XKLP1 (Xl) | Binds to DNA, involved in chromosome segregation |
| Kinesin-5                | BimC, N-II, BIMC, Bipolar, tetrameric | KIF11 | EG5 (Xl) BIMC (Sc) | Homo-tetrameric, involved in spindle formation |
| Kinesin-6                | MKLP1, N-VI, MKLP | KIF20A | MKLP1 (Xl) | Recruits regulatory proteins required for the completion of cytokinesis |
| Kinesin-7                | CENP-E | KIF10 CENP-E | KIP2p (Sc) | Involved in chromosome alignment during metaphase |</p>
<table>
<thead>
<tr>
<th><strong>Standardised Family Name</strong></th>
<th><strong>Previous Names</strong></th>
<th><strong>Example Sequences—Mammalian</strong></th>
<th><strong>Example Sequences—Non Mammalian</strong></th>
<th><strong>Properties and Functions</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinesin-8</td>
<td>Kip3, N-IIX</td>
<td>KIF19A</td>
<td>KIP3p (Sc)</td>
<td>Involved in spindle orientation</td>
</tr>
<tr>
<td>Kinesin-9</td>
<td>MKLP</td>
<td>KIF9</td>
<td>KLP1 (Cr)</td>
<td>KIF9 interacts with the GTPase Gem and may be involved in cytoskeletal dynamics</td>
</tr>
<tr>
<td>Kinesin-10</td>
<td>Chromokinesin</td>
<td>KIF22</td>
<td>Nod (Dm)</td>
<td>Regulation of spindle formation and chromosomal segregation during cell division</td>
</tr>
<tr>
<td>Kinesin-11</td>
<td>Divergent Kinesin I</td>
<td>KIF26A</td>
<td>VAB-8 (Ce)</td>
<td>VAB-8 involved in posteriorly directed axon outgrowth and cell migration</td>
</tr>
<tr>
<td>Kinesin-12</td>
<td>MKLP</td>
<td>KIF15</td>
<td>Xklp2 (Xl) PaKRP1 (At)</td>
<td>KIF15 is implicated in growth cone and dendritic migration, and also in cell division.</td>
</tr>
<tr>
<td>Kinesin-13</td>
<td>MmKif2, MCAK/Kif2, M, I-type, KinI</td>
<td>KIF2</td>
<td>XKCM1 (Xl) MCAK (Cg)</td>
<td>Central motor domain, involved in axonal growth and also has microtubule depolymerising activity</td>
</tr>
<tr>
<td>Kinesin-14</td>
<td>C-terminal motor, C-type</td>
<td>KIFC1, KIFC2/C3</td>
<td>NCD (Dm) KAR3p (Sc)</td>
<td>C-terminal motor domain, has minus-end polarity movement, generally involved in mitosis/meiosis, Kar3p moves nuclei in mating cells for fusion</td>
</tr>
<tr>
<td>Orphans</td>
<td>Orphans</td>
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Table 6.1: Standardised name and properties of each kinesin family, with examples of mammalian and non-mammalian members. *At*: Arabidopsis thaliana; *Ce*: Caenorhabditis elegans; *Cg*: Cricetulus griseus (Chinese hamster); *Cr*: Caenorhabditis reinhardtii; *Dm*: Drosophila melanogaster; *Gg*: Gallus gallus; *Sc*: Saccharomyces cerevisiae; *Sp*: Strongylocentrotus purpuratus (purple sea urchin); *Xl*: Xenopus laevis. Adapted from Lawrence et al. (2004).
6.1.4 The motor domain and kinesin processivity

Amino acid alignment of all the kinesins reveals that the ATP and microtubule binding sites are conserved through evolution (Dagenbach and Endow, 2004; Hirokawa and Takemura, 2005). The motor domain of kinesin-1 has been studied extensively and there are many theories as to how these molecules move along microtubules (reviewed in Asbury et al., 2003; Yildiz and Selvin, 2005). Studies of single kinesin molecules reveal that kinesin moves at a rate of 800 nm s\(^{-1}\), in which time it completes 100 ATP turnovers. The kinesin moves 8.3 nm per single ATP hydrolysis reaction which is the distance between each tubulin monomer (Howard et al., 1989; Svoboda et al., 1993; Schnitzer and Block, 1997; Hua et al., 1997). Biochemical analysis of the ATP cycle revealed that the two heads of a kinesin dimer co-ordinate with each other to move along microtubules. The head is attached to the microtubule in its ATP-bound state whilst the second trailing head is in the ADP-bound state. The neck region is docked pointing towards the plus-end of the microtubule, and pulls the trailing head forward. The trailing head then binds to the microtubules, now in an ATP-bound state, and this accelerates the dissociation of the first head, now in the position of the trailing head, and the cycle starts again (Hancock and Howard, 1999).

Two models have been proposed for the mechanism by which the two heads move along the microtubules, the first is the inch worm model, in which one head always leads and the other trails behind (Hua et al., 2002). The second model is the hand-over-hand model in which the two heads exchange roles with each step, one leads and the other trails, and vice versa (Yildiz et al., 2004). There are two variations of this model. The symmetric variation is that in which the ATP cycle of each head begins in the same physical position whilst the asymmetric variation of the models suggests that the heads alternate between two distinct conformations at each ATP cycle. There is much evidence for and against both models, although there is a growing consensus that the most favourable model is the asymmetric hand-over-hand model for kinesin-1 processivity (reviewed in Yildiz and Selvin, 2005).

Kinesin-2 (formally heterotrimeric kinesin composed of KIF3A and KIF3B) is a processive motor that moves at 200 nm s\(^{-1}\). Although it is slower than kinesin-1, it can move longer distances without dissociating (up to 3.6 \(\mu\)m). The two heads co-operate with each other and both contribute to the movement of kinesin-2 along microtubules similar to the hand-over-hand model described above (Zhang and Hancock, 2004).
6.1.5 The tail domain and directional transport of cargo

The tail domain is the most divergent part of the kinesin molecule, and these domains can vary greatly between members of the same family. There is little homology conserved throughout evolution and it is the diversity of the tail domain which enables kinesin to engage many different and varied cargoes for transportation. Within neurons, transport can be divided into cargoes which are axonal and those that are dendritic. Kinesin-1 has a variety of cargoes, all of which are transported to specific destinations. The mechanisms involved appear to be governed by the means in which the cargo binds to kinesin-1. Generally, the cargo has three options by which it can bind — either directly to the C-terminal of the motor subunit, directly to kinesin light chains (KLC) or via an adaptor protein to the kinesin holoenzyme.

6.1.5.1 Kinesin light chains

Two kinesin light chains contribute to, and form an integral part of the kinesin-1 holoenzyme. They are non-motor proteins of 60-70 kDa and are thought to be involved in cargo binding. They have a highly conserved structure with a N-terminal coiled-coil region of about 100 amino acids and 6 C-terminal tetra trico peptide repeats (TPR; Pfam accession number: PF00515) regions which are implicated in protein-protein interactions (D'Andrea and Regan, 2003). KLC binds to the C-terminal of KIF5 through its globular N-terminal (Verhey et al., 1998). It has been proposed that the KLCs have a role in keeping kinesin-1 in an inactive state, and that an interaction between cargo and the tail domain induces a conformational change which activates the motor domain (Verhey et al., 1998; Coy et al., 1999; Friedman and Vale, 1999). Mutations in KLC results in the accumulation of membranous organelles in the axon, suggesting that KLCs are important for the axonal transport of cargo by KIF5 (Gindhart et al., 1998). In many cases, the binding of cargo to KLC does not appear to be direct, but mediated by a number of linker proteins belonging to the c-jun N-terminal kinase (JNK) interacting protein (JIP) family (Verhey et al., 2001; Inomata et al., 2003; Matsuda et al., 2003).

6.1.5.2 Cargo recognition and directional transport

It is quite evident that there are many ways in which the different kinesin complexes can recognise and bind to their respective cargo, what remains to be elucidated is how the path that the kinesin and its cargo takes is determined. Cargo recognition seems to have
some influence over directional transport, although the mechanisms involved in this remain unknown. The neuron is the most studied system for directional sorting and transport due to its highly polarised nature. The intrinsic polarity of microtubules differs between axons and dendrites and may be exploited for the directed transport of cargo into axons and dendrites. Plus-ended motors transport membrane proteins such as neurotransmitter receptors to axons and dendrites. Some motors referred to as ‘smart motors’ may be able to differentiate between axons and dendrites whereas other motors may be guided by the mode in which the cargo is attached. For example, KIF5 has the ability to transport cargo to both the axons and dendrites, but the method by which this differentiation occurs is unclear. It appears that cargoes which are bound to KIF5 via KLC and linker proteins such as JIP are directed towards the axon (Kamal et al., 2000; 2001), whereas cargoes that are directly bound to KIF5, such as GRIP/AMP A receptors, are steered towards the dendrites (Burack et al., 2000; Setou et al., 2002).

Amongst the many identified cargoes of KIF5 is a dendritic 1000 S (Svedburg unit) granule which contains over 42 proteins and the mRNA for MAP2, α calcium/calmodulin-dependent kinase II (αCAMKII) and activity-related cytoskeleton associated protein (ARC). The latter two proteins are involved in long-term potentiation (Kanai et al., 2004). This complex is thought to be involved in mRNA transport, since it contains proteins associated with mRNA transport and protein synthesis. These granules are transported bi-directionally suggesting a ‘tug of war’ between kinesin and dynein, but distally directed transportation is enhanced by increased KIF5 expression and reduced by functional blockade of KIF5. The transport of mRNA is fundamental for local protein synthesis, especially in neurons and it is thought that dendritic protein synthesis accompanies changes in synaptic strength and may contribute to long term potentiation (Gardiol et al., 1999; Kanai et al., 2004). Glycine receptor subunit mRNAs have been detected at postsynaptic sites in mature neurons and it may be possible that KIF5 may also be responsible for the trafficking of glycine receptor mRNA to synapses (Racca et al., 1997).

The initial segment of the axon is packed full of microtubules which appear to provide a signal for KIF5 motors to enter the axon in preference to the dendrites. A study by Nakata and Hirokawa (2003) employed a mutant KIF5 motor which binds to, but does not move along or dissociate from, microtubules (rigor KIF5) to establish that the KIF5 motor distributes on microtubules from the cell body into the initial segment of the
Rigor KIF17 appeared to preferentially distribute to dendritic microtubules. Rigor KIF2 did not localise to the microtubules in the initial segment. Tail-less mutants of KIF5 also displayed preferential distribution to the initial segment and axon. These results indicate that KIF5 has a preference for the initial segment, however the cue that directs it there remains unknown. A possible mechanism may be one similar to that observed between end binding protein 1 (EB1) and microtubules. EB1 binds to the plus-end of microtubules in many cell types. The mechanism of this end binding remains unknown, but may involve dynamic or structural cues found at the end of microtubules. Like KIF5, EB1 also displays a high affinity for the microtubules in the initial segment. The pattern of EB1 distribution when overexpressed in cultured hippocampal neurons indicates that there are differences between the microtubules in the initial segment and those in the dendrites. In these experiments, EB1 fully decorated the initial segment, whilst only binding to the tips in dendrites and the cell body. The binding sites for KIF5 and EB1 on microtubules overlap suggesting that both proteins may recognise the same signalling cue which results in the distinct distribution of these proteins. The signalling cue is likely to be within the structural components as opposed to a cytosolic diffusible signal. It appears that microtubules and their associated proteins may play an important role in the directional transport of kinesin cargo (Nakata and Hirokawa, 2003).

Two mechanisms that have been proposed for the directional transport of cargo are selective retention and directed targeting. Proteins that are non-selectively transported into axons and dendrites and then eliminated at sites where they are not required by selective endocytosis and retained at sites where endocytosis is prevented, is referred to as selective retention (Sampo et al., 2003). Studies carried out in hippocampal neurons showed that vesicular associated membrane protein 2 (VAMP2, also known as synaptobrevin, is a synaptic vesicle protein which is required for Ca\(^{2+}\)-dependent exocytosis at synaptic terminals) was targeted by selective retention. The authors found that VAMP2 was delivered to axonal and dendritic surfaces, but preferentially endocytosed from dendritic membranes. Mutation of a well defined endocytosis signal within it cytoplasmic tail resulted in its accumulation within axons and dendrites. This domain is probably required for the binding of other proteins involved in endocytosis (Grote and Kelly, 1996).
Neuron-glia cell adhesion molecule (NgCAM - thought to be involved in axonal path finding and fasciculation) appears to be directly targeted. It is found abundantly in distal axons and also contains a well defined endocytosis signal within its cytoplasmic tail (Bonifacino and Traub, 2003), however, mutations in this region do not affect the axonal distribution of the protein (Sampo et al., 2003). Instead, NgCAM targeting depends on fibronectin type III-like (FnIII) repeats in its ectodomain which, when deleted, results in a loss of axonal localisation. Therefore, these sequences seem to be the signal provider for preferential targeting of the protein to the axon. It is evident that the targeting and trafficking of proteins is a carefully controlled process, with many factors contributing to the recognition, sorting and transport of cargo by kinesins.

6.1.6 Kinesin-1

Kinesin was initially isolated from squid giant axon (Vale et al., 1985), and homologues have since been found in a variety of species ranging from protists and plants to mammals. The most widely studied kinesin is kinesin-1 (previously referred to as conventional kinesin or kinesin 1). This is a heterodimer composed of two heavy chains (KIF5) and two light chains (KLC) and is involved in plus-end directed transport of cargo along microtubules (Fig. 6.2). To date, three mammalian isoforms of KIF5 have been identified, KIF5A, KIF5B and KIF5C. These heavy chains have a N-terminal motor domain, a neck region and a C-terminal globular domain which is involved in cargo binding and also mediates interactions with KLC. These isoforms share a high degree of similarity, especially in their motor domain. KIF5A and 5C are neuronal specific whereas KIF5B is ubiquitously expressed. KIF5C, isolated in the CB2SH3-library screen was described in Chapter 5.

Fig. 6.2: Kinesin-1 holoenzyme – The motor domain is in red, the linker neck region in green, the cargo binding domain in blue and the kinesin light chains in orange.
KIF5A is 1,032 amino acids long with a predicted molecular weight of 117 kDa (Fig. 6.4A). It can form homo- and hetero-dimers with KIF5B and KIF5C. Immuno-blot studies using a KIF5A-specific antibody revealed that this isoform displays a pan neuronal distribution. It was found in most brain areas including the olfactory bulb, cortex, hippocampus, cerebellum and spinal cord. It does not seem to be present in glial cells. Subcellular studies show that the protein is seen within the neuronal cell body, axon and dendrites (Aizawa et al., 1992; Kanai et al., 2000).

KIF5B is 899 amino acids long with a predicted molecular weight of 109 kDa (Fig. 6.5A). Expression studies in tissues including brain, spinal cord, heart, lungs, liver, spleen, kidney, adrenal glands, testis, ovary, uterus and muscle have revealed that KIF5B is ubiquitously expressed (Nakagawa et al., 1997). Within the nervous system it is found in both neuronal and glial cells. It forms homo- and heterodimers with KIF5A and KIF5C, and although these two isoforms are neuronal specific, KIF5B accounts for 90% of KIF5 in total brain extract (Kanai et al., 2000).

6.1.6.1 Kinesin-1 knockout mice

KIF5A and KIF5B knockout mice are neonatal and embryonic lethal, respectively (Xia et al., 2003; Tanaka et al., 1998). However, conditional KIF5A knockout mice survive birth, but gradually degenerate and most die within three weeks. Immunohistochemical staining of brain and spinal cord of these mutant mice showed that there was a progressive age-related loss of sensory neurons, and to a lesser extent of motor neurons. Axons with larger diameters (>4 μm) seemed to be more affected than those with
smaller diameters. This suggests that KIF5A, although not essential for embryonic development, is required for the maintenance of neurons (Xia et al., 2003).

KIF5B is ubiquitously expressed and cultured cells taken from the extra-embryonic membrane of Kif5B null mutants revealed that the distribution of mitochondria was different compared to wild-type cells (Tanaka et al., 1998). In wild-type cells, the mitochondria were bound to microtubules and distributed throughout the cytoplasm. In Kif5B'''' cells the mitochondria were still anchored to microtubules (this could be disrupted by incubating the cells in nocodazole), but were clustered at perinuclear sites. This unusual mitochondrial distribution was rescued by the introduction of recombinant KIF5B into the null mutant cells, indicating that KIF5B was involved in the movement of mitochondria but not necessarily their anchoring to microtubules (Tanaka et al., 1998). Kif5B'''' cells were also devoid of normal lysosome transportation. KIF5A and KIF5C were upregulated in the null mutant cells, but this was still lower than the levels seen in the brain and therefore may only be involved in the occasional dispersal of mitochondria and not enough to compensate for fundamental KIF5B function (Tanaka et al., 1998).

Unlike Kif5A and Kif5B null mutant mice, the Kif5C null mutant mouse was viable from birth. The mice were fertile and normal in body size although they had smaller brains compared to the wild-type and heterozygous mice. The expression levels of KIF5A and KIF5B did not change significantly in the KIF5C knockout mouse indicating that they did not need to be upregulated in order for the mice to survive. The number of motor neurons in KIF5C null mutants was significantly decreased compared to those in wild-type mice, although there was no significant reduction in the number of sensory neurons. The Kif5C'''' brain, although smaller and lacking in motor neurons, displayed no adverse conditions and had a normal structure, suggesting that the fundamental function of KIF5C can be performed by other proteins, most probably the other KIF5 isoforms (Kanai et al., 2000).
6.1.7 Aims

The membrane activation model stated that collybistin is active at the synapse and catalyses GDP-GTP exchange activity on Cdc42, which in turn regulates the actin cytoskeleton, thus influencing the accumulation and clustering of gephyrin and glycine receptors. Chapter 3 of this study showed that deletion of the PH domain disrupted the formation of collybistin-gephyrin submembranous microaggregates, indicating that collybistin may be involved in the trafficking of gephyrin, in addition to being a GEF as suggested in the membrane activation model. This was supported by the identification of PI3P as the phosphoinositide to which the PH domain binds. This phosphoinositide is involved in constitutive membrane trafficking. In Chapter 5 of this thesis, a brain cDNA library screen was conducted to identify collybistin interacting proteins that could potentially be involved in trafficking and clustering gephyrin at postsynaptic sites. Two kinesin motor proteins were identified, KIF5C and KIF3A. The aims of this chapter were to:

- Establish whether collybistin interacts with kinesin motor proteins, other than KIF5C and KIF3A.

- Observe the interactions between kinesin, collybistin and gephyrin, and the effects of disrupting kinesin activity on gephyrin clustering.
6.2 Methods

6.2.1 Yeast expression constructs

To investigate whether collybistin interacts with other kinesin heavy chains, KIF5A and KIF5B yeast constructs were generated and tested against collybistin in yeast.

6.2.1.1 pACT2-KIF5A

The cargo-binding domain of KIF5A was amplified from human whole brain first strand cDNA using the primer combinations hKIFSAl and hKIF5A2. Amplifications were performed for 21 cycles at 94°C for 1 min, 65°C for 1 min and 68°C for 12 min using *Pfu* Turbo proof-reading polymerase. The PCR product was cloned into the BamHI/XhoI sites of pACT2. The plasmid was sequenced using the GAL4AD sequencing primer.

6.2.1.2 pACT-KIF5B

The cargo-binding domain of KIF5B was amplified from human whole brain first strand cDNA using the primer combinations hKIF5Bl and hKIF5B2. Amplifications were performed for 21 cycles at 94°C for 1 min, 65°C for 1 min and 68°C for 12 min using *Pfu* Turbo proof-reading polymerase. The PCR product was cloned into the BamHI/XhoI sites of pACT2. The plasmid was sequenced using the GAL4AD sequencing primer.

6.2.2 Mammalian expression constructs

Two KIF5C constructs were made to observe the interaction between collybistin and kinesin in HEK293 cells - a full-length wild-type and a dominant negative mutant. There are two ways in which a dominant negative kinesin can be generated, the headless mutant and the rigor mutant. The headless mutant, as the name suggests, lacks the head domain which contains the motor region. This mutant does not have the ability to bind to ATP or microtubules thus rendering it immobile (Fig. 6.3). The rigor mutant contains a point mutation in the ATP-binding site, therefore it has the ability to bind to the cytoskeleton but can not hydrolyse ATP, and therefore the ATP-bound kinesin becomes irreversibly bound to the cytoskeleton in a state which is described as a rigor complex (Gelfand *et al.*, 2001). The headless KIF5C was chosen as this mutant still retains the ability to bind to other kinesin molecules via the neck region and to cargo via
the cargo binding domain, thus producing single headed complexes. Previous studies have successfully employed this method to determine kinesin functions in a variety of organisms (Gelfand et al., 2001).

**Fig. 6.3:** Schematic representations of wild-type and headless KIF5C generated in pEGFP, so that the EGFP tag was at the N-terminus. The wild-type KIF5C has the motor, neck and cargo binding domains, whereas the headless mutant lacks the motor domain which contains the ATP and microtubule binding sites, however, it still has the linker (neck) region and cargo binding domain. Both constructs should be able to bind to collybistin via the cargo binding domain. The recombinant KIF5C (orange) should still be able to interact with endogenous kinesin molecules via the neck region (purple).

### 6.2.2.1 pEGFP-KIF5C-wt (wild-type, full-length)

KIF5C-wt was amplified from pBluescript-KIAA0531 (Accession number: ABO11103 HUGE protein database, Kasuza cDNA project http://www.kazusa.or.jp/huge/) using the primer combinations hKIF5C1 and hKIF5C3. Amplifications were performed for 30 cycles at 94°C for 1 min, 60°C for 1 min and 68°C for 10 min using Advantage 2 Taq DNA polymerase. The PCR product was digested with BclI and Sall and cloned into the BglII/Sall sites of pEGFP C1, so that the EGFP tag was N-terminal. The plasmid was sequenced using the GFP seq1 sequencing primer. The addition of 2 µl of GC melt appeared to aid the sequencing reaction and elicit a clearer signal.

### 6.2.2.2 pEGFP-KIF5C-hdl (headless mutant)

KIF5C-hdl was amplified from pBluescript-KIAA0531 using the primer combinations hKIF5C2 and hKIF5C3. Amplifications were performed for 30 cycles at 94°C for 1 min, 60°C for 1 min and 68°C for 10 min using Advantage 2 Taq DNA polymerase. The PCR product was digested with BclI and Sall and cloned into the BglII/Sall sites of pEGFP C1, so that the EGFP tag was N-terminal. The plasmid was sequenced using the GFP seq1 sequencing primer. The addition of 2 µl GC melt appeared to aid the sequencing reaction and elicit a clearer signal.
Chapter 6: Collybistin and Kinesins

6.2.2.3 pRK5Flag-CB$_{2SH3-}$ and pRK5Flag-CB$_{2SH3+}$

CB$_{2SH3-}$ and CB$_{2SH3+}$ were excised from the BamHI/EcoRI sites of pRK5Myc-CB$_{2SH3-}$ and pRK5MycCB$_{2SH3+}$, respectively (see Chapter 3), and sub-cloned into the BamHI/EcoRI sites of the vector pRK5Flag, so that the Flag epitope was expressed at the N-terminus. The plasmids were sequenced using the pRK5 sequencing primer.

6.2.2.4 pCisMyc Gephyrin P1

Full-length gephyrin, isoform P1 was cloned into the vector pCisMyc, and expression was detected using the 9E10 antibody. (Gift from H. Betz; Prior et al., 1992)

To study kinesin and collybistin interactions in mammalian cells, different construct combinations were transfected into HEK293 cells using effectene (see 2.3.4). The cells were incubated at 37°C, 5% CO$_2$/air and fixed and stained 18-24 h post transfection. For fixing and staining procedures see section 2.3.5. Cells were imaged by confocal microscopy. Table 6.4 shows the constructs and antibodies used throughout this chapter, and the colour the constructs appear as in the confocal images.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Tag</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
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<tr>
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<td>Myc</td>
<td>Mouse monoclonal 9E10 antibody</td>
<td>Alexa Fluor 594 conjugated goat anti-mouse</td>
<td>Red</td>
</tr>
<tr>
<td>pRK5Myc CB$_{2SH3+}$</td>
<td>Myc</td>
<td>Mouse monoclonal 9E10 antibody</td>
<td>Alexa Fluor 594 conjugated goat anti-mouse</td>
<td>Red</td>
</tr>
<tr>
<td>pRK5Flag CB$_{2SH3-}$</td>
<td>Flag</td>
<td>Sheep anti-Flag</td>
<td>Cy5 conjugated donkey anti-sheep</td>
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<td>Flag</td>
<td>Sheep anti-Flag</td>
<td>Cy5 conjugated donkey anti-sheep</td>
<td>Blue</td>
</tr>
<tr>
<td>pCisMyc gephyrin</td>
<td>Myc</td>
<td>Mouse monoclonal 9E10 antibody</td>
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Table 6.3: Constructs and antibodies used throughout this chapter.
6.3: Results

6.3.1 Interactions between collybistin and other kinesin heavy chains

The CB2_{SH3}^+ library screen revealed that KIF3A interacted strongly with collybistin and the CB2_{SH3}^- screen identified KIF5C as a collybistin binding partner. To ascertain whether collybistin interacted with other kinesin isoforms, the cargo binding domains of KIF5A and KIF5B were cloned into pACT2 and tested in yeast.

KIF5A, like KIF5C, is a neuronal specific kinesin heavy chain. In Y190 cells expressing pACT2-KIF5A with either pYTH9-CB2_{SH3} (Fig. 6.4C) or pGBK77-hPEM-2 (Fig. 6.4F) colonies remained white 3 h after the start of the\ LacZ\ assay. Fig. 6.4 D&E show that the colonies remained white when pACT2-KIF5A was co-transformed with either pYTH16 or pGBK77 respectively. Colonies also remained white when pACT2-KIF5A was expressed with either pYTH9-CB2_{SH3}ARhoGEF, which is the RhoGEF domain deletion mutant of collybistin (Fig. 6.4G), pYTH16-RhoGEF domain (Fig. 6.4H) or pYTH16-PH domain (Fig. 6.4I).

KIF5B is the ubiquitously expressed isoform of the kinesin heavy chain. In Y190 cells expressing pACT2-KIF5B with either pYTH9-CB2_{SH3} (Fig. 6.5C) or pGBK77-hPEM-2 (Fig. 6.5F), colonies began to change from white to blue 30 min after the start of the\ LacZ\ assay and when it was terminated after 3 h a blue colour was observed (Fig. 6.5C&F). However, this colour was not as intense as that seen with KIF5C or KIF3A (see Figs. 5.4 and 5.5 on pages 130 and 131). Fig. 6.5 D&E show that the colonies remained white when pACT2-KIF5B was co-transformed with either pYTH16 or pGBK77, respectively. KIF5B also gave a blue colour when expressed with pYTH9-CB2_{SH3}ARhoGEF, which is the RhoGEF domain deletion mutant of collybistin (Fig. 6.5G). The Y190 colonies co-transformed with pACT2-KIF5B and either pYTH16-RhoGEF domain (Fig. 6.5H) or pYTH16-PH (Fig. 6.5I) domain remained white 3 h after the start of the\ LacZ\ assay.
Chapter 6: Collybistin and Kinesins

Fig. 6.4: KIF5A - Amino acid sequence of KIF5A with the cargo binding site underlined, this was the region cloned into pACT2. (A). Model of KIF5A Pfam protein domain - Motor domain, and also the neck and cargo binding domains (B). Interactions of pACT2-KIF5A in yeast (Y190) with pYTH9-CB2sH3, +pACT2-KIF5A (C), pYTH16 (D), pGBKTV hPEM-2 (F), pYTH9-CB2sH3 ΔRhoGEF (G), pYTH16-RhoGEF domain (H) and pYTH16-PH domain (I). Positive interactions are indicated by the blue colouring.
Chapter 6: Collybistin and Kinesins

Fig. 6.5: KIF5B - Amino acid sequence of KIF5B with the cargo binding site underlined, this was the region cloned into pACT2 (A). Model of KIF5B Pfam protein domain - Motor domain, and also the neck and cargo binding domains (B). Interactions of pACT2-KIF5B in yeast (Y 190) with pYTH9-CB2, pYTH16, pGBK7, pGBK7-hPEM-2, pYTH9-CB2ARhoGEF, pYTH16-RhoGEF and pYTH16-PH domain (C-I). Positive interactions are indicated by the blue colouring.

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<th>E-Value</th>
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<td>PF00225</td>
<td>4.3e-171</td>
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<tr>
<td>Kinesin Motor Domain; 14-326</td>
<td>PF00225</td>
<td>4.3e-171</td>
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- FIGURE 6.5: KIF5B - Amino acid sequence of KIF5B with the cargo binding site underlined, this was the region cloned into pACT2 (A). Model of KIF5B Pfam protein domain - Motor domain, and also the neck and cargo binding domains (B). Interactions of pACT2-KIF5B in yeast (Y190) with pYTH9-CB2, pYTH16, pGBK7, pGBK7-hPEM-2, pYTH9-CB2ARhoGEF, pYTH16-RhoGEF and pYTH16-PH domain (C-I). Positive interactions are indicated by the blue colouring.

161
6.3.2 Expression of transfected KIF5C in mammalian cells

Interactions between kinesin, collybistin and gephyrin were studied in mammalian cells. In Chapter 3, it was shown that the expression of CB2SH3+ and gephyrin resulted in the formation of cytoplasmic aggregates (see Fig. 3.7 on page 60) and the expression of CB2SH3- and gephyrin resulted in the relocation of the gephyrin microaggregates to submembranous positions (see Fig. 3.8 on page 61). Two kinesin constructs were made—a full-length wild-type and a headless mutant in which the motor domain had been removed (Fig. 6.6 B&E).

Fig. 6.6 C&D show HEK293 cells transfected with pEGFP-KIF5C-wt. The majority of the fluorescence was concentrated at one end of the cell. Some fibrous structures were also observed. Fig. 6.6 G&H show HEK293 cells transfected with pEGFP-KIF5C-hdl. The fluorescence in these cells had a uniform distribution throughout the cells, and appeared to decorate the cytoskeleton. Filamentous structures were observed throughout the cell.

6.3.3 KIF5C and gephyrin

Fig. 6.7A shows that colonies of Y190 cells expressing pYTH9-gephyrin and pACT2-KIF5B remained white 3 h after the start of the LacZ assay. LacZ assays were also conducted on Y190 cells integrated with pYTH9-gephyrin, expressing pACT2-KIF5A or pACT2-KIF5C. These also gave white colonies (data not shown).

HEK293 cells were transfected with pEGFP-KIF5C-wt (Fig. 6.7B), which is expressed at one end of the cell, and pCisMyc-gephyrin (Fig. 6.7C), which formed characteristic cytoplasmic aggregates. The merged image shows that these proteins were not expressed in similar areas (Fig. 6.7D). HEK293 cells were also transfected with pEGFP-KIF5C-hdl (Fig. 6.7E), which decorated the cytoplasm, and pCisMyc-gephyrin (Fig. 6.7F), which formed cytoplasmic aggregates. The merged image shows that these proteins were not co-localised (Fig. 6.7G).
Fig. 6.6: Wild-type and headless KIF5C - Two kinesin constructs were made in the vector pEGFP so that the EGFP tag was at the N-terminus of the protein, a full-length wild-type protein (A) and a headless mutant (E). B and F show schematic representations of these proteins, the headless mutant lacks motor domain, which contains the microtubule and ATP-binding sites. Confocal images of HEK 293 cell transfected with pEGFP-KIF5C-wt (C&D) or KIF5C-hdl (G&H). Scale bar: 10 μm.
Fig. 6.7: Kinesin and Gephyrin - Gephyrin was expressed in Y190 cells with pACT2-KIF5B and in the resulting *LacZ* assay colonies remained white (A). Gephyrin was also tested against KIF5A, KIF5C and KIF3A, all of which gave negative *LacZ* assays. B-G are confocal images of HEK 293 cells transfected with pCisMyc-gephyrin (B&E) and either KIF5C-wt (C) or pEGFP-KIF5C-hdl (F). Gephyrin formed cytoplasmic aggregates (B&E arrows), and kinesin expression gave a similar phenotype to those observed in Fig. 6.6. The merged images (D&G) show that these proteins do not directly interact with each other. Scale bar: 10 μm
6.3.4 KIF5C and collybistin

The interaction between KIF5C and collybistin was established in Chapter 5 (Fig. 5.4) using the yeast two-hybrid system. HEK293 cells were co-transfected with collybistin and either pEGFP-KIF5C-wt which is the full length construct or pEGFP-KIF5C-hdl which is the headless mutant. Collybistin expression was detected using 9E10 antibody (1:200), followed by Alexa Fluor 594 conjugated secondary antibody (1:200). KIF5C-wt expression was concentrated at one end of the cell, whilst KIF5C-hdl decorated the cytoskeleton. Both CB2_{sh1} and CB2_{sh2} were diffusely distributed throughout the cytoplasm, however some co-localisation with kinesin was observed. Fig. 6.8 is an example of the phenotypes observed when wild-type (A) and headless kinesin (D) were expressed with collybistin (B&E) in HEK293 cells. The merged images (C&F) show that there was some co-localisation between kinesin and collybistin, however it was more evident with KIF5C-hdl than KIF5C-wt.

**Fig. 6.8: KIF5C and collybistin** - Confocal images of HEK293 cells transfected with either pEGFP-KIF5C-wt (A) or pEGFP-KIF5C-hdl (D) and pRK5Myc-CB2_{sh3} (B&E). In the cell transfected with wild-type kinesin and collybistin, the merged image (C) shows that kinesin has accumulated at one end of the cell and collybistin has a slightly diffuse distribution, however there is some co-localisation (arrow). In the cell transfected with headless kinesin and collybistin, the merged image (F) shows that the two proteins interact with each other as co-localisation is clearly evident (arrow). Scale bar: 10 μm
6.3.5 KIF5C, collybistin and gephyrin

HEK293 cells were transfected with pRK5Flag-CB2SH3, pCisMyc-gephyrin and either pEGFP-KIF5C-wt or pEGFP-KIF5C-hdl. Collybistin expression was detected using Flag antibody (1:200), followed by Cy5 conjugated secondary antibody (1:200) and gephyrin expression was detected using 9E10 antibody (1:200), followed by Alexa Fluor 594 conjugated secondary antibody (1:200).

Fig. 6.9A shows a series of confocal images taken through a HEK293 cell transfected with KIF5C-wt, collybistin and gephyrin. Fig. 6.12B-D are of a single section taken from the centre of this cell. KIF5C-wt was expressed throughout the cell and decorated the cytoskeleton (B), whereas gephyrin (C) and CB2SH3 (D) formed submembranous microaggregates. The merged image (E) shows that CB2SH3 and gephyrin microaggregates were colocalised at submembranous positions, but outside the boundary of KIF5C-wt expression.

Fig. 6.10A is a series of confocal images taken through a HEK293 cell transfected with KIF5C-hdl, collybistin and gephyrin. Fig. 6.10B-D are of a single section extracted from this series of images near the centre of the cell. KIF5C-hdl is expressed throughout the cell and has clearly decorated the cytoskeleton (B), gephyrin (C) and CB2SH3 (D) have formed co-localised microaggregates. The merged image (E) shows that these microaggregates are within the boundary formed by KIF5C-hdl expression.

The cells used in Figs. 6.9 and 6.10 were reconstructed into Z-stacked images, as this enhanced the clarity of the pictures and are presented in Fig. 6.11 for comparison. In both cells gephyrin and collybistin had a tight association indicated by the purple signal in the merged images and both kinesin constructs decorated the cytoskeleton. The merged images in Fig. 6.11 clearly show that microaggregates have formed between gephyrin and collybistin but in the presence of KIF5C-wt, these are located at submembranous sites outside the periphery formed by kinesin expression. In the presence of KIF5C-hdl, these gephyrin-collybistin microaggregates are trapped within the microtubular network, suggesting that the headless KIF is impeding the trafficking of these aggregates to submembrane sites.
Fig. 6.9: Wild-type KIF5C, gephyrin and CB2$_{SH3}$. Panel A shows a series of confocal images taken through a HEK293 cell transfected with EGFP-tagged KIF5C-wt, myc-tagged gephyrin and flag-tagged CB2$_{SH3}$. B-D shows the distribution of the three proteins as seen at the centre of the cell and E is a merged image of the three, clearly showing that collybistin and gephyrin aggregates are co-localised outside the periphery of KIF5C-wt. Scale bar: 10 μm
Fig. 6.10: Headless kinesin, gephyrin and CB2$_{SH3}$. Panel A shows a series of confocal images taken through a HEK293 cell transfected with EGFP tagged KIF5C-hdl, myc-tagged gephyrin and flag-tagged CB2$_{SH3}$. B-D shows the distribution of the three proteins at the centre of the cell and E is a merged image of the three, showing that collybistin and gephyrin aggregates are trapped within the periphery of KIF5C-hdl. Scale bar: 10 μm
Fig. 6.11: CB2<sub>SH3</sub>, gephyrin and KIF5C - Z-stacked confocal reconstructions of HEK293 cells transfected with pRK5Flag-CB2<sub>SH3</sub> (A&E), pCisMyc-gephyrin (B&F) and either pEGFP-KIF5C-wt (C) or pEGFP-KIF5C-hdl (G). In images D and H, the cytoskeleton which was decorated by recombinant kinesin is encircled by a white dotted line. These images clearly show that gephyrin and collybistin formed microaggregates that appear to have a tight interaction (purple signal). KIF5C-wt appeared to enhance the co-expression of gephyrin and collybistin at submembranous sites (arrows, D), whereas the headless KIF5C trapped the microaggregates within the confines of the cytoskeleton (arrows, H). Scale bar: 10 μm.
6.4: Discussion

6.4.1 Kinesin-collybistin-gephyrin complex

Amongst the proteins isolated in the collybistin library screen described in Chapter 5 were two kinesin heavy chains – KIF3A and KIF5C. The kinesins comprise a superfamily of motor proteins that utilise ATP to transport cargo along microtubules. Microtubules have intrinsic polarity with a fast growing plus-end and a slow growing minus-end. In neurons, the plus-end is generally distal to the cell body, except in proximal dendrites where the polarity is mixed. The majority of kinesins travel towards the plus-end of the microtubules, although kinesin-13 members travel towards the minus-end (reviewed in Hirokawa and Takemura, 2005).

Kinesins function as holoenzymes, generally composed of two motor domain containing subunits and a number of accessory proteins that mediate interactions with cargo. KIF5C is a neuronal specific motor protein for kinesin-1 (Kanai et al., 2000). This is the most widely studied kinesin and is composed of two heavy chains that contain the motor domains and two light chains which mediate interactions with cargo. The heavy chains are divided into three structural domains, the N-terminal motor domain, which binds to microtubules and hydrolyses ATP, the central neck region, which mediates dimerisation, and the C-terminal cargo binding domain, which interacts directly with cargo and kinesin light chains (Goldstein, 2001). In neurons, it is thought that if cargo binds directly to the cargo binding domain of the heavy chain, it is directed to dendrites, whereas if it binds to kinesin light chains, it is directed to the axon (reviewed in Hirokawa and Takemura, 2005). The precise mechanism determining this process is not established.

To establish whether collybistin interacted with other kinesin heavy chains, the cargo binding domains of KIF5A and KIF5B were cloned into pACT2 and tested in Y190 cells using the LacZ reporter gene assay to identify positive interactions. Neither collybistin or hPEM-2 gave a positive reaction with KIF5A suggesting that KIF5A does not interact with these proteins. However, collybistin and hPEM-2 both gave a positive reaction when tested against KIF5B, indicating that they interact, albeit not with the robustness observed with KIF5C and KIF3A. KIF5B is the ubiquitously expressed isoform of the kinesin-1 heavy chain (Kanai et al., 2000), whereas KIF5A and KIF5C are neuronal specific, although they have different patterns of distribution (Kanai et al.,
KIF5C expression levels were found to be five times higher than KIF5A in the cortex, hippocampus, cerebellum and spinal cord (Kanai et al., 2000). Collybistin expression was also found to be abundant in these areas, especially in the spinal cord and adult hippocampus (Kneussel et al., 2001).

Two KIF5C proteins were cloned into pEGFP so that the interactions between kinesin and collybistin could be observed in mammalian cells. KIF5C-wt was the full-length protein and KIF5C-B was a headless mutant in which the motor domain of the protein was deleted. Upon transfection of KIF5C-wt into HEK293 cells, the majority of protein expression appeared to be concentrated towards one end of the cell. In some cells fibrous structures were observed implying that the cytoskeleton was decorated. The headless KIF5C also appeared to decorate the cytoskeleton when expressed in HEK293 cells. Although the headless KIF5C no longer contained the microtubule binding site, it did possess the neck region, thus retaining its ability to dimerise with other KIFs. A recent study showed that KIF5B is endogenously expressed in HEK293 cells which suggests that the headless mutant may interact with endogenously expressed KIF5B, but as the holoenzyme will only contain one motor domain, it would be unable to move (Brickley et al., 2005), hence, KIF5C-hdl appeared to decorate the cytoskeleton throughout the cell. Fig. 6.12, is a schematic representation of this explanation for what may be occurring in HEK293 cells transfected with wild-type and headless KIF5C.

Wild-type KIF5C
Retains ability to 'walk' along microtubules and appears concentrated at one end of the cell.

Headless KIF5C
Cannot 'walk' along microtubules and appears to decorate the cytoskeleton.

Fig. 6.12: Wild-type KIF5C has the ability to bind to other kinesin molecules via the neck region, to cargo via the cargo binding domains and to bind and move along microtubules as it has a fully functioning motor domain. However, the headless KIF5C only has the ability to bind to cargo and other kinesins. It can not bind to or travel along the microtubules but can interact with endogenous kinesin which would bind to microtubules, but be unable to move along them as these motor complexes would only have one active motor domain.
Experiments conducted in Y190 yeast cells and HEK293 cells revealed that there was no direct interaction between kinesin and gephyrin. This suggests that, if kinesin does have a role in the trafficking of glycine receptors, then, gephyrin is not its directly interacting cargo and a linker protein would be required. An analogous system has been observed for the trafficking of AMPA receptors containing the GluR2 subunit, which are transported by KIF5 via an interaction with GRIP1 (Setou et al., 2002). The authors found that all three KIF5 isoforms bind to the GRIP1-GluR2 complex which is preferentially transported to dendrites instead of axons.

The interaction between collybistin and KIF5C was quite robust in yeast cells (see Fig. 5.4). Upon co-expression of KIF5C-wt and collybistin in HEK293 cells, KIF5C-wt accumulated at one end of the cell, and collybistin was diffusely distributed throughout the cell. Kinesin and collybistin did, however, show co-localisation in some areas of the cell but the interaction between the two proteins did not appear to be as tight as that observed between collybistin and gephyrin (Fig. 3.7 and Fig. 3.8, pages 60-61). Co-expression of KIF5C-hdl and collybistin gave a stronger signal for co-localisation, although collybistin expression was still slightly diffuse (Fig. 6.8).

The interactions between kinesin, collybistin and gephyrin were also assessed in HEK293 cells. In cells that were co-transfected with KIF5C-wt, CB2SH3 and gephyrin, collybistin and gephyrin formed microaggregates, which were localised outside the periphery of the cytoskeletal network as identified by KIF5C. When CB2SH3 and gephyrin were co-expressed in HEK293 cells, they formed submembranous microaggregates, however, collybistin still retained a slightly diffuse distribution (Fig. 3.10). In the presence of kinesin, collybistin no longer displayed a diffuse distribution, but showed an intense co-localisation with gephyrin. Cells that expressed KIF5C-hdl, CB2SH3 and gephyrin gave an interesting phenotype. In these cells, the headless kinesin appeared to decorate the cytoskeleton, and gephyrin and collybistin formed microaggregates. However, these microaggregates appeared to be trapped within the microtubule network, suggesting that by disrupting the activity of kinesin, gephyrin and collybistin can not reach submembranous sites. Once again, the association between collybistin and gephyrin was tighter than previously observed. It appears that the overexpression of kinesin with collybistin and gephyrin enhances the interaction between the two proteins. These data suggest that kinesin may be involved in the trafficking of gephyrin and that collybistin is the linker protein (see Fig. 6.13).
6.4.2 Kinesins and receptor trafficking

There is accumulating evidence that motor proteins are involved in the trafficking of neurotransmitter receptors (reviewed in Kneussel, 2005). Table 6.4 shows proteins which are thought to be a part of the postsynaptic scaffold or involved in receptor trafficking.

<table>
<thead>
<tr>
<th>Motor Protein</th>
<th>Cargo/Adapter protein</th>
<th>Receptors or receptor subunits</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIF5A/B/C</td>
<td>GRIPl</td>
<td>AMPA GluR2</td>
<td>Setou et al., 2002</td>
</tr>
<tr>
<td></td>
<td>GRIF1</td>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; β2 (?)</td>
<td>Brickley et al., 2005</td>
</tr>
<tr>
<td>KIF17</td>
<td>Lin2, Lin7, Lin10 complex</td>
<td>NMDA NR2B</td>
<td>Setou et al., 2000</td>
</tr>
<tr>
<td>KIF1Bα</td>
<td>PSD-95, PSD-93, Chapsyn 110, SAP-97, S-SCAM</td>
<td>NMDA; S-SCAM binds to NR2B (?)</td>
<td>Mok et al., 2002</td>
</tr>
<tr>
<td>Dynein light chains - Dlc1 and Dlc2</td>
<td>Gephyrin</td>
<td>GlyR β subunit</td>
<td>Fuhrmann et al., 2002; Hanus et al., 2004; Maas et al., 2006; Naisbitt et al., 2000</td>
</tr>
<tr>
<td></td>
<td>GKAP</td>
<td>NMDARs</td>
<td></td>
</tr>
<tr>
<td>Myosin V</td>
<td>GKAP, PSD-95</td>
<td>NMDARs (?)</td>
<td>Naisbitt et al., 2000</td>
</tr>
<tr>
<td>Myosin VI</td>
<td>SAP97</td>
<td>AMPA GluR1</td>
<td>Wu et al., 2002</td>
</tr>
</tbody>
</table>

Table 6.4: Motor proteins and their cargo which may be involved in the trafficking of neurotransmitter receptors (adapted from Kneussel, 2005).

As shown above, a number of proteins known to be part of the postsynaptic scaffold have also been identified as motor protein cargo. The most extensively studied are the KIF5-GRIPl-GluR2 and KIF17-Lin10-Lin2-Lin7-NR2B complexes (Fig. 6.14). As discussed in Chapter 5, GRIPl acts as an adapter protein between KIF5 and GluR2, and appears to influence the direction of transport towards the dendrites (Setou et al., 2002). GRIPl is also a component of the submembranous scaffold which supports AMPA receptors at postsynaptic sites (Sheng, 2001). The NMDA receptor NR2B subunit transport complex consists of Lin7, Lin2 and Lin10. The KIF17 motor protein binds to cytosolic Lin10 through a PDZ-domain mediated interaction, which in turn binds to Lin2, which interacts with Lin7, which is bound to the C-terminal of the NR2B subunit.
There is insufficient evidence to determine whether the trafficking of neurotransmitter receptors by motor proteins via scaffold-adapter proteins is a general trend, or whether it is specific for certain receptors. However, there is some circumstantial evidence to suggest that this method of trafficking may apply to receptors for inhibitory amino acid neurotransmitters.

The GABA$_A$ receptor interacting factor (GRIF1) has been shown to bind to the GABA$_A$ receptor β2 subunit (Beck et al., 2002), and also to KIF5C (Brickley et al., 2005), but it is not known whether GABA$_A$ receptors and KIF5 are linked by GRIF1. Gephyrin binds to Dlc1 and Dlc2, which are light chains of both dynein and myosin Va motor protein complexes (Fuhrmann et al., 2002). It appears that this interaction contributes to the retrograde transport of gephyrin, as studies on neurons derived from gephyrin deficient mice have shown that the expression of a gephyrin deletion mutant that no longer binds to Dlc1 and 2 does not affect the synaptic localisation of gephyrin. This suggests that Dlc mediates retrograde transport, which is consistent with dynein moving towards the cell body. A study carried out by Hanus et al. (2004) showed that the movement of glycine receptors was dependent on microtubule integrity. Using a chimeric glycine receptor α1 subunit which contained the gephyrin binding site, they showed that upon co-expression gephyrin and the chimeric glycine receptor subunit formed intracellular microaggregates, and that the movement of these receptor clusters was affected by nocodazole treatment, suggesting that their movement may be reliant on microtubules. They also showed that glycine receptors associated with gephyrin en route to the plasma membrane (Hanus et al., 2004). Recent data presented by Maas et al. (2006), showed that the disruption of dynein-gephyrin interactions interfered with retrograde transport of gephyrin.

Consistent with the idea that gephyrin transports glycine receptor vesicles from intracellular sites to the plasma membrane is the interaction between gephyrin and GABARAP. GABARAP is a post-Golgi transport factor which may be involved in the trafficking of GABA$_A$ receptors via an interaction with the γ2 subunit (Wang et al., 1999; Kneussel, 2002). GABARAP also interacts with NSF which is essential for intracellular trafficking events (Kittler et al., 2001). GRIP1 has also been shown to interact with GABARAP and the GABA$_A$ receptor γ2 subunit, however the
Chapter 6: Collybistin and Kinesins

physiological consequence of this interaction is not known, but could involve receptor trafficking (Kittler et al., 2004).

It is well established that neurotransmitter receptors accumulate opposite presynaptic terminal boutons releasing their respective neurotransmitter (Craig and Boudin, 2001). However, in isolated micro-island neuronal cultures, there is evidence for the spontaneous formation of connections between neurons which contain mis-matched appositions, for example GABA_A receptors are found opposite non-GABAergic terminals (Rao et al., 2000). This suggests that there are selective mechanisms for the correct insertion and targeting of receptors which are lacking in these artificial systems. In addition, there is evidence to show that gephyrin is required for the formation of glycine receptor clusters opposite glycine terminals in hippocampal cultures (Levi et al., 2004). Gephyrin, however, is not required for the correct positioning of GABA_A receptors in this system (Levi et al., 2004).

The cytoskeleton is an integral part of the synapse as it provides a base upon which the synaptic scaffold can be established. Cytoskeletal components can either bind to the scaffolding proteins or to receptor subunits via linker proteins, and can help to stabilise the scaffold protein-receptor complex. In some cases, the cytoskeleton appears to regulate the entry and exit of receptors from the synapse, and alter the local density of receptors and the shape of the dendritic spine (reviewed in Triller and Choquet, 2003). In the case of glycine receptors, depolymerisation of the actin cytoskeleton results in the generation of small receptor clusters and increased gephyrin accumulation (Kirsch and Betz, 1995). The disruption of microtubules induces the lateral spread of glycine receptor enriched microdomains with a decrease in gephyrin density. This suggests that the local concentration of glycine receptors is controlled by the cytoskeleton.

Typically, excitatory amino acid receptors are targeted to synapses on dendritic spines and inhibitory amino acid receptors are targeted to synapses on dendritic shafts (Kneussel, 2005). Delivery of receptors to these distinct sites may be achieved by two methods – direct targeting or selective retention, in which receptors are transported to synapses but only retained where they are required (see section 6.1.5.4). The general mode in which receptors are inserted into synapses is not known. However, Rosenberg et al. (2001) demonstrated that glycine receptor clusters are formed after insertion into the plasma membrane. They also showed that glycine receptor exocytosis was not
directed or synapse specific and occurred predominantly at extrasynaptic sites in the soma and initial portions of dendrites, and that clusters subsequently diffused laterally to distal areas of the plasma membrane (Rosenberg et al., 2001; Triller and Choquet 2003).

In summary, there is evidence in the literature to show that:

- the cytoskeleton is important for the accumulation and clustering of glycine receptors
- gephyrin trafficking is microtubule-dependent
- glycine receptors interact with gephyrin en route to the plasma membrane
- insertion of glycine receptors into synaptic sites appears to be by selective retention

It is becoming increasingly evident that molecular motors associate with receptor cargo through adaptor/scaffold proteins, and taken together with the evidence discussed above, it is proposed that the KIF5C-collybistin-gephyrin complex presented in this chapter could be an integral part of the mechanism by which glycine receptors are trafficked from the Golgi bodies to synapses. Fig. 6.13 is a simplified representation of this trafficking complex, the AMPA and NMDA receptor trafficking complexes are also shown for comparison.

**Fig. 6.13: Receptor trafficking complexes** - A schematic representation of scaffold protein-motor protein complexes which may transport neurotransmitter receptors from intracellular sites to the plasma membrane along microtubules. KIF17 transports the NMDA receptor NR2B subunit to dendritic synapses by interacting with a Lin10-Lin2-Lin7 complex (Setou et al., 2000). KIF5 transports the AMPA receptor GluR2 subunit to dendrites via interactions with GRIP1 (Setou et al., 2002). It is proposed that KIF5, collybistin and gephyrin form a trafficking complex to convey glycine receptors to the plasma membrane.
**Chapter 6: Collybistin and Kinesins**

### 6.5: Conclusions

The membrane activation model states that collybistin is active at the synapse and catalyses GDP-GTP exchange activity on Cdc42, which in turn regulates the actin cytoskeleton. This then influences the accumulation and clustering of gephyrin and glycine receptors. However, disrupting the PH domain of collybistin, inhibits the accumulation of gephyrin at submembrane and synaptic sites indicating that collybistin may also be involved in trafficking gephyrin. In accordance with this, two kinesin motor proteins were identified in Chapter 5, and in this chapter it has been shown that:

- Collybistin binds to KIF5B and KIF5C.

- The overexpression of KIF5C in HEK293 cells enhances the interaction between collybistin and gephyrin.

- The headless KIF5C traps collybistin-gephyrin aggregates within the cytoskeleton, preventing them from reaching their submembranous sites in HEK293 cells.

- KIF5C is involved in the transport of gephyrin to submembranous sites via interactions with collybistin.
Chapter 7: General Discussion
7. General Discussion

7.1 Membrane activation model...revisited

The membrane activation model was proposed by Kneussel and Betz (2000) to address the molecular mechanisms underlying the accumulation of gephyrin and subsequent glycine receptor clustering at developing postsynaptic sites. They suggested that Ca\(^{2+}\) influx and the local activation of PI3K by an unknown signal triggers gephyrin accumulation. It was postulated that PI3K phosphorylates PI4,5P\(_2\) to PI3,4,5P\(_3\), which then recruits the gephyrin binding protein collybistin to the ‘activated’ membrane via its PH domain. Collybistin then catalyses GDP-GTP exchange on Cdc42, which, in turn, regulates local actin cytoskeletal dynamics to bring about the apposition of the gephyrin scaffold opposite the relevant presynaptic terminal, thus immobilising glycine receptors at the postsynaptic membrane. This branch of the model was based upon the findings of Kins et al. (2000) who had identified two isoforms of collybistin, one of which had the ability to translocate gephyrin the submembranous sites in transfected HEK293 cells, and Reid et al. (1999), who demonstrated that the human isoform of collybistin (hPEM-2) catalysed GDP-GTP exchange on Cdc42. The aim of the work presented in this thesis was to investigate the role(s) played by collybistin in the apposition of gephyrin at postsynaptic membranes and Fig. 7.1 shows the part of the membrane activation model investigated in this study.

![Diagram](image)

**Fig. 7.1:** Collybistin-gephyrin actions depicted in the membrane activation model

The membrane activation model states that collybistin catalyses GDP-GTP exchange on Cdc42, which then regulates the actin cytoskeleton at synapses. However, in Chapter 4,
data from the YEA suggests that collybistin does not activate Cdc42. Actin rearrangement assays in NIH3T3 (Fig. 4.5, page 94) and REF52 cells (Fig. 4.6, page 96) showed that the expression of collybistin in fibroblasts does induce rearrangements of the actin cytoskeleton, but these are not clearly indicative of any of the GTPases for which phenotypes are already established. Further, the expression of CB2\textsubscript{SH3} and CB2\textsubscript{SH3} in NIH3T3 cells gave no discernible differences in phenotype, suggesting that the factors influencing SH3 domain mediated regulation of collybistin activity may be present in these cells. However, slight differences were seen between CB2\textsubscript{SH3} and CB2\textsubscript{SH3} expression in REF52 cells but not dramatically enough to provide sufficient evidence to determine which GTPase was activated.

The SH3 domain negatively regulates the ability of collybistin to translocate gephyrin in transfected HEK293 cells. However, the molecular mechanism which underlie SH3 domain activity are not understood. The majority of collybistin isoforms found \textit{in vivo} harbour the SH3 domain (Harvey \textit{et al.}, 2004b). Expression of CB2\textsubscript{SH3} and CB2\textsubscript{SH3} in cortical neurones did not have a significant effect on the distribution of endogenous gephyrin clusters (Harvey \textit{et al.}, 2004b). This suggests that collybistin function may be mediated by protein-protein interactions at the SH3 domain, which are not present in HEK293 cells. Some GEFs are auto-regulated or function as dimers. The GEF Vav1 was found to be constitutively active when truncated at the N-terminal. Removal of the first 66 amino acids results in a constitutively active form. It is thought that auto-inhibition is structural, and upon GEF activation, certain residues at the N-terminal are phosphorylated, thus destabilising the structure and removing inhibition (Aghazadeh \textit{et al.}, 2000; Han \textit{et al.}, 1997; Crespo \textit{et al.}, 1997). The GEF p115-RhoGEF homooligomerises through its C-terminus, and the deletion of this region also results in a gain of function, indicating that the C-terminus may be involved in the negative regulation of this GEF, similar to that seen with collybistin and its SH3 domain. Collybistin does interact with itself and may function as a dimer, however this has not been clarified.

What is evident is that the SH3 domain is imperative for collybistin function \textit{in vivo} and that its disruption affects the clustering of inhibitory neurotransmitter receptors at synapses (Harvey \textit{et al.}, 2004b).

The catalysis of small GTPases is usually attributed to the RhoGEF domain, and in some cases under the influence of the PH domain. However in the case of collybistin, this region was found to mediate interactions with gephyrin, directing attention towards
Chapter 7: General Discussion

a new role for collybistin, in addition to its role as a GEF. Like Tiam1 which participates in a multi-protein signalling complex (Buchsbaum et al., 2002, 2003), collybistin could act as a scaffold protein to mediate interactions between gephyrin and other proteins at synaptic and nonsynaptic sites. This concept was further supported by the phenotypes displayed by the PH domain deletion mutant of collybistin in both HEK293 cells and cultured cortical neurons (Fig. 3.12, page 68). CB2SH3APH retained the ability to bind to gephyrin, but was unable to translocate it to submembranous sites in HEK293 cells, and trapped endogenous gephyrin in the neuronal cell body. These observations suggest that collybistin is involved in trafficking gephyrin to synapses. The identification of PI3P as a binding partner for the PH domain reinforced this idea (Fig. 3.13, page 70). This phosphoinositide is predominantly found on endosomes which are involved in constitutive membrane traffic. Taken together, this evidence suggests that collybistin is involved in the trafficking of gephyrin from intracellular sites to the synapse.

The collybistin two-hybrid screen, presented in Chapter 5, identified several proteins involved in trafficking, including GATE-16, which shares 57% amino acid identity with GABARAP, a protein involved in the intracellular trafficking of GABA_A receptors. In the membrane activation model, Kneussel and Betz (2000) suggested that GABARAP was involved in the clustering of GABA_A receptors at inhibitory synapses via interactions with gephyrin. However, recent findings suggest that GABARAP may be involved in intracellular trafficking as opposed to synaptic clustering and stabilisation of GABA_A receptors. GABARAP interacts with tubulin, microtubules and gephyrin, however, as it is not abundantly found at synapses it does not appear to be a core component of the subsynaptic scaffold. It also interacts with NSF which is involved in vesicular transport, thus suggesting that GABARAP is involved in receptor trafficking. GATE-16 interacts with NSF and GOS-28 and is involved in intra-Golgi transport, thus strengthening the case for collybistin as a mediator of gephyrin trafficking.

The identification of KIF5C and KIF3A as collybistin interactors provided a physical link between collybistin and the microtubular network, thus reinforcing the idea of collybistin in gephyrin trafficking. Kinesins are molecular motor proteins which convey cargo towards the plus-end of microtubules, which in neurons is generally directed away from the cell body. These motors utilise energy derived from ATP to move along microtubules, thus distributing various proteins and lipids to their sites of
function. In addition to interacting with the kinesin isoforms isolated in the library screen, collybistin and hPEM-2 also interact with the cargo binding domain of the ubiquitously expressed KIF5B. Fig 7.2 is a modified scheme of the membrane activation model encompassing the idea that collybistin may play a role in trafficking gephyrin to the synapse along microtubules within the axon and/or dendrites via interactions with kinesin motor proteins.

![Diagram](image)

**Fig. 7.2:** A new role for collybistin in receptor clustering

7.2: Kinesin-collybistin-gephyrin as a trafficking complex

It is well established that the accumulation and correct synaptic localisation of neurotransmitter receptors is a prerequisite for fast synaptic transmission. Receptors are concentrated at postsynaptic specialisations through interactions with specific scaffolding proteins and cytoskeletal elements. Like many intracellular proteins, neurotransmitter receptors are transported from the trans-Golgi network to the plasma membrane, be it axonal or dendritic. Once the distinction is made between presynaptic and postsynaptic receptors, the postsynaptic receptors have to be further sorted. Most glutamate receptors are located at dendritic spines, whereas glycine and GABA receptors are found in the dendritic shaft, and have to be sorted accordingly. Delivery may be executed in two ways, selective retention or directed targeting. There is some evidence to suggest that receptors are delivered to the plasma membrane and are then incorporated at postsynaptic sites by selective retention and lateral diffusion (Rao et al., 2000; Triller and Choquet, 2005).

Presynaptic active zones are transported along developing axons as discrete, prefabricated ‘transport packets’ composed of cytoplasmic and membrane associated
proteins (Ahmari et al., 2000), suggesting that proteins which interact with each other at synapses are united quite early on in their formation and sorting pathways. The exact mechanisms governing the delivery of receptors to specific sites is not well understood, but there are many mechanisms which may contribute to it (reviewed in Kneussel, 2005).

Once at the plasma membrane, receptors are dispersed throughout it, at extrasynaptic sites as well as postsynaptic sites, and recent evidence reviewed by Triller and Choquet (2005) indicates that glycine, GABA, AMPA and NMDA glutamate receptors are mobile at synapses and can alternate between synaptic and extrasynaptic sites through lateral diffusion in the plane of the plasma membrane (Meier et al., 2001; Dahan et al., 2003). The idea that receptors are inserted into the plasma membrane at synapses by an exocytic pathway and then stabilised there is therefore being questioned. Changes in synaptic receptor numbers were thought to be governed by cycling of receptors between surface and intracellular compartments (Sheng and Kim, 2002). However, some receptors are not inserted directly at synapses during constitutive or activity dependent exocytosis, and some studies have shown that endocytosis occurs outside the synaptic areas (Park et al., 2004). Taken together, these observations indicate that the trafficking of receptors and their subsequent apposition at synapses are two different but interrelated processes. These observations support the idea that collybistin could mediate the interaction between gephyrin and kinesin to transport glycine receptors to the plasma membrane, but not necessarily to postsynaptic sites (Triller and Choquet, 2005). The evidence summarised in 6.4.2 shows that the cytoskeleton is important for the accumulation and clustering of glycine receptors, that gephyrin trafficking was microtubule-dependent, that glycine receptors interact with gephyrin en route to the plasma membrane and that the subsequent insertion of glycine receptors at synaptic sites appears to be by selective retention and not directed targeting.

7.3 Could collybistin play other roles in glycine receptor clustering?

Collybistin was identified as a GEF for Rho-family GTPases, and it is evident that collybistin expression in fibroblasts induces rearrangements in the intracellular distribution of actin (Fig. 4.5 and Fig. 4.6, pages 94 and 96). This influence of collybistin on the actin cytoskeleton may be important not at the synapse, as suggested by Kneussel and Betz (2000), but within the cytosol. The actin and microtubule
networks are both integral to the intracellular trafficking of proteins. There is increasing
evidence to suggest that Rho-family GTPases play a role in intracellular membrane
trafficking, which in some cases, is independent of their role in actin dynamics
(reviewed in Qualmann and Mellor, 2003). They have been localised to various
compartments of the endocytic pathway, for example, RhoD has been localised to early
endosomes (Murphy et al., 1996), which is also the primary location of PI3P.
Constitutively active RhoD causes the realignment of these early endosomes, a process
thought to be mediated by the sequential activation of Dia and the tyrosine kinase c-Src
(Gasman et al., 2003). Dia has been shown to reposition microtubules so that they are
parallel with actin filaments (Palazzo et al., 2001) and as Dia is abundant in these
compartments (Tominaga et al., 2000), RhoD appears to play a role in early endocytic
traffic.

The most prominent role for a Rho-GTPase in intracellular trafficking is that of TC10 in
the insulin-induced transportation of facilitative glucose uptake transporter 4 (GLUT4)
vesicles to the plasma membrane (Chiang et al., 2001). Insulin increases the rate of
glucose transport into fat and muscle cells by stimulating the trafficking of GLUT4
from specialised intracellular compartments to the plasma membrane (Bryant et al.,
2002). TC10 is activated by the GEF C3G which is recruited to plasma membrane lipid
rafts by insulin activated PI3K products. Activated TC10 is proposed to recruit N-
WASP and cause actin rearrangements, thereby facilitating the movement of GLUT4
vesicles (Chiang et al., 2001).

Interestingly, KIF5B and KIF3 have both been implicated in the insulin-induced
trafficking of GLUT4 vesicles to the plasma membrane (Imamura et al., 2003; Semiz et
al., 2003). KIF5B-mediated transport appears to be PI3K-independent, whereas KIF3-
mediated transport appears to be PI3K-dependent. KIF3 interacts with the GTPase
Rab4 in an insulin-dependent manner. The Rab-family GTPases are traditionally
associated with intracellular trafficking (Goody et al., 2005) and Rab4 is thought to
regulate the interaction between KIF3 and GLUT4 vesicles (Imamura et al., 2003). The
precise mechanisms governing the trafficking of GLUT4 are still not established, but it
is evident that kinesins, Rho- and Rab-family GTPases and a number of other regulatory
proteins are involved.
Chapter 7: General Discussion

It is possible that a similar number of proteins are involved in the trafficking of glycine receptors from intracellular sites to the plasma membrane. Collybistin could also be involved in regulating the transfer of gephyrin and glycine receptors from microtubules to the actin cytoskeleton at the plasma membrane. In neurons, meshworks of actin microfilaments are found mainly in the cytoskeleton underlying the plasma membrane in cell bodies, axons and dendrites (Fath and Lasek, 1988). Microtubules do not extend to the ends of axons and dendrites, instead they stop slightly short of the plasma membrane, therefore membrane proteins need to be transferred to the submembranous actin cytoskeleton for the final part of their journey. At synapses collybistin could be involved in regulating the actin cytoskeleton for the purpose of transferring gephyrin and glycine receptors to the membrane, as opposed to facilitating gephyrin clustering.

7.4: A new model

In the five years since Kneussel and Betz (2000) proposed the membrane activation model to address the molecular mechanisms underlying the clustering of inhibitory neurotransmitter receptors at developing postsynaptic membranes, a wealth of data has been published showing that receptor clustering is a carefully regulated and complex process, and that synapses are in a dynamic state with receptors cycling between synaptic and extrasynaptic sites. A recent review by Triller and Choquet (2005) discussed the insertion of glycine receptors into the plasma membrane and their subsequent diffusion in the plane of the membrane. Hanus et al. (2004) showed that the glycine receptor trafficking was dependent on microtubule integrity and glycine receptors associated with gephyrin from the trans-Golgi network to the plasma membrane.

In this study, it was shown that collybistin mediates an interaction between the KIF5C motor protein and gephyrin which appears to facilitate the transportation of gephyrin to the plasma membrane. In addition, the kinesin motor protein-scaffold protein trafficking complexes identified for AMPA and NMDA receptors support the proposal that collybistin can mediate an interaction between KIF5 and gephyrin to transport glycine receptors from the trans-Golgi network to the plasma membrane. Fig. 7.3 is a model depicting the roles which collybistin may play in the trafficking of glycine receptors to neuronal plasma membrane.
Control of membrane traffic?
Regulated transfer of cargo from microtubules to actin?

Collybistin interacts with kinesin motor proteins to transport gephyrin and possibly glycine receptors along microtubules to the plasma membrane.

The PH domain of collybistin binds to PI3P, which is found on early endosomes and involved in constitutive membrane traffic.

Collybistin interacts with GATE-16, a late acting intra-Golgi transport factor, which is closely related to GABARAP.

Fig. 7.3: Model for glycine receptor trafficking by kinesin-collybistin-gephyrin complex - A schematic representation of glycine receptor trafficking by a kinesin-collybistin-gephyrin complex. Kinesin transports cargo along microtubules to destinations throughout the cell. In neurons, this can be over long distances along axons and to distal dendrites. Kinesin ‘walks’ along tracks provided by microtubules. The kinesin heavy chains KIF5B, KIF5C and KIF3A interact with collybistin, which acts as a chaperone to transport gephyrin-glycine receptor complexes to the plasma membrane. Collybistin also interacts with GATE-16, which is an intra-Golgi transport factor, and the PH domain of collybistin binds to PI3P, both implicating collybistin in membrane trafficking events. Collybistin does appear to activate a GTPase, however its identity remains elusive. The GTPase may contribute to glycine receptor trafficking by regulating membrane trafficking, or may be involved in the transfer of the receptor complex from microtubules to the actin cytoskeleton.
This study showed that:

- The isoform of collybistin found in humans and most abundant in rats is that harbouring the SH3 domain, which negatively regulates the ability of collybistin to translocate gephyrin to submembranous sites in HEK293 cells. In cortical neurons there was no discernible difference observed in gephyrin clustering by the expression of recombinant CB2<sub>SH3</sub> and CB2<sub>SH3+</sub>, however the functional importance of this domain was underlined by the fatal mutation identified in a hyperekplexic patient (Harvey et al., 2004b).

- The RhoGEF domain of collybistin binds to gephyrin.

- PH domain deletion mutants result in a trafficking mutant which impedes the translocation and clustering of gephyrin in HEK293 cell and cortical neurons.

- Collybistin does activate a GTPase, whose identity remains unknown, but it does not appear to be Cdc42.

- Collybistin interacts with a number of proteins, which may aid it in its role in gephyrin clustering, including NGEF and GATE-16, an intracellular trafficking factor which is closely related to GABARAP, known to be involved with the trafficking of GABA<sub>\text{A}</sub> receptors.

- Collybistin interacts with three kinesin motor protein heavy chains – KIF3A, KIF5B and KIF5C.

- The interaction between collybistin and gephyrin appears to be enhanced in the presence of KIF5C.

- Disrupting kinesin movement appears to trap the translocation of collybistin-gephyrin microaggregates to submembrane sites.

Although this study develops some of the proposals made by Kneussel and Betz (2000) in the membrane activation model, it also raises a number of other questions which will need to be addressed in order to fully understand the molecular mechanisms underlying the trafficking and clustering of inhibitory amino acid neurotransmitter receptors at postsynaptic sites.
Appendix 1
<table>
<thead>
<tr>
<th>No.</th>
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<th>Insert Size</th>
<th>Backcheck pYTH16</th>
<th>Backcheck CB2SH3</th>
<th>In Frame</th>
<th>Genbank accession number</th>
<th>Sequence ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.2</td>
<td>+++</td>
<td>0.9kb</td>
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**Appendix 1:** A summary of the proteins isolated from the mammalian brain cDNA library screen performed using pYTH9-CB2_{SH3} as bait (see Chapter 5). All potential interactors were rescued from the yeast, digested with EcoRI/XhoI to elucidate the size of the isolated cDNA insert, and sequenced for identification purposes. Preys were back-checked against pYTH16 and pYTH9-CB2_{SH3}, in order to eliminate any false positives and ensure the veracity of the interaction. *LacZ* assays were graded upon the intensity of the colour gained: - represents no change in colour after 3 h; changes were graded + to ++++, with ++++ indicating a change in colour occurring within 30 min of the start of the assay and a very intense colour observed after 3 h. (M)/Mixed: Colonies derived from one single colony appeared blue and white in the *LacZ* assay. Sequencing also confirmed whether the prey cDNA was expressed in the correct open reading frame. *common for all YTH screens. The interactors which were selected for further analysis are discussed in Chapter 5.
References
References


References


References


References


References


References


Langosch D., Hoch W., Betz H. (1992) The 93 kDa protein gephyrin and tubulin associated with the inhibitory glycine receptor are phosphorylated by an endogenous protein kinase. FEBS Letters 298: 113-117.


References


References


References


205
References


References


